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The Role of EPHB3 in a Human Model of Neural Crest Development and Palate Formation

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This dissertation is dedicated to my family, especially my mother for all of her sacrifices, support and encouragement that made it possible for me to pursue my education.

ABSTRACT OF THE DISSERTATION

The Role of EPHB3 in a Human Model of Neural Crest Development and Palate Formation

by

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The neural crest (NC) is a population of cells unique to vertebrates that arise early in development, migrate extensively and differentiate into a wide variety of derivatives. Neural crest cells (NCC) give rise to many derivatives which include melanocytes, adipocytes, neurons and glia, and cartilage and bone of the craniofacial region, amongst other derivatives. To form these intricate three-dimensional structures and derivatives NCC development must be spatiotemporally accurate, errors in these processes result in a wide range of clinical conditions known as neurocristopathies, which include cancers, rare syndromes and common craniofacial disorders like cleft palate. Oral clefts occur in about 1 in every 700 live births, ensue a considerable economic burden, and have functional and social implications for the child, including the ability to feed, and speak. A deep understanding of NCC development, including the early steps

of formation, differentiation potential, and spatiotemporal contributions are likely to aid in diagnostic and therapeutic efforts to treat NCC related conditions.

Early NCC development is initiated by multiple signaling pathways, including WNT, BMP, and FGF amongst others; however, EPH/EFN signaling has not been associated with early NCC development in human. EFN signaling has been associated with early NCC development in *Xenopus*, and mouse models implicate EPH signaling in of oral clefts. To study for the first time, the role of EPH receptors in early NCC development I used an in *vitro* model based on human pluripotent stem cells. Additionally, I engineered an in *vitro* model of palate fusion using a NCC-derived osteoblast core surrounded with an epithelial layer, that allows for an effective, animal free method to study palate fusion events. This platform will hopefully enable the testing and characterization of environmental (including chemical) and genetic insults associated with facial clefts, including EPH/EFN signaling.

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Introduction: Overview of Neural Crest Cells, Ephrin Lignads and Receptors and Their Respective Roles in Oral Palate Formation

Abstract

Neural crest cells (NCC) have played a critical role in vertebrate evolution, as they give rise to many derivatives of the body which include the peripheral nervous system, pigment cells, and the tissues of the craniofacial skeleton, such as bone, cartilage and connective tissue. Numerous studies have started to look at the gene regulatory network necessary for the formation of vertebrate NCC, much has been unveiled, but a lot more remains to be uncovered. Due to the vast contributions of NCC, abnormalities in their formation, migration, differentiation and/or maintenance lead to a wide range of pathologies broadly known as neurocristopathies. The contributions of signaling pathways such as WNT, FGF and BMP, amongst others have been widely studied in the context of early NCC formation.[1,2]Additionally, cell surface receptors such as EPH receptors have been shown to play a critical role during migration. Numerous studies carried out in mouse, zebrafish, *Xenopus* and chick embryos, have addressed the signaling contributions responsible for NCC formation and have established significant roles for the BMP, FGF and WNT pathways.

To date, no studies have addressed the possible role of EPH receptor mediated signaling during early NCC formation. Importantly, while human embryo studies lag considerably behind those performed in model organisms, human embryonic

stem cell based models offer an appealing alternative to advance our understanding of human NCC development.

Here I describe, for the first time, studies addressing the role of EPH receptors during early NCC development, focusing on the early stages of their formation. I report that EPH receptors and their EFN ligands are dynamically expressed during hNCC formation. I found that EPH function is critical for the early steps in hNCC formation. Specifically, EPHB3 is upregulated early on in NCC formation, and its inhibition either through small molecules or through siRNA knockdown strategies prevent NCC formation. Additionally, given the known contributions of NCC to craniofacial structures and their involvement in craniofacial clinical conditions, including frequent orofacial clefts, we engineered a model of palatal development based on hESC and hNCC derivatives.

Cleft lip/palate are amongst the most common craniofacial defects, and can be seen in 1 out of 700 live births. Cleft lip and palate can lead to eating or speaking impairments, can carry social alienation and have a considerable financial burden. The ability to study the effects of environmental toxins and gene mutation in the developing human palate remain limited.

Currently, the ability to study the effects of environmental toxins and gene mutation in the developing human palate are limited due to technical and ethical implications. The development of an engineered 3D spherical structure-based model is an ideal method for carrying out these studies. My 3D model relies on

spherical NCC-derived osteoblasts spheres, surrounded by an epithelial cell coating derived directly from hESC.

This model can be used to test the effects of EPHB3 receptor inactivation at different time points in the developmental process from NCC formation to osteoblast differentiation and fusion in oral plate formation. This model provides a useful platform to broadly study the effects of gene mutations and/or environmental toxins in clefting of the oral palate in an accessible, efficient, and economic manner, and could lead to novel diagnostic and therapeutic approaches.

Introduction to Neural Crest Cells

NCC are a multipotent population of cells that are unique to vertebrates. In a majority of species that have been studied (mice being the exception), premigratory NCC have been found to arise from the dorsal region of the neural tube.[3] In frog, fish and chicken, NCC begin to migrate upon closure of the neural tube; however in mice migration precedes full closure of the neural tube.[4] These premigratory NCC undergo epithelial to mesenchymal transition (EMT), after which they begin to migrate and give rise to a multitude of derivatives which include: neurons and glia, cartilage, melanocytes and bone of the craniofacial region.[5,6] NCC are key for vertebrate evolution and diversity, they are a vertebrate specific population that provide the color and craniofacial structures of these animals. It is thanks to these structures that all of these vertebrates, including humans can afford our predatory lifestyle.[7,8,9]

Based on their axial position along the anteroposterior axis, NCC procure their identity as cranial, vagal, trunk or sacral.[10,11] Generally cranial NCC will form cranial ganglia, connective tissue and craniofacial cartilage and bone, whereas vagal NCC will form smooth muscle, melanocytes, and enteric ganglia, trunk NCC form pigment cells, adrenal medulla, dorsal root ganglia, and sympathetic ganglia, and sacral NCC form enteric ganglia.[12] Experiments done in avian embryos have shown that when pre-migratory NCC from a given axial position are transplanted into a new spot the NCC from the grafts will migrate and differentiate according to signals from their new position.[13]

NCC were first identified in chick embryos in 1868 by Wilhelm His.[14] They were found between the neural tube and the region which would become the epidermal ectoderm. They were found to give rise to the cranial and spinal ganglia.[15] In 1878, Arthur Milnes Marshall termed these cells NCC.[16] Even though the existence of NCC has been known for more than 150 years, and they have been the subject of intense research, there is still a lot to be understood. When there are defects in NCC formation, migration, differentiation and/or maintenance, we get diseases known as neurocristopathies.[17,18] In order to better understand how the defects in NCC lead to these disorders, we must advance our understanding of NCC biology.

Neural Crest Cell Formation

Neural crest cells are specified following gastrulation, at the border between the neural plate and the non-neural ectoderm.[19,20,21] The neural tube is formed when

the neural folds meet during neurulation, then NCC migrate out from the dorsal most region.[22] Recent work from the Garcia-Castro lab suggests that NCC specification can be identified during the epiblast stage, prior to the formation of definitive mesoderm and ectodermal tissues. [23] This work suggests that NCC specification can proceed without the presence of the mesoderm or definitive neural ectoderm.[24,25] It has been shown that when explants were taken from a region prior to the expression of the transcription factor PAX7 (an early NCC marker) or any other NCC markers, the explants were able to express pre-migratory and migratory NCC markers following incubation, which shows that the involvement of neural nor mesodermal interactions is not needed for NCC formation.[26] Additionally, it has been shown *in vitro* that NCC can differentiate from human embryonic stem cells(hESC) into NCC without the presence of neuroectodermal cells, which would be marked by PAX6. Additional work has shown that NCC can arise without contributions from mesodermal tissue.[27] Taken together this information supports the idea that once specified, NCC precursors can advance in their NCC path of development without other tissue interactions, offering an alternative to the classic theory of NCC induction.

Work done in frog and chicken embryos provides evidence for the role of WNT, BMP, FGF, and Notch in NCC induction.[28] During formation of the neural plate border, WNT and FGF interactions play a role.[29,30,31] Next, during neurulation, BMP, WNT and Notch have been shown to be essential in the activation and maintenance of transcription factors necessary for the specification of NCC.[32,33,34]

Signaling Pathways Involved in Neural Crest Cell Formation

WNT, BMP, FGF and Notch signaling have been shown to play essential roles in the formation, migration and differentiation of NCC.

WNT Signaling

WNTs are signaling ligands that are evolutionarily conserved, they are essential for development. In humans and mice there are 19 WNT ligands and 10 Frizzled (Fz) receptors.[35] The WNT pathways are divided into the canonical and non-canonical WNT pathways, canonical WNT has been shown to play a key role in neural crest cell induction.[36,37,38,39] The WNT/ß-Catenin pathway regulates stem cell pluripotency and plays an essential role in embryonic development, such as cell fate decisions, and is involved in degenerative diseases and cancer progression.[40,41] Canonical WNT signaling functions to stabilize and nuclearize ß-catenin, which is essential for cytoskeletal arrangement and for the initiation of NCC regulatory transcription factors. When WNT is not present, the destruction complex involving APC and Axin is allowed to form, sequestering ß-catenin, and marking it for degradation by GSK3ß through phosphorylation. When the WNT ligand is bound to the Frizzled/LRP receptor, Dishevelled prevents the formation of the destruction complex which allows for the stabilization of ß-catenin and for ß-catenin entry into the nucleus, thus promoting the transcription of target genes and DNA bound TCF/LEF protein needed for NCC induction.[42,43]

Numerous studies have shown that WNT signaling is critical for NCC induction in the mouse, chick, *Xenopus* and zebrafish model systems. In *Xenopus* it is been shown that ectopic expression of wnt increases NCC induction, whereas the inhibition of wnt prevents NCC induction.[44,45,46] The wnt ligands that are involved in NCC induction have been shown to vary by species. In chick, Wnt3a and Wnt8a have been shown to be present in the lateral epiblast at the blastula stages. In zebrafish, Wnt8a is involved in NCC induction.[47,48] In *Xenopus* wnt3a and wnt8a are first expressed during the gastrula stage in the paraxial mesoderm and the caudal neural plate.[49,50] It has been shown that when the wnt ligand is not present, NCC specific genes are not induced.

In mouse, no significant evidence suggesting the importance of WNT signaling in NCC induction has been shown.[51] In mouse embryos the necessity of WNT signaling has been shown in the migration and formation of NCC derivatives.[52,53] Due to ethical and technical limitations of working with human embryos, the use of human pluripotent stem cells provides a good alternative. There are several groups who have reported hESC derived NCC models, these models make it possible to study signaling at the early stages of NCC development. The García-Castro lab has reported a robust system for the formation of NCC from hESC, it is a time efficient system that provides a high efficiency of NCC, which are able to differentiate into the expected NCC derivatives. This model uses CHIR, a small molecule inhibitor for GSK3, which permits ß-catenin nuclear localization thus mimicking Wnt activation, which is necessary for human NCC induction.[54]

Recent reports in *Xenopus* have shown that matrix metalloprotease ADAM13 is involved in wnt signaling. ADAM13 inhibits EFNB signaling, which has been shown to inhibit wnt signaling; by inhibiting EFNB signaling, ADAM13 activity leads to an increase in wnt signaling. It has been shown that ADAM19 functions to stabilize ADAM13 and promotes wnt signaling in *Xenopus* NCC induction.[55]

Numerous studies have shown that WNT signaling is needed for NCC induction, but the exact details explaining how WNT signaling works to activate NCC transcription factors is still largely unknown. Studies have started to address this gap in knowledge. In chick it has been shown that *Axud1*, a transcription factor, works downstream of WNT and directly regulates *FOXD3* expression, through interactions with *MSX1* and *PAX7*, which are neural plate specifiers.[56] A study in *Xenopus* has shown that DKK2, a wnt antagonist, works through the LRP5/6 receptor and activates ß-catenin which is needed for NCC specification.[57] Another study in *Xenopus* has demonstrated that *hes3*, a transcription factor, negatively regulates wnt signaling.[58]

BMP Signaling in Neural Crest Cell Formation

Bone morphogenetic proteins (BMPs) are growth factors belonging to the transforming growth factor beta (TGFß) signaling pathway. TGFß signaling has been shown to play an important role in embryonic development including differentiation of cells, apoptosis, and other cellular functions. Dysregulations of BMP signaling can lead to many pathologies including cancer.[59,60,61] In chick and

Xenopus models, gain-of-function studies have shown that in addition to Wnt signaling, the modulation of BMP signaling is necessary for NCC formation. There are two models which describe the crucial role of BMP signaling regulation for NCC induction. These two models are referred to as the gradient model and the “zone of competence” model.[62]

The gradient model argues that an intermediate level of BMP, in conjunction with WNT and FGF lead to induction of NCC between the border of the neural and the non-neural ectoderm.[63,64] Studies done in *Xenopus* and chick have shown that dysregulation of BMP from the appropriate levels prevent NCC formation. [65,66,67] At the neural plate border antagonistic interactions lead to an intermediate gradient of BMP signaling. The nonneural ectoderm provides high levels of BMP signaling and BMP inhibitors from the neural plate dampen the levels of BMP signaling.[68,69]

The “zone of competence” model suggests that attenuation of BMP signaling, during gastrulation, in conjunction with WNT and FGF signaling promotes NCC induction. This model suggests that WNT and FGF signaling can promote NCC induction, after which BMP signaling is reactivated at the neural plate border, this leads to NCC genes expression at the neural plate border.[70,71]

Mammalian systems have been used to investigate the role of BMP signaling in NCC induction. In inducible knockout mice, which were derived by Wnt1-cre or Pax3-Cre it has been shown that a knockout of the BMP receptors Alk1/3/5 or

Tgfbr2 leads to defects in regions derived from NCC, such as cardiac, pharyngeal and the craniofacial region.[72,73] At early stages (E8.5), neural plate border genes (*Msx1/2, AP2a, Pax3*, and *Sox9*) were not affected, but by E9.5 their expression was downregulated. Additionally, a NCC model originating from embryonic stem cells via WNT signaling activation has shown that BMP regulation is necessary for NCC induction.[74] Taking the data together from the different model systems, it is suggested that first WNT and FGF signaling are activated for NCC induction, then WNT and BMP are needed for progression of NCC induction.[75]

FGF Signaling in Neural Crest Cell Formation

Fibroblast growth factors (FGF) are signaling proteins required for development, any aberrations in FGF signaling can lead to a wide range of defects.[76,77] FGF is necessary for mesoderm formation, embryo patterning , and gastrulation.[78,79] In vertebrates, 22 members of the FGF family have been identified. FGF genes transduce signaling via FGF receptors, and are modulated by extracellular matrix components.

Studies using chicken embryos have shown that FGF signaling is essential for NCC induction. When FGF signaling is inhibited, in the gastrula epiblast, specifically in the neural plate border region, expression of NCC markers Pax7 and Snai2 are inhibited. When FGF signaling is inhibited after gastrulation, Pax7 and Snai2 are not inhibited. Studies suggest that FGF/MAPK play a direct role in

the formation of the cells at the neural plate border, and provide evidence that ERK signaling is needed during NCC formation. In perspective NCC epiblast, FGFR1/4 expression is present, but is absent from the mesoderm.[80,81] A different study showed that when FGF4 soaked beads were placed in the non-neural ectodermal region, BMP4 and Wnt8c and NCC markers were upregulated, before NCC associated transcripts were detected. [82,83,84]

In a study done in rabbit, explants were taken from gastrula rabbit embryos at the prospective NCC region; when an FGF inhibitor was added they were unable to express Pax7 and Sox10 (both of which are NCC markers).[85] Another study in which FGF signaling was inhibited in a hESC derived NCC model, found that NCC induction was inhibited, despite the presence of Wnt signaling.[86] The precise role of FGF signaling in zebrafish and mouse embryos remains to be established.

Notch/Delta Signaling in Neural Crest Cell Formation

In Notch/Delta signaling, Notch is the receptor and Delta is a membrane-bound ligand, when the ligand binds to the receptor in the Notch signaling pathway, a cascade of proteolysis is activated, and as a result the Notch intracellular domain (NICD) is released. Translocation of the NICD to the nucleus functions as a transcription factor in interaction with the CSL protein. Notch/Delta signaling has been reported to play an important role in trunk NCC development in species including chick, zebrafish and frog embryos; however it has not been shown to

affect cranial NCC formation.[87] Studies in chicken and frog embryos have shown that Notch functions upstream of BMP4.[88] To date, studies showing the role of Notch signaling in mouse have not been conducted.

WNT, FGF, BMP Signaling Pathway Interactions in Cranial NCC Formation

Taken together, information from numerous studies suggests that NCC induction heavily relies on appropriate levels and timing of WNT, FGF, and BMP activity. These studies suggest that NCC induction is initiated by WNT and FGF signaling, but maintained by BMP signaling. In chick embryos it has been shown that WNT can induce BMP expression, and inhibition of WNT can lead to attenuation of BMP signaling. WNT induced BMP activation is necessary to provide instructive cues at the neural plate border for NCC specifier activity.[89] Using *Xenopus*, zebrafish and chick models, it has been shown that the presence of FGF signaling decreases BMP signaling, through negative regulation of the BMP ligand.[90,91] These signaling pathways are essential to induce NCC.

Transcription Factors and Gene Activation for Neural Crest Cell Formation

WNT, BMP, FGF, and Notch signaling are postulated to be responsible NCC induction, through the activation and maintenance of the gene regulatory network. Studies have shown that they are responsible for the development of the neural plate border, and the initiation and development of NCC. The neural plate border is marked by the presence of transcription factors which include

PAX3, PAX7, ZIC1, MSX1 and *TFAP2A.*[92,93] Many model species including human embryonic stem cell-based systems, rabbit, hagfish, lamprey, *Xenopus*, mouse, chicken and zebrafish have contributed to understanding the gene regulatory network involved in NCC induction.

Transcription Factors Necessary for Neural Plate Border Specification

During the formation of NCC, signals from the previously discussed signaling pathways are received by the neural plate border cells, and transcription factors referred to as the neural plate border specifiers are expressed, which include *TAP2A, ZIC1, MSX1, MSX2, GBX2, PAX3, PAX7, SP5* and *ZEB2.*[94,95,96] These neural plate border specifiers are typically regarded as some of the early influencers in NCC formation. In *Xenopus* it has been shown that BMP and FGF can separately induce expression of *zic1* and *pax3* [97] Wnt, FGF and BMP can lead to the induction of *zic1, pax3*, and *msx1.*[98,99] Wnt and FGF8 activity can lead to the induction of *pax3.*[100] Wnt signaling induces *gbx2*, which then induces additional neural plate border specifiers, *msx1*, and *pax3.*[101] In Drosophila and mouse, intermediate levels of BMP have been shown to activate the *msx2* promoter.[102]

A study in chick has shown that *PAX7* is the earliest NCC marker and has been shown to be essential for the formation of NCC. Another study done in rabbit has shown that *Pax7* is the earliest NCC factor expressed at the neural plate border.[103,104] Studies done in *Xenopus* have demonstrated that *pax7* expression

is regulated by Wnt, FGF, and retinoid.[105] In chicken embryo experiments using beads that soaked in BMP or WNT inhibitors were placed on the prospective PAX7 region, and Pax7 expression was negatively affected.[106] In chicken embryos it has been shown that FGF/Erk signaling is necessary for the expression of PAX7.[107] Studies using chicken embryos have shown that *Pax7* is expressed upon binding of *cMyb* to a *Pax7* enhancer.[108]

The role of *pax3* and *pax7* have been shown to be different in *Xenopus* and amniote model systems. In *Xenopus* it has been proposed that *pax3* function influences NCC induction through its expression domain in the ectoderm and that *pax7* influences NCC induction via the paraxial mesoderm.[109] Studies in mice suggest that *Pax3* and *Pax7* play a redundant role in NCC formation.[110] In mice studies have also suggested that NCC formation can occur without the function of *Pax3* or *Pax7*.[111] Results taken from the varying model systems provide us with a strong understanding of how the signaling pathways work to induce the neural plate border transcription factors, but these results bring to light the issue of varying roles of the signaling pathways and neural plate border transcription factors amongst different species.

Transcription Factors Necessary for Neural Crest Cell Induction

Following the formation of the neural plate border, NCC specifiers are activated, which allows for induction of NCC. NCC specifier genes are activated in response to the spatial-temporal control of the previously discussed signaling

pathways and the neural plate border specifier genes. Amongst the functions of NCC specifiers is their role in the emergence and maintenance of NCC in an undifferentiated state.[112]

NCC specifiers include *SOX5, SOX8, SOX9*,*SOX10, ID* genes, *FOXD3*, *SNAI1, SNAI2, ETS1, TWIST* and others.[113,114,115]Additionally, genes such as *MYB* and *MYC* have been suggested to play a role in NCC formation.[116,117,118] *PAX3, PAX7*, and *TAP2A* are maintained from the neural plate border specifier genes through NCC formation. In chick, it has been suggested that *ETS1, SNAI1/2* and *FOXD3* are the first neural crest specifiers to come into play.[119]

Neural plate border specifiers *PAX3/7* and *MSX1* are directly responsible for the expression of F*OXD3* and *ETS*1 expression in chick embryos.[120] In *Xenopus* it has been demonstrated that *pax3/7* and *zic1* directly contribute to the emergence of *snail1/2* expression.[121,122] Studies done in lampray have demonstrated that when *pax3/7, ssx1* and *zic1* are knocked down *foxD* and *soxE1* expression is lost.[123] From these studies it can be postulated that *pax3/7, ssx1,* and *zic1* play a critical role in NCC induction and maintenance.[124]

It has been shown that *TFAP2A* acts in combination with nuclear receptors NR2F1 and NR2F2 to regulate neural crest specifiers. This study suggests that *TFAP2A*, NR2F1 and NR2F2 lead to a permissive chromatin state which allows for activation of neural crest genes.[125] Additionally, functional studies done in chicken, *Xenopus* and zebrafish embryos have shown that *TFap2A* is essential

for NCC specification.[126,127,128] In lampray, *ets1* has been shown to regulate foxD3, through a positive regulatory loop.[129] In *Xenopus twist, sox9, foxd3*, *snail1/2* appear to be positively regulated by *snail1/2*.[130] In mouse *FoxD3* has been shown to upregulate *Sox10* expression.[131] In *Xenopus*, *snail2* and *sox10* have been shown to be positively regulated by expression of *sox10*.[132] In *Xenopus sox8* has been shown to be essential for expression of other *soxE* genes.[133]

Id and cmyc have been found to be crucial in NCC maintenance by the regulating the genes necessary for the proliferation and differentiation. *Id* genes are downstream of *cmyc*, and downstream of the BMP signaling pathway.[134,135] In *Xenopus* it has been shown that when *id3* is knocked down, NCC formation is negatively effected.[136] Cmyc maintains *id* gene expression in pre-migratory NCC.

Other transcription factors such as *cMYB, ETS1*, and *GLI2* have been shown to regulate NCC specification. *CMYB* regulates *PAX7* and *SNAI2,*[137] in chick it has been shown that when *cMYB* is knocked down, *PAX7* and *TWIST* are downregulated.

Using *Xenopus* embryos it has been demonstrated that Notch signaling regulates twist1 express.[138] *Ets1* has been found to transduce the FGF/Erk pathway, as a downstream effector of the pathway. *Ets1* plays a regulatory role during NCC delamination.[139] *Gli2*, also a transcription factor, has been suggested to play a role in both neural plate border and neural crest genes,[140,141] its direct role

remains to defined. However, it has been shown that *gli2* effects the expression of genes such as *foxD3, pax3, msx1, zic1* and *snai2.*[142] Additional studies are needed to determine the direct roles of many of these transcription factors in regards to NCC formation. By the time NCCs are specified, premigratory markers such as *pax3/7, foxD3, snail2, sox8, sox9,* and *sox10* are expressed. Additionally, expression of select neural plate border genes are expressed, including *zic1, TFap2* and *msx1.*[143,144]

Neural Crest Cell Migration and Differentiation of Terminal Derivatives

Following delamination NCC assume a migratory character. Many neural crest specifier genes, including *Ets1, FoxD3*, and *Sox8/9/10* are maintained. Through cis regulatory analysis it has been shown that the NCC specifiers do not all act thorough the same mechanism.[145,146] *FoxD3* is regulated by *NC1*, which is an early enhancer, and it is active when the cranial neural tube first closes, but upon delamination of the NCC, *NC1* activity ceases.[147] During cell migration, the enhancer *NC2* maintains expression of *FoxD3*.[148] Although many of the same genes are active during the premigratory and migratory state of NCC, the mechanism of regulation varies.

Analysis of cis-regulatory genes involved in NCC migration suggests that expression of these transcription factors is controlled by the interaction of NCC specifiers with tissue-specific enhancer elements. In chick embryos it has been shown that *SOX10* expression during the migratory phase of NCC is mediated by

SOX10E2 in cranial NCC, and *SOX10E1* in trunk NCC.[149] NCC specifier genes *cMYB, SOX9* and *ETS1* are responsible for the regulation of the cranial enhancer, *SOX10E2. ETS1* is suggested to be a critical regulator in the development of cranial NCC, and has been shown to be responsible for NCC migration from the neural tube and provides the migration patterns seen in cranial NCC.[150]

In mice, cis-regulatory analysis has demonstrated involvement of NCC specifiers in the regulation of NCC migration, *PAX3/7*, *SOXE*, *TFAP2A*, and LEF/TCF transcription factors seem to play a regulatory role here.[151] It has been shown that *SOX10* regulates itself, in conjunction with *TFAP2a* and *FOXD3.*[152] In zebrafish *FOXD3* alongside an intronic enhancer in the *SOX10* locus, containing LEF/TCF, *SOXE/D*, and *FOXD3* binding sites.[153] In mice, *SOX9* has been shown to regulate itself through an upstream enhancer.[154] Cis-regulatory analysis has shown that many genes that are expressed in migratory NCC are self-regulating. It has been demonstrated that *SOX10* plays a critical and abundant role in the regulation of migratory NCC.[155] However, an abundance of information on the transcriptional activity underlying NCC migration remains to be explored.

Following cell migration, NCC undergo mesenchymal-to epithelial transition (MET), and give rise to numerous NCC derivatives which include bone of the craniofacial region, peripheral neurons and glia, cartilage, and melanocytes. Through functional analysis it has been shown that regulation of enhancers is

critical for the development of the craniofacial region, and aberration can lead to craniofacial malformations, which include cleft lip and palate.[156] For example, it has been shown that in mice with a *Pax7* null allele have craniofacial defects that can be linked to NCC dysregulation. In these mice, the heterozygous animals appear to be free of dysregulation of any sort and they are fertile; however, their homozygous counterparts, die within 3 weeks of birth. A common trait amongst all of the mutant animals in this study is that they all have defects of the craniofacial region, which include defects of the nasal tubules of serous glands, the maxilla and the nasal capsule.[157] In addition to control of transcription factor activity expressed by the migratory NCC themselves, environmental factor play a critical role. The first trimester of pregnancy is the most fragile, and the developing embryo is most susceptible to malformations, due to factors such as teratogens. Factors that lead to orofacial clefts include the use of tobacco and alcohol, weight, low zinc levels, and folic acid levels. Seven Genome Wide Association Studies (GWAS) have been carried out for cleft lip and palate, and two have been done for cleft palate alone.[158,159] About two dozen genes that are significant for cleft lip and plate have been identified, but only one has been identified for cleft palate alone, these genes include *PAX7, EPHA3, FOXE1, SPRY2, PAX9, TGFB3*, and N*OG* amongst others.[160] Both environmental factors and genetic factors can lead to NCC defects, including craniofacial defects.

Overview of EPH Receptors

The EPH receptor (erythropoietin-producing human hepatocellular receptors) family accounts for approximately a fourth of the receptor tyrosine kinases (RTK) superfamily, which has a kinase domain is in the transmembrane region and has a cytoplasmic region. EPH receptors and EFN ligands (EPH family receptor interacting proteins, also known as the ephrin ligands) are divided into two classes, class A which are membrane bound proteins and class B, which are transmembrane proteins. In the human genome there are nine EPHA receptors, and they bind to the five EFNA ligands, and there are five EPHB receptors which bind to the three EFNB ligands. Typically, class A ligands bind to class A receptors and class B ligands bind to class B receptors; however, the binding of these receptors has been found to be promiscuous.[161] EPHA4 can bind to the EFNB ligands and EPHB2 can bind to the EFNA ligands.[162] Spliced forms of multiple EPH receptors have also been found. [163]

The conserved receptor-binding domains in EFNA and EFNB are connected by a linker segment to the plasma membrane, the length of the linker segment can be altered through alternative splicing. A glycosylphosphatidylinositol (GPI) anchor attaches EFNA ligands to the cell surface, but they can be released and they can bind EPHA receptors, including those that are not on adjacent cells. EFNB are bound by a transmembrane segment and a short cytoplasmic region.[164]

EPH receptors and EFN ligands are involved in the development and organization of almost all tissues, including neuronal connections in the brain.

The expression of EPH receptors and EFN changes over time as the tissue develops; this typically occurs in combination with multiple EPH and EFN proteins. In many cells both EPH receptors and EFN are expressed in the same cell, in other cases they are exclusively expressed which allows for complementary expression gradients and activity.[165]

EPH receptors and EFN lignands are involved in many stages of development, which include the morphology of the cells, movement, adhesion, proliferation, survival and differentiation. As a result, EPH and EFN interactions play a crucial role during development, which includes spatial organization of cell populations, such as neural crest cells (NCC), the guidance of axons, angiogenesis, and neuronal synaptic connections. Additionally, they play key roles in adult homeostasis and maintenance, in areas such as the self-renewal of stem cells, bone remodeling, proper immune function, maintenance of proper synaptic, and epithelial turnover. Dysregulation of EPH receptor and EFN ligand activity can lead to diseases such as cancer and craniofacial malformations, amongst other developmental pathologies. Craniofacial malformations, include improper formation and closure of the oral lip and palate, which lead to pathologies known as craniofacial defects.[166,167]

Forward Signaling in EPH Receptors

Generally, RTK signaling is activated when a ligand binds to a receptor and the kinase domain is activated, this is known as forward signaling. Unlike most RTK, EPH-EFN interactions lead to both forward and reverse signaling.[168] Reverse

signaling is when a signaling cascade is initiated via the ligand. When the EFN and EPH receptors bind on juxtaposed cell surfaces there is oligomerization through the ligand and the receptor cis interfaces.[169,170] When there is EFN binding, EPH receptor clusters can also incorporate EPH receptors that are unbound to EFN.[171] Additionally, EPH receptor clustering can be effected by the manner in which it is interacting with the actin cytoskeleton.[172] Due to the promiscuous nature of EPH/ EFN interactions, clustering can include receptors of either class.

Depending on the proximity of clustered EPH receptors, trans-phosphorylation can also occur. Inhibitory trans-molecular interactions with the kinase domain can lead to inhibition. When two conserved tyrosine regions in the juxta-membrane domain are phosphorylated kinase activity is allowed to proceed. [173,174] In the EPH receptor family variation exist in key residues within the kinase domains, known as gatekeeper residues. The gatekeeper residues are responsible for allowing access to the hydrophobic pocket located adjacent to the ATP-binding site, between kinase domain lobes in the hinge region. In a majority of EPH receptors the gatekeeper is threonine with the exception of EPHA6 and EPHA7, in which it is valine and isoleucine, respectively.[175,176]

EPH receptor activation is responsible for the regulation of many of the same downstream targets as the other RTK family members. These adaptor and effector proteins include Nck and Crk and non-receptor tyrosine kinases of Src and Abl which are necessary for signal transduction. EPH receptor activation

allows for the recruitment of SH2 domain containing proteins that are downstream of the EPH receptor tyrosine auto-phosphorylation sites in Eph receptors are activated.[177] Additionally, when proteins which contain the PDZ domain, bind to carboxy-terminal tails on EPH receptors signaling is activated. Unlike most RTK families, EPH receptors can use central regulators such as Akt/mTORC1, and Rho and Ras family GTPase effectors to achieve cell repulsion and prevent cell growth.[178] An increased understanding in these interactions may allow for advancements towards therapeutics in areas such as suppression of tumors, and therapeutics for other neurocristopathies.

EPH receptor signaling can be inconsistent, largely due to alternatively spliced forms of EPH receptors, which lack particular kinase domains and as a result can lead to decreased clustering or they can illicit varying signals. For example, in the forming neural tube when a truncated membrane anchored variation of EPHA7 is present in the extracellular domain, instead of cell repulsion, cell-cell adhesion can be prompted. A truncated EPHA7 can bind to EPHA2 and it can suppress tumors in follicular lymphoma.[179] Depending on size of the EPH clusters, their ability to recruit signaling molecules can be affected. Positive and negative feedback loops can also play a role in the abilities of EPH receptors.

Reverse Signaling in Ephrin Ligands

Unlike most RTK families, in EPH-EFN signaling there is also reverse signaling which is signaling in the EFN expressing cells.[180] This occurs when the Src family kinases are activated, Src kinases phosphorylate EFNB when EPH receptors

bind. EFNB phosphorylation allows for the creation of SH2 binding sites for adaptors proteins including Grb4, which is responsible for axon pruning, formation of synapses, and in the hippocampus of the developing mouse it has been shown to be responsible for dendritic spine morphogenesis.[181,182] When the EPHB receptor binds to the ligand, the serine closest to the EFNB carboxyl terminus is phosphorylated, and the AMPA neurotransmitter receptor is stabilized.[183] With this taken together EFNB has been shown to play a role in the regulation of synaptic plasticity.[184]

The presence of PDZ domain containing proteins at the EFNB carboyl terminus is essential. Neuronal cell migration and neural progenitor self-renewal are controlled by the interaction between the adaptor protein PDZ-RGS3 and EFNB, by connecting EFNB to the G-protein coupled receptors.[185,186] Axon guidance and synaptic plasticity are regulated by EFNB and PDZ interactions, additionally, these interactions promote angiogenesis and lymphangiogenis through VEGF receptor endocytosis.[187] Studies in mouse have shown that neural migration is controlled through EFNB signaling, via crosstalk with Reelin, a secreted glycoprotein.[188] It also activates Rac1, which increases the ability of glioma cells to invade.[189]

It is not well understood how EFNA function to initiate intracellular signaling, as they do not have a cytoplasmic domain. It has been suggested that the p75 neurotrophin receptor, TrkB and Ret RTKs act as transmembrane binding partners for ephrin A, which then allows for ephrin A dependent reverse signaling

that takes place in axonal guidance and branching.[190,191] Also, EFNA2 reverse signaling has the ability to prevent neural progenitor cells from proliferating.[192] Glial EFNA3 alongside EPHA4 has been shown to uptake neurotransmitter glutamate which promotes synaptic plasticity, in the adult hippocampus.[193] Through the activation of Akt and Src family kinases, EFNA4 has been shown to prevent apoptosis in Jurkat immune cells.[194] Although, it is not well understood how EFNA functions to activate intracellular signaling, many studies have shown that they do play an essential role and should be further studied.

Role of EPH in Rho, Ras, mTor/AKT

EPH and Rho Interactions in Cell Regulation

A well-established role of EPH receptors is their regulatory effects on the actin cytoskeleton, which in turn plays a role in determining cell shape, cell-cell adhesion, and movement of the cell. Rho GTPases, including RhoA, Rac1, and Cdc42 are essential for cytoskeleton regulation.[195] GTPases can either be in an inactive state, where they are GDP bound, or they can be in an active state where they are GTP bound. The GTP state is when they are able to bind to downstream effectors. EPH receptors are able to regulate guanine nucleotide exchange factors and the hydrolysis of GTP to GDP. EPH receptors can play a regulatory role in GTPase-activating protein and guanine nucleotide exchange factors through tyrosine phosphorylation, ubiquitination induced degradation, EFN-induced association or through constitutive functions.[196]

RhoA is responsible for the formation of focal adhesions and stress fibers, and actomyosin cytoskeletal contraction. Rac1 and Cdc2 are responsible for lamellipodia and filopodia formation respectively.[197] When there is more RhoA activity present than Rac1/Cdc42 activity, it has been shown that this may contribute to the repulsion activity that is seen as an effect of EPH receptor forward signaling, which contributes to cell migration inhibition.[198,199] An example of repulsive effects resulting from EPHA receptors, through the Rho family activation and Rac1 inactivation are the collapse of dendritic spines and neuronal growth cones[200]. During gastrulation, it has also been shown that Eph repulsive signaling is involved in mesodermal and ectodermal tissue separation, through Rho family GTPases[201]. Amongst others it has also been shown to be necessary for segregation of astrocytes and Schwann cells in injured nerves.[202] EPH receptors can also regulate Rho family GTPases in manners other than repulsion. Through activation of Cdc442, EPHB activated by EFNB in stromal cells can increase HGF-dependent activity in metastatic PC3 prostate cancer cells.[203] To fully understand the mechanisms that describe EPH receptor function a lot more work is needed.

EPH and Ras Interactions in Cell Regulation

When the adaptors Grb and/or Shc are bound to an activated RTK H-Ras GTPase is activated by GEF Sos. Next Erk1 and Erk2 serine/threonine kinases are activated due to phosphorylation events that result from H-Ras-GTPas activation.[204] H-Ras is inactivated through p120Ras-GAP activation which leads

to inhibition of EPH receptor-dependent Erk activity.[205] Additionally, integrin activation needed for malignancy control and cell process retraction can be controlled via R-Ras, which can be inhibited by EPH receptors through p120RasGAP.[206] The Ras-Erk pathway is responsible for many physiological processes, including cell survival, proliferation, adhesion, migration and differentiation.

Ras-Erk pathway activation can be inhibited by EPH receptor forward signaling, even if it has been previously activated by other RTK. In ascidian embryos it has been shown that asymmetric division and fate determination results from EPH receptor activation in a polarized manner, through the reduction of FGF RTK induced Erk activation.[207]

Additionally, EPH receptors can also work to activate the Ras-Erk pathway. It has been shown that EPHB-EFNB1 interactions can work to activate Erk signaling in mouse mesenchymal cells, which can increase proliferative activity of the cells, and function to regulate early gene expression.[208] In MCF7 breast cancer cells, Erk1/2 activation by EFNB2 activation of EPHB4 occurs through PP2A serine/threonine phosphatase. EPHB2/EFNB1 signaling can be strengthened and EPHA2 gene transcription upregulated, through feedback loops in which Ras-Erk reciprocally effects EPH receptors.[209] Although, information on the effects EPH receptors on Rho GTPases and vice versa is available, the specific mechanism has yet to be identified.

EPH and AKT/mTOR Interactions in Cell Regulation

MTor is a downstream effector of the serine/threonine kinase, Akt. Akt plays a vital role in the survival, proliferation and the size of cells. Akt is activated through PI3, which is a lipid kinase, and activation of Akt results from the phosphorylation of T308 and S473.[210] However, Akt activation can be inhibited by EPH receptor forward signaling. In many cases mTORC1 can be inactivated, which leads to diminished cell migration and growth, this occurs in cancer cells when T308 and S473 are dephosphorylated though EphA2 activation.[211,212,213] In some cases, lung cancer cell migration and metastasis can be deactivated through inhibition of Akt through EphB3 activation.[214] In pancreatic cancer EFNA1 can bind with EPH receptors to activate Akt. Similar to Ras, Akt signaling can use feedback loops to reciprocally influence EPH receptors.[215] For example, S897 of EphA2 can be phosphorylated by Akt, on the other hand S897 can be dephosphorylated when by EFNA1 is activated.[216] More work is needed to determine exactly how EPH and AKT/mTor interactions work in regulating cell activity and function.

EFN Independent EPH Activity

EPH receptors can be activated in certain cases without EFN ligand binding. EPH receptors can be activated through other receptor systems and through cytoplasmic signaling molecules. When EPH receptors are activated through alternative routes, rather than through EFN ligand binding opposite effects can be seen. Studies have shown that EPHA2 is often upregulated in cancerous cells, and this occurs when EFNA is expressed at low levels or is unable to elicit

forward signaling from EPHA2 expressing cells.[217,218] When EPHA2 is overexpressed, even in the absence of an EFN binding partners, oncogenic genes can be activated.[219] The mechanism behind this is not fully understood, but studies have shown that Akt phosphorylates the S897 site on EPHA2.[220] Another study has shown that glioblastoma cells display an upregulation in invasive properties when the S897 on EPHA2 is phosphorylated, this happens when Akt is induced by extracellular Hsp90 binds to LRP1 receptors.[221] Interactions between EPHA2 and Ephexin4 increase activation of RhoG, which leads to the recruitment of ELMO2, which is a RhoG-GTP-binding protein, and of Rac-GEF DOCK4 binding to EPHA2, which leads to the activation of Rac1, and ultimately increases the invasive properties of cancer cells.[222] Another study has shown that when EPHB3 is overexpressed, lung cancer cells can become more tumorigenic, due to kinase independent mechanisms.[223] Further studies need to be done to increase understanding of how EFN and kinase independent activation of EPH receptors occurs.

Role of EPH-EFN Interactions in Embryogenesis

A lot of cell coordination is necessary in the developing embryo; one such aspect is the cell patterning that occurs during embryogenesis. EPH receptors play a large role in the distribution of cells that leads to the formation of these cell patterns. EPH receptors are present in all germ layers, and play a role in processes such as cell positioning and segregation in the formation of tissue boundaries. EPHB/EFNB complementary expression can lead to the segregation

of cells and creates a boundary between cells expression EPHB and EFNB.[224,225] Segregation and positioning due to complementary EFN and EPH protein expression takes place in numerous tissues, including hindbrain rhombomers and somites.[226] For example, in zebrafish, there are two sets of interactions that place, boundaries are formed at rhombomere boundaries, between ephA4 expressing cells and efnB expressing cells, but these cells also have adhesive interactions.[227]

In mammalian epithelial tissues, ADAM10-mediated cleavage and shedding of E-cadherins (adhesion proteins). This mechanism is initiated through EphB3 and EfnB1 interactions.[228] In mice, between epithelial cells EphB/EfnB interactions control cell positioning in the small intestine, along the crypt-villus axis. EphB2 and EphB3 expression is seen in the intervillus pockets of newborn mice, but expression of EphB proteins and complementary EfnB1 leads to segregation of these cells. In adults, proliferating progenitor cells express EphB2, and expression levels of EphB2 decrease closer to the top of the crypts.[229] EphB2 expression is present in stem cells towards the bottom of the crypt, and differentiated Paneth cells obtain EphB3 expression.[230,231] Abnormalities in EphB/EfnB signaling can cause dysregulation of cell proliferation and positioning in both embryonic and adult intestines. In the intestine Wnt signaling regulates expression of EphB-Efn. EphB2 and EphB3 are increased by Wnt.[232]

During development EPH/EFN interactions play a significant role in vascular remodeling. Functioning blood vessel systems are developed early on during the

formation of the embryo. Throughout embryogenesis blood vessels are remodeled and new ones are generated. Studies done in zebrafish embryos have shown that formation of the dorsal aorta (DA) and the cardinal vein (CV) involve ephB4 and efnB2 signaling.[233] Additionally, forming lymphatic vessels express efnB2 and ephB4. EphB4 activated efnB2 reverse signaling has been shown to be necessary for vascular sprouting from primitive capillary plexus through the PDZ-binding motif.[234]

Eph-efn signaling provides a balance between adhesive and repulsive forces during cell migration and other cell processes. These interactions permit controlled migration of cells, while inhibiting improper invasion of territories. EphB and efnB expression has been shown to affect how the mesoderm migrates over the ectoderm, during *Xenopus* gastrulation. Mesodermal cell detachment is initiated by ephB forward signaling.[235] On the other hand, efnA mediated attraction and adhesion through the ephA ectodomain is necessary for sensory axon guidance along the motor axons expressing ephA. EfnA expressing sensory axons progress along motor axons expressing ephA proteins, in the peripheral nervous system. When sensory efnA or motor ephA3/4 are not present there are repulsion events that take place, the mechanism by which this occurs is unknown.[236] Although, studies are making headway in explaining which eph-efn interactions and mechanisms are necessary in embryogenesis, many gaps in the information remain to be explored.

Role of EPH/EFN Interactions in Neural Crest Cell Formation and Migration

EPH/EFN interactions provide extracellular cues to NCC which allow them to migrate in a specific manner. Regulated NCC migration is critical for the migration patterns of the branchial arches for craniofacial development and for formation of the peripheral nervous system via migration of trunk crest. EPH/EFN interactions have been shown to play a regulatory role in NCC migration.[237]

In the vertebrate embryo NCC from the branchial arches follow a migration pattern that resembles a stream of cell migration.[238]

In chick embryos it has been shown that NCC migrate through the rostral half, while avoiding the caudal half of each somite, on their path to ventral portions of the embryo. These NCC go on to give rise to dorsal root and sympathetic ganglia. Spinal motor neuron axons are only projected through the somites on the rostral half. EPH/EFN play a key role in segmentation of trunk motor axons and segmentation of NCC, due to attractive signaling from the rostral portion of the somites and/or repulsive signals from the caudal portion of the somites.[239]

NCC migrate out from the brachial arches and differentiate into bone, cartilage, and ganglia of the cranial region. In the chicken embryo model system, NCC migrate in 3 waves, which leads to segmentation of the hindbrain. First, NCC migrate into the first branchial arch from midbrain rhombomeres r1 and r2, then they migrate into the second branchial arch from rhombomere r4, after which they migrate into the third and fourth branchial arches from rhombomere r6.[240] In the chicken embryo, NCC from the first branchial arch differentiate into skeletal

structures.[241] Although, migration of these cells is based on EPH-EFN interactions these cells acquire their positional identity, in part due to *HOX* genes.[242] In the case of the cells that differentiate into skeletal structures front the first branchial arch, they have acquired a rostorocaudal identity. In mouse it has been shown that when *Hoxa-2* is inactivated. Typically, *Hoxa-2* is expressed in the second branchial arch, but is not expressed in the first branchial arch. On the other hand, when Hoxa-2 is expressed in the first branchial arch, NCC taken from the second arch will migrate into the first branchial arch.[243,244] Taken together we can see that rhomobomere specific migration of NCC is necessary. EPH-EFN signals provide both repulsive and attractive cues. EFNB2 is present in the caudal half of somites, and EphB2 expression is present in migratory trunk NCC. In chick, EFNB1 is present in the caudal half of somites. Both in *vivo* and in *vitro* studies have shown that when EPH-FN signaling is blocked NCC do not migrate to the caudal somites.[245,246]

In *Xenopus* embryos it has been shown that efnB2 is expressed in the mesoderm and in the NCC of the second branchial arch. Efn interacts with ephA4 and ephB1, which are found in NCC in the third brachial arch and in mesoderm, this leads to repulsion of cells from neighboring branchial arches, which prevents them from invading other territories. For example, in NCC of the third branchial arch it has been shown that in the second branchial arch mesoderm expression of efnB2 prevents NCC from the third branchial arch from entering into second branchial arch regions; here, efnB provides repulsive cues.

Eph receptors and efn are expressed differently depending on tissue, but there is some overlap in these expression patterns.[247]

Traditionally studies look at the expression of eph-efn pairs in adjacent tissues; however, this does not hold true in every physiological case, often times many eph and efn are expressed by a single cell.[248] The presence of eph and efn on the same cell means that there is a possibility that cis signaling in addition to trans signaling is taking place on a given cell. Additionally, it means that there may be cross talk with other signaling pathways downstream of the eph-efn interactions. A study using *Xenopus* embryos shows that multiple eph and efn are expressed in the ectoderm-mesoderm boundary in the developing embryo.[249] It has been shown that there are direct interactions between eph and efn at the tissue interface, and this leads to transient Rho GTPase activation, and thus attachment and detachment events in a cyclic manner, through repulsion events due to cell contact. Antiparallel forward signaling at the tissue interface is required through efn mediated stimulation of eph in both tissues.[250] Interactions between eph and efn can be restricted to the boundaries of tissues, when there are multiple ligands and receptors present.

Boundary formation in embryogenesis results due to extracellular interactions, in which the receptors and ligands are semi-selective pairs in the network. Most of the molecules can interact with more than one partner, for example efnB2 can bind with all ligands.[251] For example, both ephrinB2 and ephrinB3 could interact with EphA4. An experiment where ephrinB3 and EphA4 interactions were studied

shows that when the receptors and ligands are switched for others that can bind in the same manner and maintain the same boundaries, separation of the tissues would still occur.[252] This study done during *Xenopus* gastrulation showed that efnB3/ephA4, efnB1/ephB2 and efnB2-ephB4 pairing could lead to this boundary formation, through the formation of antiparallel ligand-receptor boundaries. EphB receptors are expressed prior to gastrulation, and the dorsal ectoderm and mesoderm are established at the start of gastrulation. This separation begins to occur as soon as efnB2 expression begins in the mesoderm. Although most studies focus on the repulsive effects of eph/efn interactions, attractive forces may play an additional role. For example, ephA4/efnB2 interactions may be acting as adhesive forces, this warrants further studies.[253]

During the gastrula stage of *Xenopus,* 7 of the 8 efn and 11 of the 14 eph known to exist in Xenopus, are present. These eph/efn are involved in formation of the eye field,[254] somites,[255],the hind-brain,[256] notochord-presomitic mesoderm,[257] the ventral and dorsal ectoderm-mesoderm boundaries.[258] The exact timing for initiation and boundary formation is still unknown. In the notochord it is believed that they play a role throughout the maintenance of boundaries.[259] In somites, segmentation has been shown to go hand in hand with reorganization at the source of the boundaries. Some eph and efn have been shown to be maintained throughout, whereas others are transient.[260] The timing of eph/efn expression in hindbrain formation is not well characterized.

A study done in *Xenopus* has shown the expression of eph and efn range from the late blastula to early neurula. There is dynamic regulation in a majority of eph and efn. In addition to up/downregulation between germ layers, there are changes in the expression of eph and efn in dorsal and ventral specific activation and repression.[261] An example of this is that ephA2 is much higher on the dorsal side of the neurula. EfnB3 expression is significantly upregulated in both the gastrula and neurula stages. There is complementary distribution of ephB receptors in the ectoderm and ephA4 receptors in the mesoderm. Eph/efn expression in presomitic mesoderm largely resembles that in the early ectoderm. Studies done in chick embryos have shown that EphB3 and EphA4 are upregulated in the primitive streak, but they are downregulated in the mesoderm. EphrinB2 expression is upregulated upon ingression of the cells, and in the mesoderm this expression is maintained.[262]

Paraxial mesoderm segmentation takes place in an anterior to posterior manner. The position of the tissue is determined by EPH receptors and EFN before physical separation occurs.[263] EPHA4, EFNA1 and EFNB2 are present in the 4 available vertebrate models (zebrafish, *Xenopus*, chicken and mouse). EPHA4 and EFN in some models seems to be complementary, but in other cases they form opposite gradients, which suggests a model specific regulatory manner. EPHA4 is expressed during segmentation, others are continuously expressed, and the expression of additional ligands and receptors comes on when the somites begin to mature.[264] Here these receptors and ligands are responsible for

regulating processes such as NCC migration. EPHA3 expression is first upregulated at a posterior location in the segmenting somites and then in the anterior position.[265]

In the hindbrain EPH/EFN expression seems to be conserved in all 4 of the model systems. There are 7 rhombomeres/segments total, r3-5 show complementary patterns, but in r4-3 of the EFNB ligands are expressed, and r3 and r5 express high levels of EPH receptors. R1 and r7 are high in EFN expression, r2 and r6 have a mixture of EPH and EFN that are adjacently expressed. Upstream *H*OX genes are responsible for hindbrain segmentation. *Krox20* regulates the expression of EPHA4 in r3 and r5, *HOXA* and *HOXB1* regulate the expression of EPHA2, and *HOXB4* and *HOXD4* are responsible for the expression of *EPHA7.*[266] This suggests that the segmentation process is controlled by the activation and repression of EPH and EFN by *HOX*.

The role that EPH/EFN interactions play in boundary formation has been established; however, their additional roles must be studied. It is likely that EPH/EFN interactions also play parallel complementary and compensatory roles. It has been shown that EFNB2 can bind to EPHA4 and all of the EPHB. EFNB1 binds to selected EPHB, and EFNB3 binds to EPHB3 and EPHA4.

When there are large clusters of EPH and EFN binding, with high affinity, there is an adhesive force between the two cells that are in physical contact. For these adhesive forces to be disrupted proteolytic cleave of the EPH or EFN through

ADAM must take place or the EPH/EFN clusters must be endocytosed. The rationale behind why some EPH/EFN interactions are adhesive while others are repulsive is not clear, most likely the quantity of ligands and receptors present on the cell surface may play a key role in determining these forces. Less signaling is required for adhesive forces versus repulsive forces.[267,268]

A study done in zebrafish shows that when ephA4 is knockdown in the hindbrain, the depleted and the wildtype cells mix together in the rhombomeres that express efnB, but they avoid the rhombomeres expressing ephA4. The authors of this study concluded that ephA4 has a role that is not depended on efnB2; they hypothesized that ephA4 expressing cells have an adhesive affinity towards efnB2 expressing cells.[269] Another study showed that when cells were depleted of efnB2, the cells negative for efnB2 were repelled by cells expressing efnB2.[270] This study suggests that adhesive forces are at play, not just repulsive forces in eph-efn interactions, when these adhesive forces are taken away it leads to repulsion of cells. In *Xenopus* gastrula cells both overexpression and depletion of eph or efn lead to a decrease in the aggregation abilities of ectodermal cells. When eph/efn are present in a low quantity ectodermal cells displayed an adhesive nature, but if eph/efn interactions are depleted or overexpressed the cells become repulsive.[271]

Studies done in the chick, mouse and *Xenopus* model systems show that NCC of the cranial region use eph and efn receptors to provide migratory cues for the

cells. Each one of the branchial arches expresses different Eph receptor combinations, and EfnB1 and EfnB2 play opposing roles in the migration of NCC in mice. A study done in chicken embryos has shown that NCC expressing EPH receptors migrate along cells which express EFNB1 in the hindbrain. Also, cranial NCC in the avian embryo express EFNB2 and these cells migrate along cells which express EphB2.[272]

Although a lot of information has been generated on the role of EPH/EFN receptors in different cell types, including information on the migration and differentiation of NCC, the role of EPH and EFN in isolation and in conjunction remains to be studied. Furthermore, crosstalk between EPH, EFN and the classical signaling pathways known to be involved in NCC formation, such as BMP, WNT, FGF, and Notch/Delta remain to be studied in detail.

Oral Palate Differentiation: The Role of NCCs and EPH/EFN Interactions

NCC are responsible for the development of facial structures in vertebrates, as they give rise to craniofacial bone and cartilage. Due to the extensive role of NCC in the development of craniofacial structures, craniofacial defects are often times attributed NCC donysregulati. NCC development is composed of formation, migration and differentiation. Defects in the developmental process due to NCC defects are known as neurocristopathies. Approximately one-third of all birth defects are craniofacial syndromes of the head and neck, these defects include cleft lip and palate, or other improper development of the skull or facial region,

such as malformation of the teeth, eyes, ears or nose. Oral clefts occur in about 1 in every 700 live births. Clefts can occur in the primary palate, referring to the alveolar process and the lip, and/or they can occur in the secondary palate.[273] Current treatments involve surgeries beginning at 6 months of age and continue for a decade or longer without a guarantee of success.

It has been estimated by the Center for Disease Control and Prevention that it costs $697 million US dollars each year to treat children affected with clef lip and/or palate.[274] Despite these efforts and costs oftentimes the child still struggles with social, functional and aesthetic issues over their lifetime. The best strategies for fully corrective methods would be therapeutic options of prevention. Before this can be done, the normal events and mechanisms in which craniofacial formation takes place must be fully understood. An in *vitro* fusion model will allow for an effective, comparatively cheap, animal-free method to study fusion events. This model can be used to study dysregulation of EPH in the formation and fusion of the oral palate.

Currently, literature suggests that in the palate mesenchyme EphB2 and EphB3 serve as receptors for EfnB10. However, a study has shown that only 26% of ephEfnB2/LacZ allele homozygous mice result cleft palate.[275] This suggests that there are other Eph interactions that are involved in palate formation, one reason might be that there are 9 EphA receptors which bind to 5 EfnA ligands, and 5 EphB receptors which bind to 3 EfnB ligands. The interactions are promiscuous and some Eph/Efn of the other class can also bind,[276] this leaves the question

which Eph interactions are responsible for palatal fusion. This model could provide us with a controlled system in which to study the effects of Eph on oral palate formation and fusion.

A majority of the connective and skeletal tissues in the face are derived from cranial NCC. To form this intricate three-dimensional structure all steps of NCC induction and derivative formation must be temporally and spatially accurate. If these processes are not properly regulated diseases of the NCC, known as neurocristopathies arise[277]. As a result, genetic disorders or failures in NC processes are more likely to be evident in the craniofacial region.[278] A better understanding of the role of EPH in NCC induction may result in improved gene therapies, and the construction and integration of implantable tissues, such as the oral palate. This understanding may allow for the induction of NCC in large quantities, which would allow for making large quantities of the derivatives needed to make implantable tissues, and to construct a fusion model of the oral palate which would allow for an effective, comparatively cheap, animal free method to study oral palate fusion in *vitro*.

The Palatogenesis Process

Both mechanical and chemical cues play a role in the formation of the oral palate. The oral palate consists of the primary palate (anterior part of the palate and the upper lip), and the secondary palate (the hard roof of the mouth, which contains bone). Cranial NCC from the dorsal edge of the rostral neural tube occupy the facial prominences. As the nasal pits form the frontonasal

prominences divide into lateral and medial process. When the nostrils form resulting from fusion of the medial and lateral processes, the upper lip forms, dysregulation in this process lead to cleft lip.[279] The primary and secondary palate are formed from five facial prominences that arise from the tissue in the oral cavity. For primary palate formation, the frontonasal prominence and the two maxillary prominences fuse and form the lip, upper jaw and the anterior palate. Next, the secondary palate is established from the two outgrowths originating from the maxillary prominence. These outgrowths project out from the sides of the oral cavity and grow in a vertical manner that flanks the tongue. After elevation the shelves reposition to a horizontal orientation over the tongue, which leads to formation of the palatal shelves. The palatal shelves grow, and fuse at the midline, and the epithelial midline seam is formed. From an anterior direction, the secondary palate fuses with the primary palate, from an anterodorsal direction it fuses with the nasal septum, and thus the roof of the oral cavity is formed. In human embryos, palatogenesis begins at week 6 and by week 12 of gestation the palate is fused. In the mouse model, palatogenesis begins at embryonic day 11.5 and palatogenesis is complete by E17.[280,281,282] When dysregulation of these processes occurs, clefting of the oral palate is seen.
At the center of the palatal shelves is the neural crest-derived mesenchymal core, which is surrounded by an epithelial layer derived from the ectoderm. A basement membrane separates the mesenchyme and the epithelial layer. The epithelial cells have many adhesion molecules and extracellular matrix

proteins(ECM), which include hyaluronic acid, fibronectin, integrins, collagen and laminin.[283,284] Both the neural crest-derived mesenchyme and the epithelial cells proliferate as the palatal shelves grow. At the point of fusion, the mesenchyme has differentiated and the mesenchyme cells have become osteoblasts, which create the bone that segregates the roof of the mouth and the nasal cavity.[285] At this time the outer epithelial layer covering the medial surface where the palatal shelves fuse, has become a surface of single layer basal cells, and is known as the medial edge epithelium. This event occurs when the periderm (the layer on the outside) has become discarded and the MEE (medial edge epithelium) is no longer proliferating. In human embryos, at the point right before fusion, the palatal shelfs are 150-220 microns thick if the measurements are taken from the medial edge on the oral side to the nasal cavity.[286] The midline epithelial seam (MES) is formed when the two palatal shelves fuse, after which cells of the MES apoptosis. If the epithelial seam is unable to be removed, the mesenchyme is unable to merge, which can lead to the formation of cleft palate.[287]

There are numerous pathways that must be modulated a spatially and temporally controlled manner for proper palatogenesis to occur, such as BMP, FGF and TGF, and sonic hedgehog (SHH). These pathways are necessary for cell growth including proliferation and migration, differentiation into terminal derivatives. SHH and FGF10 are necessary for outgrowth of the palatal shelfs.[288] FGF10, and its downstream targets Jag2 and Irf6 are necessary for MEE differentiation prior to fusion of the palatal shelfs.[289] EFNB2 plays a role in MEE cells before the palatal

shelfs fuse, it is expressed at high levels in MEE. Following the point when the palatal shelves make contact and adhere, epithelial cells in the MES either apoptosis, migrate, or undergo mesenchymal transition, the exact mechanism is not yet known.[290]

Palatal fusion requires a proper balance of a number of pathways. In the embryo proliferation is controlled by a number of different pathways. Amongst which epidermal growth factor (EGF) is necessary for this stage of palatal development. In the mesenchyme and in the MEE both EGF receptor (EGFR) and EGF have been found to be present, before fusion in both rodent and human models.[291] EGF receptor levels go down and proliferation in the MEE stops, right before fusion. When levels of EGF are higher, fusion is hindered. In a study where a palatal organ culture was supplemented with EGF, proliferation of the MEE continued, preventing proper palatogenesis.[292,293] TGFB3, alongside EGF are involved in fusion of the palatal shelfs. When levels of EGF are higher than levels of TGFB3 palatal shelve fusion becomes delayed.[294]

Mouse is the typical model system that is used to study cleft palate. There are many similarities between mouse and human palatogensis, however there are differences in molecular signaling, and possibly in other aspects of palatal development, which leads to a need for a model of oral palate using human cells.

Due to technical and ethical reasons an in *vitro* model that is able to recapitulate human palatal development is necessary. A good model for this would be one in which the osteoblasts originate from a neural crest derived-mesenchyme.

For oral palate development, NCC must delaminate from the neural tube and migrate through the mesenchymal layer, and then contribute to the craniofacial region. Four weeks into human development, NCC have contributed to the formation of the mandibular processes, the maxillary process and the frontonasal prominence. By the end of week, the nasal placodes are formed, and the frontonasal prominence has been divided. By week 6, the primary palate has been formed. At the point, immediately prior to the completion of palatogenesis, the lateral nasal process is undergoing massive levels of cell division making it incredibly susceptible to teratogenic substances, which can lead to craniofacial pathologies such as cleft lip and cleft palate. Dysregulation in the developmental processes can be seen at week 6, which is when there is vertical growth of the palatal shelfs, or in week 7 when you get repositioning of the palatal shelves into a horizontal position and the shelves fuse. By the week 10 of embryogenesis palatal fusion should be completed.[295,296]

Illuminating which EPH are involved in oral palate formation and fusion may assist in the development of gene therapies for cleft palate. The development of an in *vitro* oral palate fusion model is key, as it will allow for an isolated, xeno-free, cost effective and efficient method. The role of EPH/EFN signaling in bone

remains characterized only in part. Throughout osteoblast development, EFNB1, EFNB2 and EPHB receptors are expressed, in osteoclast precursors they become more restricted.[297]

Proper bone homeostasis is necessary for proper bone growth, bone repair and the generation of new bone. Bone remodeling is an ongoing process where both resorption and formation are constantly taking place. When this fails to occur we get many disease of the bone which include osteoporosis and bone cancer.[298] EPH receptors and EFN ligands have been shown to play a key role in bone homeostasis; however, these roles remain poorly understood.

Osteoblasts are terminally differentiated bone cells, and they are able to undergo bone resorption. They are multi-nucleated cells that arise from osteoblasts. Osteoblasts arise from NCC derived mesenchymal stem cells, and are responsible for the formation of bone. *RANKL* and *OPG* are responsible for the differentiation of osteoblasts into osteoclasts.[299] BMP, TGF, and parathyroid hormone (PTH) are necessary for the differentiation of the mesenchymal stem cells into pre-osteoblasts.[300] *RUNX2* is one of the key transcription factors for osteoblast differentiation. The transcription factors, *ostrerix* acts downstream of *Runx2*, which studies have implicated as necessary for osteoblast differentiation over chondrocyte differentiation.

Initially osteoblasts will proliferate and differentiate, but then they start to condense and mineralize, during which time they are positive for alkaline phosphatase and they begin to secrete collagen and matrix proteins such as osteopontin and bone sialoprotein. These osteoblasts will eventually become osteocytes.[301]

EphB1 has been implicated in skeletal development. It was first shown in EfnB1 knockout mice, using Cre-lox. When EfnB1 was knocked down one of the defects seen were skeletal abnormalities, in the appendicular and the axial skeleton, these deformities included a shortened skull, asymmetric formation of ribs, and the occurrence of cleft palate amongst other deformities.[302] In EphB2/EphB3 double knockout mice sternebral fusions and asymptomatic rib pairing was seen. Skeletal defects were only seen in mice that had a double knockouts, indicating a redundant role of the two Eph.[303]

EfnB1 is necessary for bone mass and bone size. Using a Col1a2 promoter EphB1 was targeted in mesenchymal lineage cells. In this study knockout mice were created with a muted PDZ domain, which is necessary for reverse signaling in EfnB1 positive NCC. The knockout of EfnB1 in mice lead to abnormal bone formation.

In humans, EFNB1 abnormalities have been shown to be involved in craniofrontonasal syndrome, which is a x-linked maldevelopment, which can lead to the formation of asymmetric craniofacial structures and thoracic skeletal defects. EFNB1 has been linked to many cases of craniofrontosal syndrome, amongst which cleft palate is frequently occurring.[304]

EFNB2 has also been implicated in bone homeostasis. A study has shown that EFNB2 is a target of NFAT, and it is necessary during osteoclast differentiation. The EFNB2 protein is necessary during osteoclast differentiation, and is present in osteoclasts and in osteoblasts during differentiation. In *vitro* it has been shown in osteoclasts that both EPH receptors and EFN ligands are constitutively expressed. EphrinB2 reverse signaling has been shown to restrict the formation of osteoclasts.[305] However, in mice were there was no EfnB2 in osteoclasts, but osteoclast differentiation was not inhibited, most likely due to compensation by EfnB1. It has been shown that EfnB2 is able to interact with all of the EphB receptors, but for reverse signaling to take place through EfnB2, there must be an interaction with EphB4. Mice in which there was a double knockout of EphB2 and EphB3 showed stunted palatal shelf growth which lead to cleft palate. These studies showed that an increase in EphB4 expression could lead to the formation of bone, and it could inhibit bone resorption in a mouse model.[306,307,308]

Another study showed that for the proliferation of the mesenchyme via MAPK/ERK activation, forward signaling from EfnB1 was necessary. When EfnB1 was knocked out in these mice there was failure in the formation of the midline. This hints that EphB2/3 work as EfnB1 receptors in the palate mesenchyme. When the palatal shelves from the EphB2 LacZ/LacZ mice and the EphB3 -/- mice were placed in contact they were able to form a MES and they fused, suggesting that EphB3 signaling alone is not necessary for fusion events.[309]

EphB2 and EfnB2 were present in the MES right before and during the process of MES degradation, which suggests that EfnB2 signaling may be responsible, at least in part for EMT and fusion of the palate.

Another study found that when EPHB2/FC chimera proteins and anti-Fc were added together palatal fusion in chick would occur, regardless of the absence of TgfB3, which is normally found at the fusion site. EPHA4/Fc proteins, are able to fuse to EFNB, and act as competitive inhibitors which are able to block palatal chick fusion despite TgfB3 signaling. The same was true of mouse palatal shelves in EphA4/Fc treated mice. Ephin-B reverse signaling is crucial for palatal fusion.[310]

Our lab has recently reported a fast and efficient model of human NC (hNC) development based on induction of human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC).[311] Transcriptome analysis via RNA

sequencing in this model suggests significant changes in multiple EPH/EFN molecules throughout early hNCC development, and experiments with specific chemical inhibitors for EPHB3 further support the contribution of EPH/EFN signaling in early NCC development. An in *vitro* model to study the role of EPH in the fusion of the oral palate may aid in understanding and overcoming defects caused by abnormal EPH expressions and interactions. The in *vitro* model developed by our lab to mimic oral palate fusion provides us with a controlled system in which to study the effects EPH have on the oral. The in *vitro* model will provide an effective and cost-efficient way to study the role of EPH interactions in the formation and fusion of the oral palate. Work from this study may illuminate the role of EPH signaling in neurocristopathies, including facial clefts, improving our ability to diagnose, treat and manage these conditions.

Chapter 1: EPH/EFN Regulation is Essential for Neural Crest Cell Formation

Abstract

The neural crest is a multipotent population of stem cells, that gives rise to a variety of cell fates which include peripheral neurons and glia, connective tissue, cartilage, craniofacial bone and melanocytes. Neural crest cells (NCC) migrate widely during embryogenesis and malformations in NCC formation or migration can lead to a multitude of syndromes and birth defects, that are collectively referred to as neurocristopathies. NCC are responsible for the defining features of vertebrates, which include the jaw, and the predatory lifestyle of these vertebrates. Understanding, NCC is an essential part of fully understanding embryogenesis, development and consequently neurocristopathies. For proper NCC formation and migration singling must be tightly regulated. FGF and WNT signaling are required at the neural plate border to initially induce NCC progenitors, after which BMP and WNT signaling is required for the expression of definitive NCC markers. In addition to FGF, WNT signaling, and BMP, ephrin receptor (EPH) and ephrin ligand(EFN) signaling is imnvolved in cell fate determination and maintenance. Using RNA-sequence data I determined that expression of several EPH and EFN fluctuated throughout the NCC induction process, these EPH/EFN include EFNA2, EFNA5, EFNB1, EPHB2, and EPHA4. In this study I have examined the role of EPH and EFN signaling in NCC formation.

Introduction

Neural crest cells(NCC) are key for vertebrate development, they are a multipotent population of cells that forms and migrates extensively to give rise to a plethora of derivatives of the vertebrate body, which include connective tissue, melanocytes, peripheral neurons and glia, and craniofacial bone.[312] Due to the extensive contribution of NCC, dysregulation in the normal NCC development and migration processes lead to a vast number of abnormalities, collectively known as neurocristopathies.[313] These syndromes and birth defects include cancers such as neuroblastoma and melanoma, craniofacial malformations, and genetic conditions such as Waardenburg syndrome.[314] Craniofacial malformations, account for approximately one-third of all congenital birth defects, most specifically cleft lip and palate, which can be seen in about 1:700 live births in the United States[315]. Cleft lip and palate can occur either individually or in conjunction, and are characterized by improper formation or fusion of bones of the craniofacial region, and malformed teeth and facial characteristics.[316] To understand how these malformations occur we must start by gaining a deeper understanding of how normal NCC development takes place.

Crosstalk between several signaling pathways, which include WNT, FGF, and BMP is critical for NCC induction. These pathways play a critical role in the induction, progression and maintenance of NCC. Studies suggest that WNT modulates BMP signaling. In chick it has been shown that Wnt3a has the ability

to induce BMP expression in gastrula stage neural explants; however, when Wnt signaling in these explants was inhibited, BMP signaling was downregulated. Overall, these studies suggest that for NCC induction, inhibition of BMP and activation of Wnt is required for initial NCC induction, and activation of BMP by Wnt signaling is necessary for continued NCC specifiers to turn on at the neural plate border, so that NCC induction may continue. In *Xenopus*, zebrafish, and chick, FGF/MAPK signaling has been shown to modulate BMP signaling. Putting this information together we can determine that the neural crest gene regulatory network at the neural plate border, requires crosstalk between FGF and WNT, and these pathways modulate BMP signaling.

EPH receptors and EFN are able to communicate with other receptors, thus potentially effecting other pathways. EFN may be able to crosstalk with other RTK as well. An example of this which has been shown in *Xenopus,* the PDGF receptor can phosphorylate the efnB cytoplasmic domain. Additionally, in *Xenopus*, the activated FGF receptor phosphorylates efnB1. In *Xenopus* embryos, during cell dissociation, phosphorylation by the FGF receptors reverses the effects of efnB1. Another study also done in *Xenopus* suggests that the matrix metalloprotease ADAM13 upregulates wnt signaling activity by inhibiting efnB1 signaling. EPH and EFN signaling may play a key role in the regulation of WNT, FGF, and BMP signaling.

Studies done in the chick, mouse and *Xenopus* model systems have shown that NCC of the cranial region use EPH receptors and EFN ligands to provide migratory cues for the cells. Each one of the branchial arches expresses different Eph receptor combinations, and EfnB1 and EfnB2 play opposing roles in the migration of NCC in mice. A study done in chicken embryos has shown that NCC expressing EPH receptors migrate along cells which express EFNB1 in the hindbrain. Also, cranial NCC in the avian embryo express EFNB2 and these cells migrate along cells which express EPHB2.[317]

Due to these variations in EPH/EFN expression between species, to study the effects of EPH and EFN interactions with other signaling pathways in hNCC I used an in *vitro* model of NCC formation. Through the use of RNA sequence data I was able to determine that several EPH and EFN fluctuated significantly through the NCC induction process, which includes EFNA2, EFNA5, EFNB1, EPHB2, and EPHA4. Taking this information together with information from studies showing the involvement of these EPH and EFN in the migration of NCC, I decided to investigate the role of these EFN and EPH in early NCC formation.

Here I describe, for the first time, a study which focuses on the role of EPH receptors and EFN ligands during early NCC development, these studies use hNCC derived from hESC. This report demonstrates that EPH receptors and EFN ligands are dynamically expressed during hNCC formation. The study shows that inhibiting these EFN and EPH through the use of siRNA knockdown

strategies prevent NCC formation. Additionally, my data provides evidence suggesting that there is crosstalk between WNT signaling, FGF, BMP and EPH/EFN. When, EFNA2, EFNA5, EFNB1, EPHB2, and EPHA4 are knocked down WNt, FGF, and BMP signaling is dysregulated. Understanding the involvement of EPH and EFN in NCC formation can lead to advances in understanding embryogenesis and shed light on how dysregulation of these EPH and EFN lead to neurocristopathies. [318,319]

Results

To determine whether EPH receptors and EFN ligands are critical to NCC induction, I set out to determine which EPH receptors and EFN ligands are most upregulated in early NCC induction. I used a model of hNCC development based on the induction from hESC and induced pluripotent stem cells iPSC. This system relies on the use of GSK3 inhibition to activate canonical WNT/ß-catenin for the first 48 hours of induction in a 5-day protocol (Figure 1a). [320, 321] Using this system of NCC we can obtain an unlimited synchronized population of NCC.

Using RNA sequencing data, I was able to analyze the transcription profile of EPH and EFN levels present during the different time-points of NCC induction, which includes expression of EPHA/B and EFNA/B in hESC, and day 3/5 of NCC formation. Using RNA sequence data to look at the 14 EPH receptors that are known to be present in human cells, I found that *EPHA3, EPHA5*, and *EPHA6* were present a negligible level, and *EPHA10* levels were low relative to other

receptors, although not negligible. *EPHA1, EPHB4,* and *EPHB6* were highest in ESC. *EPHA2, EPHA7, EPHB1* were highest at day 5. RNA sequence from the cells collected at day 3 shows that *EPHA4, EPHB2* and *EPHB3* are highest, which made them the targets of interest (Figure 1b). Next, I looked at the RNA sequence data to determine which of the 8 EFN ligands are key for early NCC induction, and found that *EFNA1* was present at the highest levels in hESC and the presence of *EFNA1* and *EFNA2* decreased as NCC induction progressed to day 3 of induction. The RNA sequence data shows that *EFNA2* and *EFNA5* levels are increased by day 3 relative to hESC and this increase is maintained through day 5, indicating that they are critical in early NCC formation and in maintaining the NCC fate. *EFNB1* expression is robustly upregulated from the hESC state, and falls towards the end of induction at day 5, indicating that it might be most necessary in early NCC induction. *EFNA4* is robustly expressed in hESCs, and expression increased slightly by day 3, but falls back to the same levels as are expressed in hESC by day 5. *EFNB2* and *EFNB3* expression are lowest in hESC. The data shows an increase by day 3 of NCC induction, but expression is highest at day 5 of NCC induction. Taking this data together, I determined that *EPHA4, EPHB2*, *EPHB3* (data and extensive experiments are shown in chapter 2 for *EPHB3*)*, EFNA2, EFNA5,* and *EFNB1,* are key targets to study early NCC induction.

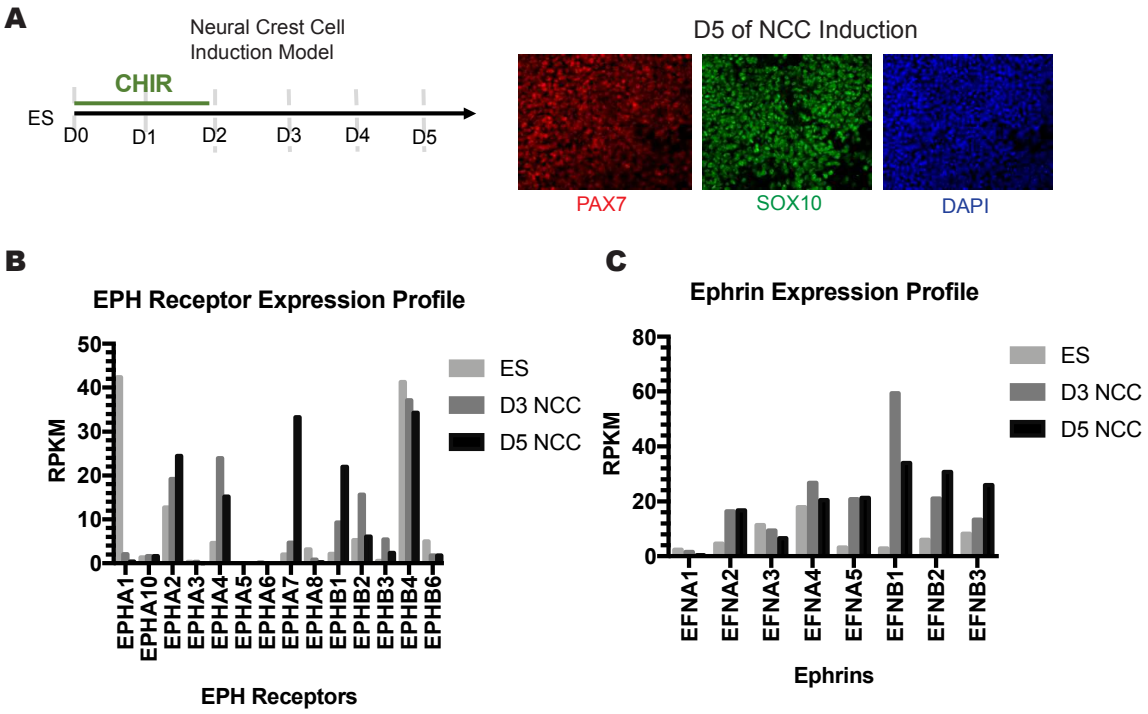


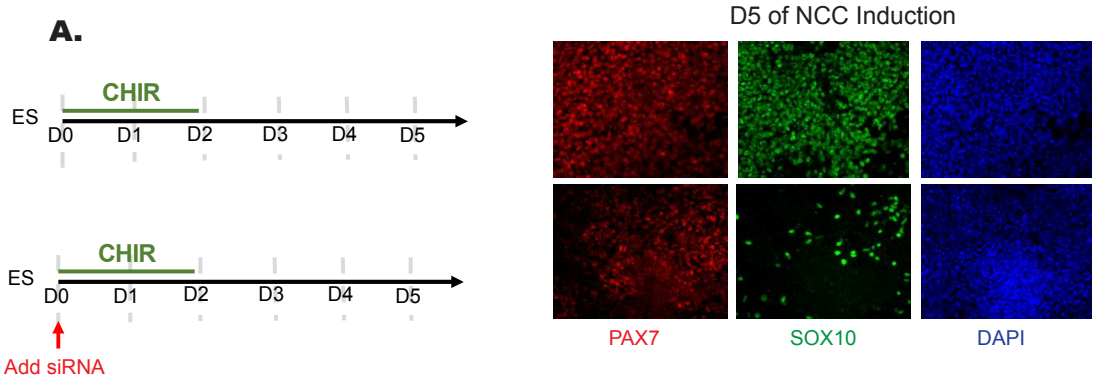
Figure 1: Expression of EPH During NCC Induction

A) Schematic showing the 5-day NCC induction protocol which uses a 2-day exposure to the small molecule CHIR99021. A panel is shown of *PAX7* (red), *SOX10* (green) and the nuclear stain, DAPI (blue).
B) RNA sequence data shows the expression profile of EPHA and EPHB, measured in RPKM in ES, 3 day NCC and 5 day NCC.
C) RNA sequence shows the expression profile of EFNA and EFNB, measured in RPKM in ES, 3 day NCC and 5 day NCC.

EFNA2 knockdown studies show that the knockdown of EFNA2 ultimately leads to an inability of the hESC to form NCC. Using immunofluorescence to compare *PAX7* and *SOX10* expression in the treated condition and untreated conditions, it is shown that *PAX7* and *SOX10* expression are diminished relative to the control condition on cells collected at D5 of the NCC induction (Figure 2A). Next using a Nikon elements software for automated cell counting, cells counts are used to quantify the presence of *PAX7* and *SOX10* positive cells and the results show that they are reduced to 12% and 10% respectively (Figure 2B), the DAPI cell counts are unchanged (data not shown). On samples collected at D5, through qPCR analysis we show that *FOXD3* (-0.55), *MSX1*(-0.33), *PAX3*(-0.13), *PAX7* (-0.17), *SNAI2* (-0.30), *TFAP2* (-0.84) and *ZIC1*(-0.20) are decreased (Figure 5C). The data shows that NCC induction is inhibited through the use of siRNA to knockdown EFNA2, at both the transcriptional and translational level.

Work done in various model systems, including *Xenopus*, chick, mouse, and hESC based NCC models, provides evidence that WNT, BMP, FGF, play a role NCC induction.[322] Interactions between WNT signaling and FGF have been shown to play a role in the formation of the neural plate border.[323,324,325]. After which BMP and WNT are crucial in the activation and maintenance of transcription factors which are required for the specification of NCC.[326,327,328] Taking this into consideration I ran qPCR for direct targets of WNT, which include *AXIN2, SP5, DKK1, LEF1*, and *c-MYC.*[329,330] Using siRNA, the data shows a reduction in the early NCC cell markers *PAX7* (-0.43) *ZEB2* (0.29), and *ZIC3*

(0.19) (Figure 5D) in the qPCR data on samples collected at day 2. At day 2, there is a reduction in *AXIN2*(-0.22), *DKK1*(-0.18), *LEF1*(-0.19) and *c-MYC* (-0.15), and SP5 (0.07) (Figure 4E). Next, I assessed *DUSP6, SPRY1*, and *SPRY2,* which are targets of FGF.[331,332] These results show that *DUSP6* (0.17) was increased*,* but *SPRY1*(-0.25) was decreased, and *SPRY2* (-0.02) are almost unaltered*,* suggesting that overall there is dysregulation of FGF. Lastly, I looked at targets of BMP, which include *Id1, Id3, Klf2* and *Klf5*.[333] These results show that *Id1*(-0.1) is slightly decreased*, Id3* (0.51) and *Klf2* (0.52) are increased, and *Klf5* (0.00) is not affected, which overall demonstrates alterations in BMP activity due to EFNA2 knockdown. Results show dysregulation in WNT, BMP and FGF activity resulting from EFNA2 knockdown. Overall, through the use of siRNA to knockdown EFNA2 I found that NCC formation was inhibited. This suggests that there is crosstalk between EFNA2 and other signaling pathways, in this case WNT, BMP, and FGF.


A.
CHIR
ES
D0
D1
D2
D3
D4
D5
CHIR
ES
D0
D1
D2
D3
D4
D5
Add siRNA
D5 of NCC Induction
PAX7
SOX10
DAPI

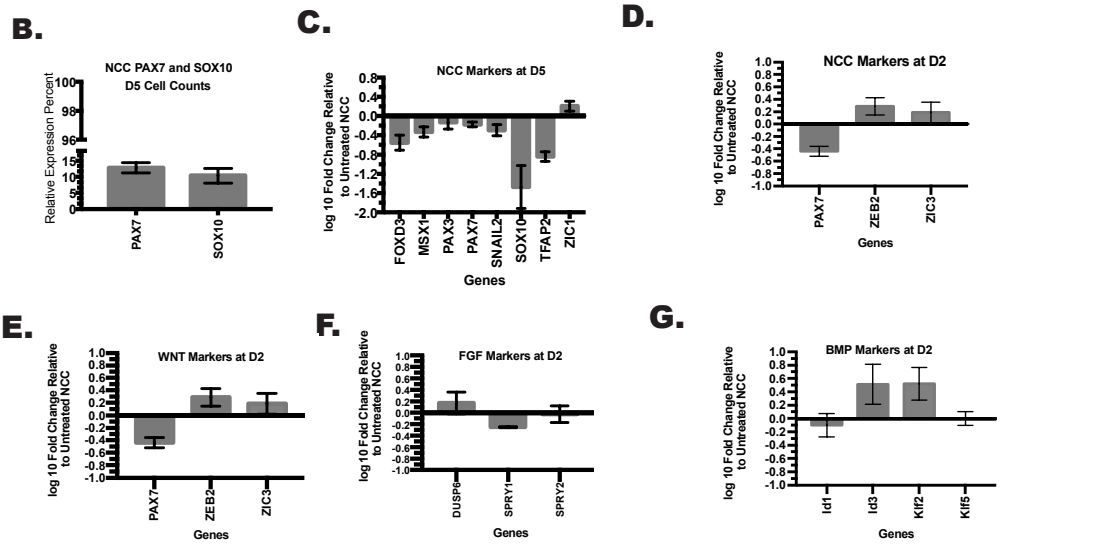

B.
NCC PAX7 and SOX10
D5 Cell Counts
Relative Expression Percent
PAX7
SOX10
C.
NCC Markers at D5
log 10 Fold Change Relative to Untreated NCC
FOXD3
MSX1
PAX3
PAX7
SNAIL2
SOX10
TFAP2
ZIC1
Genes
D.
NCC Markers at D2
log 10 Fold Change Relative to Untreated NCC
PAX7
ZEB2
ZIC3
Genes
E.
WNT Markers at D2
log 10 Fold Change Relative to Untreated NCC
PAX7
ZEB2
ZIC3
Genes
F.
FGF Markers at D2
log 10 Fold Change Relative to Untreated NCC
DUSP6
SPRY1
SPRY2
Genes
G.
BMP Markers at D2
log 10 Fold Change Relative to Untreated NCC
Id1
Id3
Klf2
Klf5
Genes

Figure 2: NCC Induction is Inhibited by siRNA Knockdown of EFNA2

A) A diagram demonstrating the results of NCC induction with and without the addition of siRNA. The first row depicts a control NCC induction done using a scrambled siRNA, the second row shows a NCC induction carried out with the addition of siRNA to knockdown EPHB3. Immunofluorescent images show expression of NCC markers *PAX7*(red), *SOX10*(green) and the nuclear stain, DAPI(blue).
B) Cell count data showing the expression of PAX7 and SOX10 markers in cells treated with siRNA, relative to a control NCC.
C) QPCR data presenting changes in fold change of NCC markers (*FOXD3, MSX1, PAX3, PAX7, SNAIL2, SOX10, TFAP2,* and *ZIC1*). Cells were collected at day 5 of the induction.
D) QPCR data showing changes in early NCC markers (*PAX7, ZEB2*, and *ZIC3*) at day 2 of collection. The gene fold is determined relative to D2 collection of untreated NCC.
E) QPCR data depicting changes in WNT targets (*AXIN2, SP5, LEF1*, and *c-MYC*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.
F) qPCR data presenting variations in FGF targets (*DUSP6, SPRY1*, and *SPRY2*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.
G) qPCR data showing alterations in BMP targets (*Id1, Id3, Klf2*, and *Klf5*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.

Knockdown of EFNA5 through the use of siRNA targeting EFNA5, leads to diminished formation of NCC marker expression. Immunofluorescence data shows that NCC collected and analyzed at D5, show diminished expression of P*AX7* and *SOX10* expression in the EFNA5 knockdown (Figure 3A). Cell counts demonstrate that *PAX7* and *SOX10* expression is reduced to 31.2% and 12.9% respectively (Figure 2B), DAPI cell counts remain unchanged (data not shown). QPCR analysis of day 5 samples shows that *FOXD3* (-1.12), *PAX3*(-0.51), *PAX7* (-0.82), *SNAI2* (-0.84), *SOX10*(-1.71) are decreased, where *TFAP2* (0.24) and *ZIC1*(0.63) are increased and *MSX1*(0.00) is unaffected (Figure 5C). The data suggests that knockdown of EFNA5 leads to alterations in NCC genes, which results in a diminished NCC population. Using siRNA, the data shows a reduction in the early NCC cell markers *PAX7* (-1.12), and *ZIC3* (-0.51), but *ZEB2* (0.00) was unaffected (Figure 5D) in the qPCR data from samples collected at day 2. At day two there is a reduction in *AXIN2*(-0.22), *DKK1*(-0.18), *LEF1*(-0.19) and *c-MYC* (-0.15), but SP5 (0.07) is largely unaffected (Figure 4E). Analyzing FGF targets, *DUSP6, SPRY1*, and *SPRY2*[334,335] results show that *DUSP6* (-0.1) is decrease; however, *SPRY1*(-0.03) and *SPRY2* (-0.06) are almost unaltered. Looking at targets of BMP, which include *Id1, Id3, Klf2* and *Klf5,*[336] I find that *Id1*(-0.14) is slightly decreased*, Id3* (0.39) and *Klf2* (0.71) are increased, and *Klf5* (-0.05) is unaffected. The results demonstrate a dysregulation in WNT, BMP and FGF activity due to the knockdown of EFNA5, which prevents NCC formation, mostly likely due to crosstalk between EFNA5 and other signaling pathways.

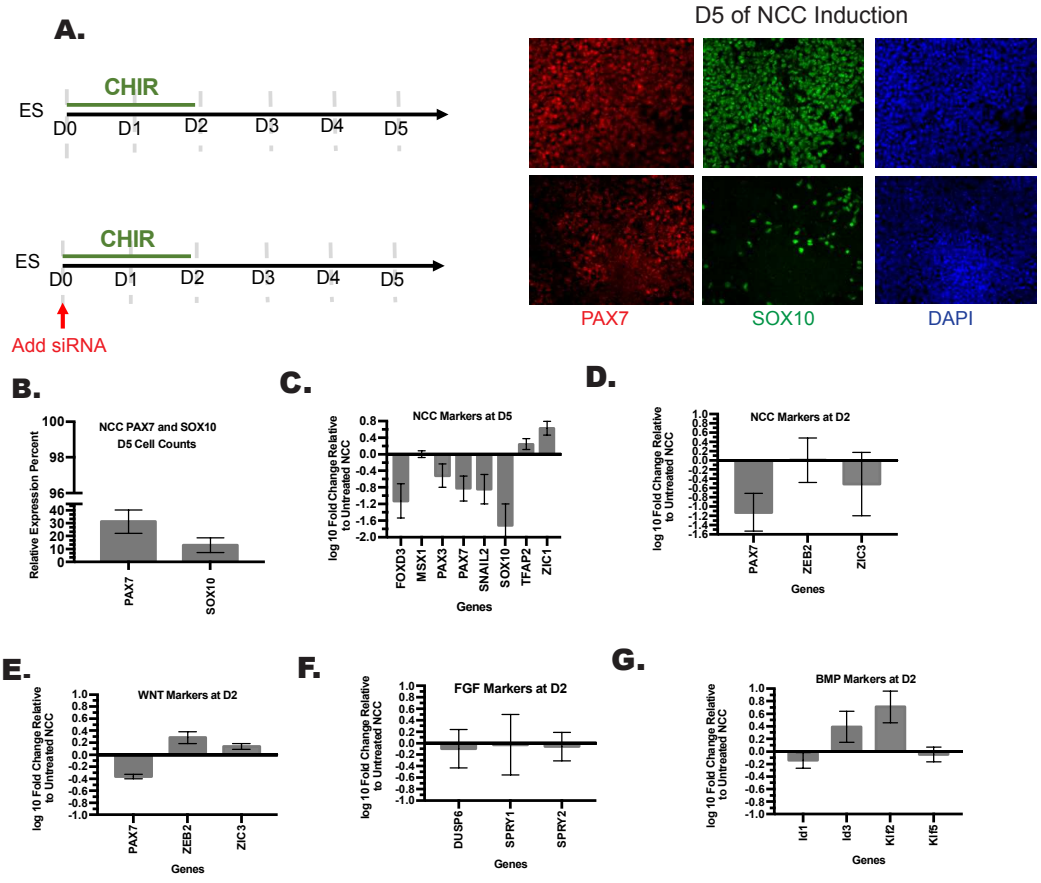

A.
ES
CHIR
D0
D1
D2
D3
D4
D5
Add siRNA
D5 of NCC Induction
PAX7
SOX10
DAPI
B.
NCC PAX7 and SOX10
D5 Cell Counts
Relative Expression Percent
PAX7
SOX10
C.
NCC Markers at D5
log 10 Fold Change Relative to Untreated NCC
FOXD3
MSX1
PAX3
PAX7
SNAIL2
SOX10
TFAP2
ZIC1
Genes
D.
NCC Markers at D2
log 10 Fold Change Relative to Untreated NCC
PAX7
ZEB2
ZIC3
Genes
E.
WNT Markers at D2
log 10 Fold Change Relative to Untreated NCC
PAX7
ZEB2
ZIC3
Genes
F.
FGF Markers at D2
log 10 Fold Change Relative to Untreated NCC
DUSP6
SPRY1
SPRY2
Genes
G.
BMP Markers at D2
log 10 Fold Change Relative to Untreated NCC
Id1
Id3
Klf2
Klf5
Genes

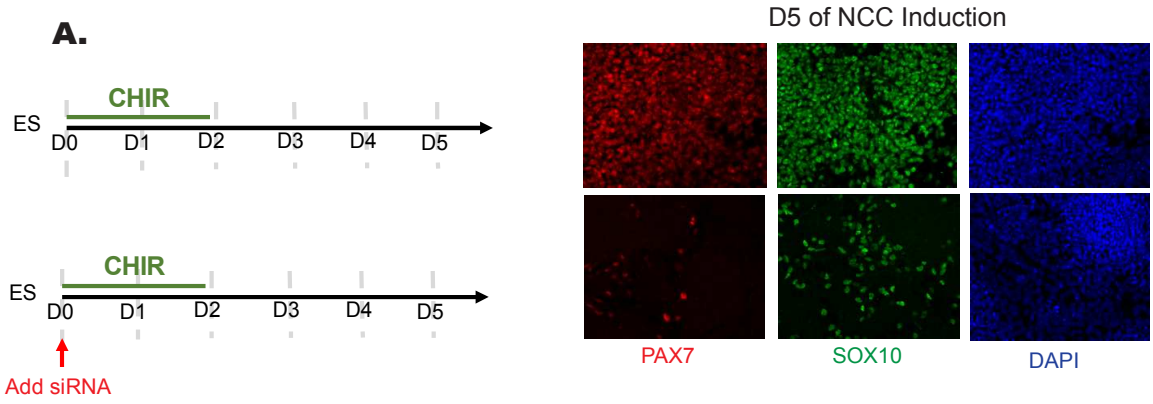
Figure 3: NCC Induction is Inhibited by siRNA Knockdown of EFNA5

A) A schematic demonstrating NCC induction with and without the addition of siRNA. The first row shows a control induction done a scrambled siRNA, the second row shows a NCC induction carried out with the addition of siRNA to knockdown EPHB3. Immunofluorescent images showing expression of NCC markers *PAX7*(red), *SOX10*(green) and the nuclear stain DAPI(blue).
B) Cell count data showing the expression of PAX7 and SOX10 expression in cells treated with siRNA, relative to a control NCC.
C) QPCR data presenting changes in fold change of NCC markers (*FOXD3, MSX1, PAX3, PAX7, SNAIL2, SOX10, TFAP2,* and *ZIC1*), for cells collected at day 5 of the induction.
D) QPCR data showing changes in early neural crest markers (*PAX7, ZEB2*, and *ZIC3*) at day 2 of collection. The gene fold is determined relative to D2 collection untreated NCC.
E) QPCR data depicting changes in WNT targets (*AXIN2, SP5, LEF1*, and *c-MYC*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.
F) qPCR data presenting variations in FGF targets (*DUSP6, SPRY1*, and *SPRY2*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.
G) qPCR data showing alterations in BMP targets (*Id1, Id3, Klf2*, and *Klf5*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.

Next using siRNA to knockdown the gene expression of EFNB1, immunofluorescence comparing *PAX7* and *SOX10* expression in the treated condition (EFNB1 knockdown) and untreated conditions demonstrates that *PAX7* and *SOX10* expression are diminished relative to the control condition on cells collected at D5 of the NCC induction (Figure 3A). Data from cell counts shows that the expression of *PAX7* and *SOX10* positive cells is reduced to 41.2% and 23.9% respectively (Figure 2B), again DAPI cell count are unaltered (data not shown). Day 5 samples show that *FOXD3* (-0.74), *PAX3*(-0.32), *PAX7* (-0.59), *SNAI2* (-0.51), *SOX10*(-1.15) expression is decreased, where *TFAP2* (0.14), *MSX1*(0.33), and *ZIC1*(0.37) are increased (Figure 5C). The data shows that NCC induction is inhibited through EFNB1 knockdown, and this can be seen at both the transcriptional and translational level.

Next I went on to look at the effects on early NCC induction, and analyzed cells collected at D2 for early NCC markers, and for direct targets and downstream targets of the WNT pathway, BMP and FGF using qPCR. The data shows a reduction in the early NCC cell markers *PAX7* (-0.74), *ZEB2* (0.33), and *ZIC3* (-0.32) (Figure 3D). WNT pathway targets show that there is a slight reduction in *AXIN2*(-0.10), but *DKK1*(-0.02), *LEF1*(-0.05) and *c-MYC* (-0.01), and SP5 (0.08) are largely unaltered (Figure 4E). Results from the targets of FGF show that *DUSP6* (-0.14) and *SPRY1*(-0.21) are decreased, but *SPRY2* (-0.05) is almost unaltered, suggesting an overall dysregulation of FGF signaling. Data from the BMP targets shows that *Id1*(-0.25) and *Klf5* (-0.27) are decreased, *Klf2* (0.34) is

increased, *Id3* (-0.05) and is not notably affected; overall, results suggest that there is dysregulation in the WNT pathway, BMP and FGF activity when EFNA5 is knocked down. Overall, through the use of siRNA to knockdown EFNB1 I found that NCC formation was inhibited, potentially resulting from a combination of the EFNB1 knockdown itself and through crosstalk between EFNB1 and other signaling pathways.


A.
CHIR
ES
D0
D1
D2
D3
D4
D5
CHIR
ES
D0
D1
D2
D3
D4
D5
Add siRNA
D5 of NCC Induction
PAX7
SOX10
DAPI

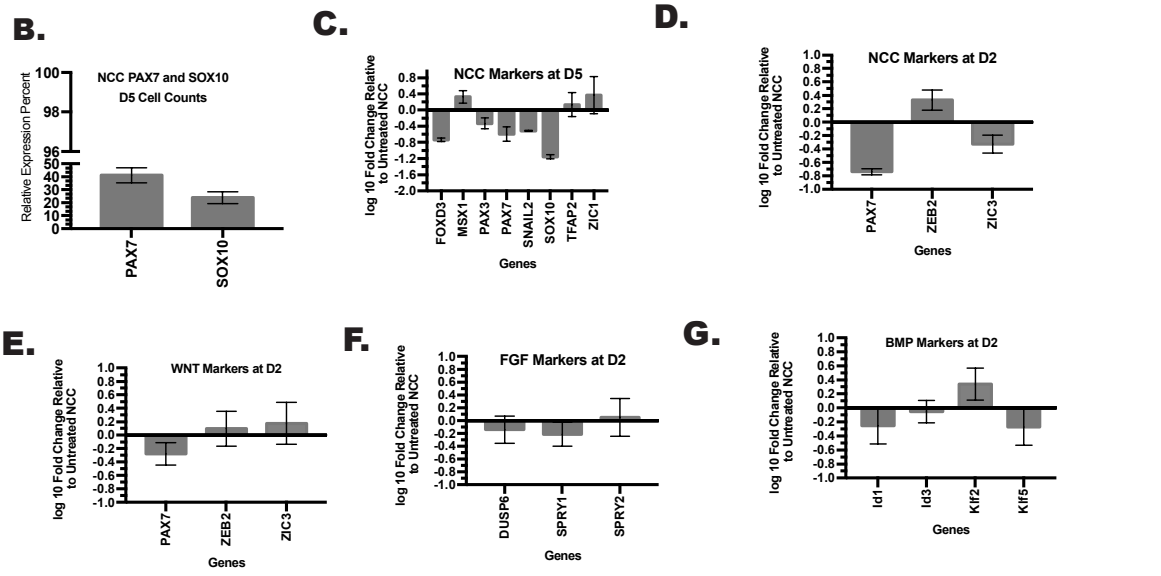

B.
NCC PAX7 and SOX10
D5 Cell Counts
Relative Expression Percent
PAX7
SOX10
C.
NCC Markers at D5
log 10 Fold Change Relative to Untreated NCC
FOXD3
MSX1
PAX3
PAX7
SNAIL2
SOX10
TFAP2
ZIC1
Genes
D.
NCC Markers at D2
log 10 Fold Change Relative to Untreated NCC
PAX7
ZEB2
ZIC3
Genes
E.
WNT Markers at D2
log 10 Fold Change Relative to Untreated NCC
PAX7
ZEB2
ZIC3
Genes
F.
FGF Markers at D2
log 10 Fold Change Relative to Untreated NCC
DUSP6
SPRY1
SPRY2
Genes
G.
BMP Markers at D2
log 10 Fold Change Relative to Untreated NCC
Id1
Id3
Klf2
Klf5
Genes

Figure 4: NCC Induction is Inhibited by siRNA Knockdown of EFNB1

A) A diagram showing a NCC induction with and without the addition of siRNA. The first row shows a control induction done a scrambled siRNA, the second row shows a NC induction carried out with the addition of siRNA to knockdown EPHB3. Immunofluorescent images showing expression of neural crest markers *PAX7*(red), *SOX10*(green) and DAPI(blue).
B) Cell count data showing the expression of PAX7 and SOX10 expression in cells treated with siRNA, relative to a control NCCs.
C) QPCR data presenting changes in fold change of neural crest markers (*FOXD3, MSX1, PAX3, PAX7, SNAIL2, SOX10, TFAP2,* and *ZIC1*). Cells were collected at day 5 of the induction.
D) QPCR data showing changes in early neural crest markers (*PAX7, ZEB2*, and *ZIC3*) at day 2 of collection. The gene fold is determined relative to D2 collection untreated NCC.
E) QPCR data depicting changes in WNT targets (*AXIN2, SP5, LEF1*, and *c-MYC*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.
F) qPCR data presenting variations in FGF targets (*DUSP6, SPRY1*, and *SPRY2*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.
G) qPCR data showing alterations in BMP targets (*Id1, Id3, Klf2*, and *Klf5*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.

Next I went on to investigate the effects of EPHB2 knockdown in the NCC induction process. Immunofluorescence data shows that *PAX7* and *SOX10* expression in the EPHB2 knockdown conditions versus and untreated conditions, is greatly reduced at D5 of the NCC induction to 12.0% and 21.3%, respectively (Figure 4B), DAPI cell counts are maintained (data not shown). QPCR analysis carried out on day 5 of the induction protocol shows that *FOXD3* (-0.78), *PAX3*(-0.28), *PAX7* (-0.57), *SNAI2* (-0.54), *SOX10*(-1.18) are all decreased, but *MSX1*(0.26), *TFAP2* (0.23) and *ZIC1*(0.70) are increased (Figure 5C). EPHB2 knockdown leads to NCC induction inhibition as is demonstrated both at the transcriptional and translational levels.

Next, I assessed the effects of EPHB2 knockdown on early NCC genes, WNT pathway targets, followed by targets of FGF and BMP at day 2. The data shows a reduction in the early NCC markers *PAX7* (-0.11) and *ZIC3* (-0.27), but *ZEB2* (0.01) expression is unchanged (Figure 3D). Data from WNT pathway targets shows a slight increase in *LEF1*(0.1) and SP5 (0.15), but *AXIN2*(-0.03), *DKK1*(0.02), and *c-MYC* (-0.06) expression is mostly, unaltered (Figure 4E). Next, the effects of EPHB2 on FGF are explored and the data shows that *DUSP6* (0.26) and *SPRY2* (0.34) are increased*,* but *SPRY1*(0.00) is unaltered*.* Lastly, results from targets of BMP show that *Id1*(0.22)*, Id3* (0.43) and *Klf2* (0.10) are increased, but *Klf5* (0.00) is not affected. Comprehensively, the data shows a dysregulation in WNT, BMP and FGF activity when EPHB2 is knocked down, leading to NCC formation inhibition.

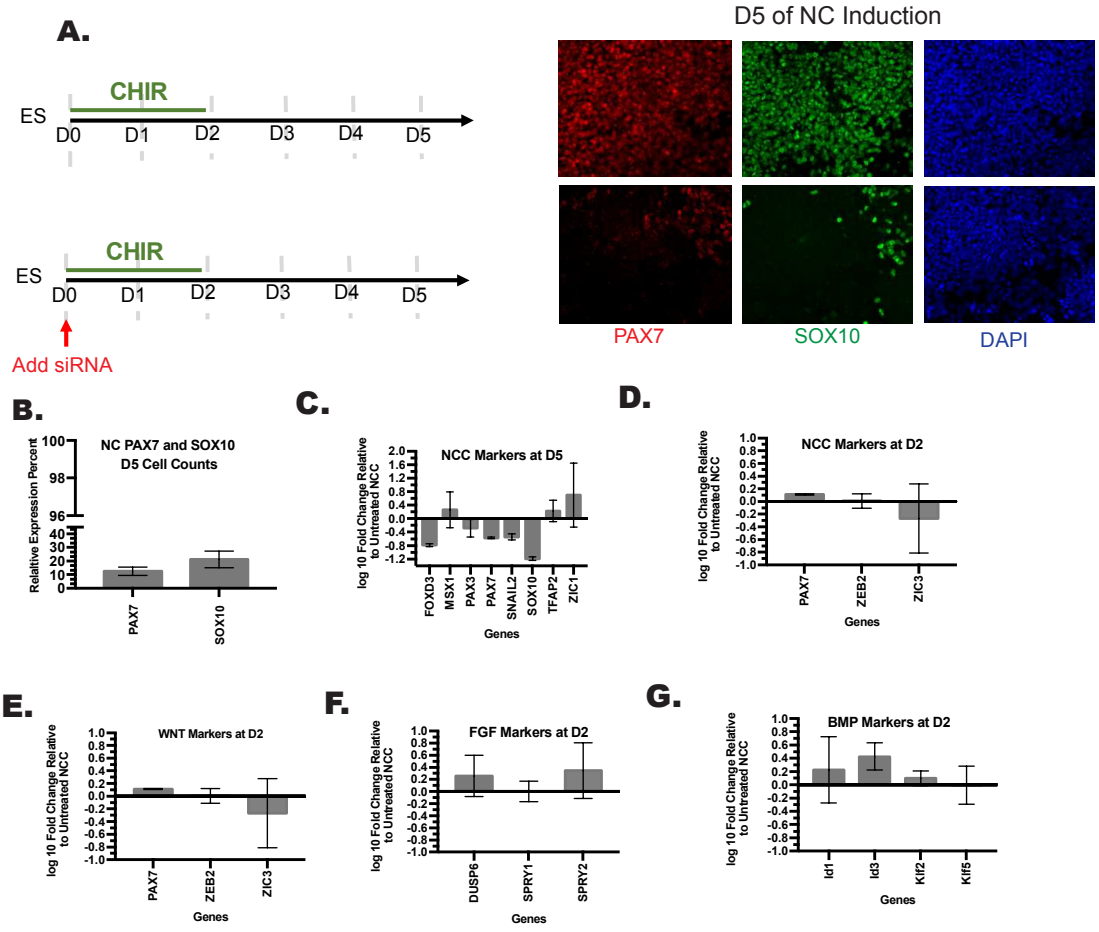

A.
CHIR
ES
D0
D1
D2
D3
D4
D5
CHIR
ES
D0
D1
D2
D3
D4
D5
Add siRNA
D5 of NC Induction
PAX7
SOX10
DAPI
B.
NC PAX7 and SOX10
D5 Cell Counts
Relative Expression Percent
PAX7
SOX10
C.
NCC Markers at D5
log 10 Fold Change Relative to Untreated NCC
FOXD3
MSX1
PAX3
PAX7
SNAIL2
SOX10
TFAP2
ZIC1
Genes
D.
NCC Markers at D2
log 10 Fold Change Relative to Untreated NCC
PAX7
ZEB2
ZIC3
Genes
E.
WNT Markers at D2
log 10 Fold Change Relative to Untreated NCC
PAX7
ZEB2
ZIC3
Genes
F.
FGF Markers at D2
log 10 Fold Change Relative to Untreated NCC
DUSP6
SPRY1
SPRY2
Genes
G.
BMP Markers at D2
log 10 Fold Change Relative to Untreated NCC
Id1
Id3
Klf2
Klf5
Genes

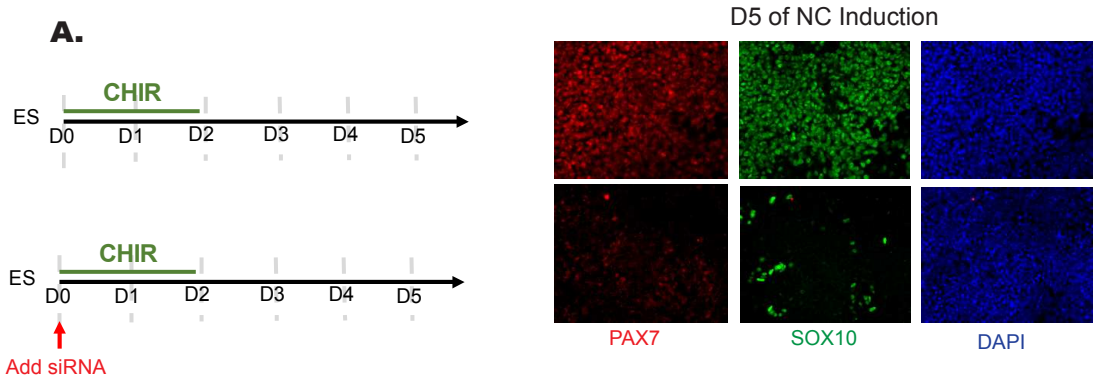
Figure 5: NCC Induction is Inhibited by siRNA Knockdown of EPHB2

A) A diagram showing a NC induction with and without the addition of siRNA. The first row shows a control induction done a scrambled siRNA, the second row shows a NC induction carried out with the addition of siRNA to knockdown EPHB3. Immunofluorescent images showing expression of neural crest markers *PAX7*(red), *SOX10*(green) and DAPI(blue).
B) Cell count data showing the expression of PAX7 and SOX10 expression in cells treated with siRNA, relative to a control NCCs.
C) QPCR data presenting changes in fold change of neural crest markers (*FOXD3, MSX1, PAX3, PAX7, SNAIL2, SOX10, TFAP2,* and *ZIC1*). Cells were collected at day 5 of the induction.
D) QPCR data showing changes in early neural crest markers (*PAX7, ZEB2*, and *ZIC3*) at day 2 of collection. The gene fold is determined relative to D2 collection untreated NCC.
E) QPCR data depicting changes in WNT targets (*AXIN2, SP5, LEF1*, and *c-MYC*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.
F) qPCR data presenting variations in FGF targets (*DUSP6, SPRY1*, and *SPRY2*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.
G) qPCR data showing alterations in BMP targets (*Id1, Id3, Klf2*, and *Klf5*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.

Lastly, siRNA knockdown of EPHA4 is used to inhibit production of EPHA4. Immunofluorescence for *PAX7* and *SOX10* expression shows that in comparison to the control condition *PAX7* and *SOX10* expression are diminished to 11.0% and 6.1% respectively, at day 5 (Figure 4B). DAPI cell counts are consistent between the experimental and control conditions (data not shown). QPCR analysis shows that *FOXD3* (-0.65), *PAX7* (-0.42), *SOX10*(-0.79) are increased, where *ZIC1*(0.30) is decreased *MSX1*(0.24), and *PAX3*(0.07), *SNAI2* (0.02), *TFAP2* (-0.08) are not greatly affected (Figure 5C). Experimental results show that at both the transcriptional and translational level NCC induction is inhibited when siRNA is used to knockdown EPHA4.

Next we went on to assessed the effects of EPHA4 knockdown on early NCC genes, and on the WNT pathway, BMP and FGF at day 2 of NCC induction. QPCR for early NCC genes shows that *PAX7* (-0.41) is decreased, but *ZEB2* (-0.05), and *ZIC3* (0.03) are mostly unaffected (Figure 3D). When assessing the targets for the WNT pathway, data shows that there is an increase in SP5 (0.30); however, *AXIN2*(-0.06), *DKK1*(-0.06), *LEF1*(0.04) and *c-MYC* (-0.07) are generally unchanged (Figure 4E). When FGF targets are evaluated, data shows that *DUSP6* (-0.28) and *SPRY1*(-0.35) are decreased, but *SPRY2* (-0.07) are almost unaltered. Lastly, targets of BMP were examined, and these results show that *Id1*(-0.61), *Id3* (-0.34), *Klf2* (-0.68), and *Klf5* (-0.57) are decreased, which illustrates alterations in in BMP activity due to EPHA4

knockdown. Altogether, WNT pathway, BMP and FGF activity are shown to be dysregulated when, EPHA4 is knocked down, which ultimately leads to NCC formation inhibition, suggesting that there is crosstalk between EPHA4 and other signaling pathways.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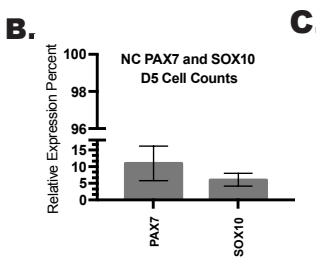
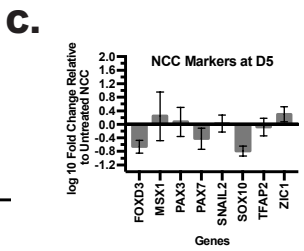
CHIR
ES
D0
D1
D2
D3
D4
D5
CHIR
ES
D0
D1
D2
D3
D4
D5
Add siRNA
D5 of NC Induction
PAX7
SOX10
DAPI


B.
NC PAX7 and SOX10
D5 Cell Counts
Relative Expression Percent
100
98
96
15
10
5
0
PAX7
SOX10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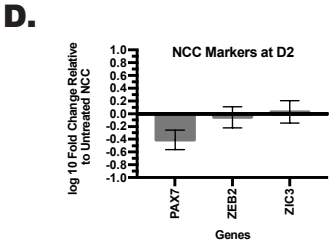
NCC Markers at D5
log 10 Fold Change Relative to Untreated NCC
2.0
1.6
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
FOXD3
MSX1
PAX3
PAX7
SNAIL2
SOX10
TFAP2
ZIC1
Genes


D.
NCC Markers at D2
log 10 Fold Change Relative to Untreated NCC
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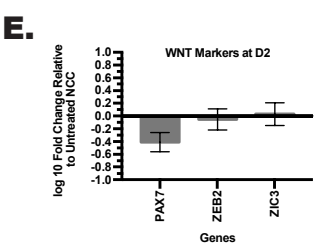
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
PAX7
ZEB2
ZIC3
Genes


E.
WNT Markers at D2
log 10 Fold Change Relative to Untreated NCC
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0.8
0.6
0.4
0.2
0.0
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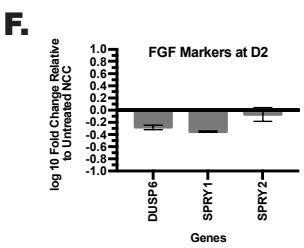
-0.4
-0.6
-0.8
-1.0
PAX7
ZEB2
ZIC3
Genes


F.
FGF Markers at D2
log 10 Fold Change Relative to Untreated NCC
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
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DUSP6
SPRY1
SPRY2
Genes

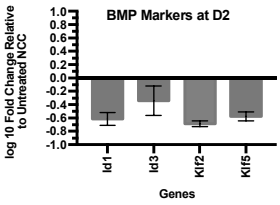

G.
BMP Markers at D2
log 10 Fold Change Relative to Untreated NCC
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Id1
Id3
Klf2
Klf5
Genes

Figure 6: NCC Induction is Inhibited by siRNA Knockdown of EPHA4

A) A diagram showing a NCC induction with and without the addition of siRNA. The first row shows a control induction done a scrambled siRNA, the second row shows a NC induction carried out with the addition of siRNA to knockdown EPHB3. Immunofluorescent images showing expression of neural crest markers *PAX7*(red), *SOX10*(green) and nuclear stain DAPI(blue).
B) Cell count data showing the expression of PAX7 and SOX10 expression in cells treated with siRNA, relative to a control NCCs.
C) QPCR data presenting changes in fold change of neural crest markers (*FOXD3, MSX1, PAX3, PAX7, SNAIL2, SOX10, TFAP2,* and *ZIC1*). Cells were collected at day 5 of the induction.
D) QPCR data showing changes in early neural crest markers (*PAX7, ZEB2*, and *ZIC3*) at day 2 of collection. The gene fold is determined relative to D2 collection untreated NCC.
E) QPCR data depicting changes in WNT targets (*AXIN2, SP5, LEF1*, and *c-MYC*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.
F) qPCR data presenting variations in FGF targets (*DUSP6, SPRY1*, and *SPRY2*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.
G) qPCR data showing alterations in BMP targets (*Id1, Id3, Klf2*, and *Klf5*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.

Overall, the results show that when either EFN (EFNA2, EFNA5, EFNB1) or EPH (EPHB2 and EPHA4) are knocked down, NCC induction is inhibited at day 5. By day 2, cells treated with siRNA for the knockdown of EFN or EPH result in diminished early NCC genes. Additionally, there is dysregulation in targets of the WNT pathway, FGF and BMP. Taking the results together the data suggests that when EFN or EPH are inhibited NCC formation is inhibited, and there is a dysregulation of the classically known pathways (WNT, BMP and FGF) that are involved in NCC formation. This suggests that there is crosstalk between EFN and EPH and other signaling pathways, in this case WNT, BMP, and FGF, that prevents the formation of NCC induction.

Discussion

NCC defects are more likely to be evident in the craniofacial region. Importantly, studies in mouse implicate Eph/Efn signaling regulation is necessary for oral palate formation. EphB2 and EphB3 serve as receptors for EFNB1 in the palate mesenchyme, and EphB3 and EphB2 null mice exhibit cleft palate. Despite this, EPH-EFN contributions to NCC formation have not been widely studied. WNT, BMP, FGF, and Notch signaling are postulated to be responsible for NCC induction, through the activation and maintenance of the gene regulatory network. Studies have shown that they are responsible for the development of the neural plate border, and NCC formation.

Taking together information from literature, I determined EPH and EFN play a significant role in NCC migration and a critical role in the formation of NCC derived structures such as the craniofacial region. I found that EPH receptors and EFN ligands are necessary in NCC induction. RNA sequence data shows that EPH receptor and EFN ligand expression vary depending on the stage of NCC induction (Figure 1). This suggests that even though EPH receptor and EFN ligands are needed throughout the induction protocol, not the same ones are needed at every point in the induction protocol. NCC induction initiation from ESC, NCC progression and maintenance are regulated in part by different combinations of EPH receptors and EFN ligands. There are several possibilities behind the mechanism by which regulation of NCC induction by EPH and EFN might be occurring, these possibilities include, cytoskeletal rearrangements and/or crosstalk leading to alterations in the WNT, BMP, and FGF pathways, which are known to be key players in NCC induction. I went on to look at the EPH and EFN that were found to be upregulated early on in NCC induction, suggesting that they play a critical role in the early formation of NCC.

Studying the effects of EFNA2 knockdown through the use of siRNA, I found that NCC formation is greatly reduced both at the protein level and at the transcriptional level. Immunofluorescent data at day 5 showed a substantial reduction of both *PAX7* and *SOX10,* so I went on to see if these effects were only translational and that inhibition of EFNA5 was preventing the translation of NCC markers to proteins or if the transcripts for these markers were also absent. From

qPCR data from day 5 it was found that the transcripts for the day 5 NCC genes were largely reduced, for the most part. Next, I wanted to study whether this reduction in NCC transcripts could be seen early on in the NCC induction, at day 2, which is the time-point where it is assumed that the NCC have been initiated from ESC and are at a point where they are maintaining the existing NCC specifier genes and acquiring additional ones. Data from day 2 cell collections shows that the early markers of NCC were dysregulated. From here I went on to look at the targets of WNT, BMP, and FGF were all dysregulated, as well (Figure 2). This suggests that there is crosstalk between EFNA2 and other signaling pathways, in this case WNT, BMP, and FGF. It is possible that either EFNA2 is effecting WNT, BMP, and FGF directly or indirectly though other pathways. Further studies must be done to explore, the role of EFNA2 in NCC maintenance and its role in the regulation of crosstalk between the pathways necessary for NCC induction.

Using siRNA, the effects of EFNA5 knockdown were studied and the results show that both the transcripts and proteins that mark for NCCs are reduced, overall. Data from day 5 immunofluorescence analysis and qPCR data from day 5 NCC genes shows that there is dysregulation; generally, inhibition of both NCC gene transcripts at the RNA level and proteins at the translational level. Effects at the transcriptional level could be seen by day 2, through dysregulation of day 2 NCC genes. Next looking at the targets of WNT, BMP, and FGF dysregulation was seen, demonstrating effects of EFNA5 inhibition on the maintenance of

WNT, BMP, and FGF (Figure 3). This suggests several explanations which include EFNA5 is effecting WNT, BMP, and FGF directly or indirectly though other pathways. This warrants additional studies to elucidate the effects of EFNA5 on NCC formation.

EFNB1 knockdown was studied in the context of NCC formation at a transcriptional and translational level, through the use of siRNA. Both day 5 immunofluorescence and qPCR data from day 5 samples display dysregulation of NCC genes. Also, dysregulation of day 2 NCC genes was seen, in addition to changes to the presence of WNT, BMP, and FGF targets (Figure 4), suggesting that EFNA5 inhibition leads to dysregulation of WNT, BMP, and FGF. Taking together this data I propose that EFNA5 is effecting WNT, BMP, and FGF, additional studies are needed to confirm this and provide a mechanism through which these effects are seen.

In addition to studying the top EFN candidates, I studied the top EPH candidates for NCC formation as determined through RNA sequence data. Knockdown of EPHB2 and EPHA4 individually showed dysregulation of NCC genes by day 5 of NCC induction through both immunofluorescence and through qPCR, suggesting that dysregulation was present at both the transcriptional and translational level. Dysregulation in NCC genes was seen by day 2, suggesting that EPHB2 and EPHA4 activity are needed for the early stages of NCC induction. Furthermore, changes in WNT, BMP, and FGF target regulation was present, indicating that

EPHB2/EPHA4 inhibition leads to dysregulation of WNT, BMP, and FGF (Figure5/6). Collectively this data suggests that WNT, BMP, and FGF interact with EPH, this warrants additional studies to shed light on the mechanisms by which EPH regulate NCC induction.

Overall, looking at EFNA2, EFNA5, EFNB1, EPHA4, EPHB2, it can be concluded that knockdown of EFN or EPH leads to abrogated NCC formation. Overall, immunofluorescence for *PAX7 and SOX10* show diminished expression of the two NCC markers. Additionally, qPCR shows general downregulation of most day 5 NCC markers, and dysregulation of day 2 NCC markers, indicating that a NCC lineage is not initiated properly when EFN/EPH knockdown takes place. In all cases, targets of WNT, FGF, and BMP show dysregulation to some extent; however, not equally so with all of the EFN/EPH knockdowns, which suggests that although EFN/EPH knockdown in general leads to a lack of NCC formation, at least partially through WNT, FGF, and BMP crosstalk, the mechanisms amongst how this occurs in different EPH and EFN varies.

Information taken together from numerous studies suggests that NCC induction heavily relies on appropriate levels and timing of WNT, FGF, and BMP pathway activity. These studies suggest that NCC induction is initiated by WNT and FGF signaling, but maintained by BMP signaling. In chick embryos it has been shown that Wnt can induce BMP expression, and inhibition of Wnt can lead to dampening of BMP signaling. Wnt induced BMP activation is necessary to

provide instructive cues at the neural plate border for NCC specifier activity.[337] Using *Xenopus*, zebrafish and chick models, it has been shown that the presence of FGF signaling decreases BMP signaling, through negative regulation of the BMP ligand.[338,339] These signaling pathways are essential to induce neural crest specifiers during gastrulation at the neural plate border. Understanding the contributions of EPH and EFN to NCC formation independently and through crosstalk with these pathways could prove to be instrumental in the development of therapies, for neurocristopathies.

Materials/Methods

1.1 hESC Maintance

HESCs are maintained in mTESR from Stem Cell Technologies (component #85851 and # SCBW6732) on Matrigel (Reference # 354277), and cells were passed using Versene from Gibco (Reference # 15040-66).

1.2 Neural Crest Induction

HESCs are dissociated using Accutase (Life Technologies), and the cells counted and plated onto vessels coated with Matrigel (Corning) at a density of 20,000cells/cm2 in media containing DMEM/F12 (Thermo Fisher Scientific no. 175020048), supplemented with 1X serum-free B27 supplement (Invitrogen, Cat. No. 17504- 044), 1X Glutamax (Thermo fisher scientific, Cat. No. 35050061), 0.5% BSA (Sigma, A7979).

1.5 siRNA

siRNAs were purchased from Dharmacon (sp) and Lipofectamine RNAiMax reagent was purchased from Invitrogen. Using reverse transfection siRNA and Lipofectamine were added. A final concentration of 1.2 pMole of siRNA and 0.05μL of Lipofectamine, siRNA was diluted to 6 pMole in 100μL OptiMEM, and Lipofectamine was diluted to 0.5μL in 100μL of OptiMEM. H1 cells were accutased and the hNCC induction protocol was carried. Prior to plating the cells the matrigel used to coat the wells was aspirated, and 10μL of Lipofectamine and 20μL of siRNA were added to each well. The Lipofectamine/siRNA mixture was incubated at RT for 10-20 minutes, and then the cells were plated. Cells were mixed at 8.9k cells/100μL and plated in 90μL (8k cells/well). \ Scrambled siRNA and Cy3-tagged siRNA were used as a control.

1.6 Immunoflorescence

Cells were fixed with 4% paraformaldehyde for 10 minutes, they were then permeablized with 0.4% Triton X-100 for 10 minutes. Cells were then blocked using 10% fetal bovine serum (FBS) with 0.05% Tween 20 for 1 hour at room temperature. Primary antibody incubations were done overnight at 4C, followed by secondary which was done at room temperature for an hour. Cells were imaged using a Nikon Eclipse Ti microscope, the images were processed using Adobe Photoshop, and cell counts were done using Nikon Elements software. Antibodies used: Mouse anti- SOX10 (Santa Cruz Biotechnology, SC271163), 1:200. Mouse anti-PAX7 (1:10).

1.7 Quantitative PCR

Total RNA was extracted using TRIzol reagent from Life Technologies (# 15596026). CDNA was generated from 500ng of RNA using the High Capacity cDNA Reverse Transcription kit (number RR014B) from Clonetech. SYBR Green (Applied Biosystems) was used in the reactions. Two biological replicates were assessed for siRNA treated samples.

Table S1. List of Primers

Transcript	Forward	Reverse
PAX7	AACGACAGAACCCGACTATGTTC	CGGCGTTGGGTGGAAA
ZEB2	CCAAGAGAGGAAGAGGAAGATGAAA	TCTTCCTTCATTTCTTCTGGACCAT
ZIC3	TCTGCAAAGTGTGCGACAAGT	TGACCCTTGAGATTCATGAACCT
AXIN2	CGGGAGCCACACCCTTCT	TGGACACCTGCCAGTTTCTTT
SP5	CTTCGGGTGTCCATGCCTC	GTGCGGTCCTGGAGAAAGG
DKK1	GCACCTTGGATGGGTATTCCA	GCACAACACAATCCTGAGGC
LEF1	ATGTCCAGGTTTTCCCATCAT	CTGAGGTGTTACAATAGCTGG
MYC	GCCACGTCTCCACACATCAG	TCTTGGCAGCAGGATAGTCCTT
FOXD3	TCATCACCATGGCCATCCT	GGAAGCGGTTGCTGATGAAC
MSX1	AAGTTCCGCCAGAAGCAGTA	GCGGTTCTGGAACCATATCT
MSX2	CCAGGTCAAAATCTGGTTCC	GCATAGGTTTTGCAGCCATT
PAX3	GAACCCGGGCATGTTCAG	ACGGCACGGTGTTTCGA
PAX7	AACGACAGAACCCGACTATGTTC	CGGCGTTGGGTGGAAA
SNAIL2	GATCCTCAGCTCAGGAGCATACA	GGAGTATCCGGAAAGAGGAGA
SOX10	GAGGCTGCTGAACGAAAGTGA	GCGGCCTTCCCGTTCT
TFAP2	GAGAGTAGCTCCACTTGGGTG	GTCGTGACGGTCCTCGC
ZIC1	CAAAACTTTCAGCACCATGCA	TCCCAGAAGCAGATGTGATTA

Chapter 2: Role of EPHB3 Receptors in Early Neural Crest Development

Abstract

Neural crest cells (NCC) are a multipotent population of cells that are unique to vertebrates. NCCs have a great differentiation potential, which allows them to differentiate into peripheral neurons and glia, melanocytes, and craniofacial bone and cartilage, amongst other cell fates. Over the past century, considerable research has been done to give us an understanding of how NCC are formed. NCC formation involves spatially and temporally controlled input from several signaling pathways and transcription factors from the gastrulation to neurulation stages. In formation of NCC, FGF and WNT signaling are required to induce NCC progenitors at the neural plate border, which activates transcription factors including *MSX, PAX* and *ZIC*. Next, BMP and WNT signaling come into play, and definitive NCC markers such as *Snail2, FoxD3*, and *SOX9/10* are activated. In addition to these signaling pathways, cell-cell signaling has been implicated in NCC formation. Bidirectional signaling mediated by ephrin receptors (EPH) and their ephrin ligands (EFN) modulate cell differentiation, actin cytoskeleton rearrangement, migration, survival, and proliferation. In this study we identify the expression and requirement of EPH receptors during the early facets of NCC formation. We find that blockade of EPH receptor function for the first 24 hours, prevents the acquisition of NCC markers, but does not alter cell survival nor proliferation. This effect is preceded by alterations in early NCC markers as soon as day 1 of induction. These results were found when using a general EPH

inhibitor and similarly when using an inhibitor specific for EPHB3. We also found that EPHB3 inhibition leads to the dysregulation of fibroblast growth factor (FGF), WNT, and bone morphogenetic protein (BMP) signaling, which is likely responsible for the effects found on NCC formation. Determining the role of specific EPH interactions during NCC formation will improve our capacity to diagnose and treat neurocristopathies.

Introduction

Neural crest cells (NCC) are a transient population of cells that originate from the neural plate border, migrate and contribute to a wide range of ectodermal and mesenchymal derivatives. Abnormalities in NCC development and homeostasis lead to pathologies, termed neurocristopathies, which include cancers such as melanoma, congenital disorders such as Treacher-Collins syndrome, and some of the most common craniofacial disorders (cleft lip and palate),[340,341,342] amongst other abnormalities. Despite their extensive role in human embryonic development, much remains to be studied in regards to exactly how NCC are formed.

WNT, BMP, and FGF signaling have been shown to play essential roles in the formation, migration and differentiation of NCC. In chick embryos it has been shown that Wnt signaling can induce BMP signaling, and Wnt inhibition leads to attenuation of BMP signaling. WNT induced BMP activation is necessary to provide instructive cues at the neural plate border for NCC specifier activity.[343] In *Xenopus*, zebrafish and chick models, it has been shown that FGF signaling can

lead to a decrease of BMP signaling.[344],[345] Taking this information together it can be postulated that these signaling pathways are essential for the formation of NCC.

WNT, BMP, and FGF signaling leads to NCC formation through activation and maintenance of the gene regulatory network. Studies have shown that they are responsible for the development of the neural plate border, after which NCC formation is initiated. Transcription factors, which include *PAX3, PAX7, ZIC1, MSX1* and *TFAP2,* are expressed at the neural plate border.[346,347].Model systems including human embryonic stem cell-based systems, rabbit, *Xenopus*, mouse, chick and zebrafish have contributed to the understanding of the gene regulatory network involved in NCC induction.

Additionally, cell-cell signaling, such as erythropoietin-producing human hepatocellular receptors (EPH) and their ligands, EPH receptor-interacting proteins (ephrins), have been suggested to play a role in NCC development. Bidirectional signaling mediated by EPH/EFN interactions modulate cell differentiation, actin cytoskeleton rearrangement, migration, survival, and proliferation. [348] EPH receptors, which are members of the Receptor Tyrosine Kinase (RTK) superfamily and their EFN ligands are divided into two classes: A and B. Typically, class A ligand will bind to class A receptor, and class B ligands will bind to class B receptors; however, in some cases they can bind across classes. The EPH subfamily of signaling molecules is a widespread group of

surface proteins found throughout the developing embryo and is responsible for directing a multitude of developmental processes.[349]

Prompted by cell-cell signaling, prospective NCC populations respond to the stimulus by undergoing structural rearrangements. For example, in mouse, EfnB2 is expressed around the branchial arches, leading to repulsion of the migrating cranial NCC which express EphA4, EphB1, and EphB3. EfnB2 initiates Eph forward signaling and guides migration of CNC cells.[350,351]

In chick it has been shown that throughout migration NCC express EPHB3, EPHA3, EPHA7, EPHB1, and EPHB3; they migrate along non-NCC that express EfnB1. In chick it has been shown that cranial NCC express EFNB2 and migrate along non-NCC that express EPHB2.[352] In chick the role of EFNB1 and EFNB2 differ from those in mice, as they seem to be switched.[353] This suggests that even though NCC are a distinguishing feature of vertebrates, not all EPH and EFN are conserved amongst species, and they play different roles. It is possible that because they act promiscuously, the orthologues are able to activate comparable pathways leading to the same downstream effects.[354]

While many studies focus on the role of EPH and EFN interactions in cell migration, it remains to be studied which intracellular signaling cascades are directed by these surface interactions and their role in NCC formation. Recently a report has shown that matrix metalloprotease ADAM13 is needed for WNT signaling. ADAM13 inhibits EFNB signaling, EFNB signaling has been shown to

inhibit WNT signaling. By inhibiting EFNB signaling, ADAM13 leads to an increase in WNT signaling. It has been shown that ADAM19 functions to stabilize ADAM13 and promotes wnt signaling in *Xenopus* NCC induction.[355]

Due to differences in the expression of EPH/ EFN and the exact roles they play in different species there is a need to study EPH/EFN interactions in a human model system to understand their precise role in human embryonic development. An in *vitro* model of hNCC formation from hESCs is the best approach to carry out these studies due to ethical and technical limitations in using human embryos. Using a protocol which provides a fast and efficient model of hNCC development based on human embryonic stem cells (hESC) and induced pluripotent stem cells(iPSC),[356,357] using qPCR I was able to determine that EPHB3 expression is upregulated early on in NCC induction, and decreases as NCC formation proceeds. Taking this information together with information from studies showing the involvement of EPHB3 in the migration of NCC, I decided to investigate the role of EPHB3 in early NCC formation.

Here I describe, for the first time, studies addressing the role of EPH receptors during early NCC development, using hNCC derived from hESC. I report that EPH receptors are dynamically expressed during hNCC formation. I found that EPH function is critical for the early stages of hNCC formation. Specifically, EPHB3 is upregulated early on in NCC formation, and its inhibition through the use small molecules or through siRNA knockdown strategies prevents NCC formation. I have found that when EphB3 inhibited for the first 24 hours NCC

induction is diminished. Additionally, the data provides evidence suggesting that there is crosstalk between WNT, FGF, BMP and EPHB3. In the absence of EPHB3 signaling, WNT, FGF, and BMP signaling is dysregulated. Overall this study suggests that EPHB3 plays a crucial role in the first 24 hours of NCC development. Understanding the role that EPHB3 plays in NCC formation can lead to advances in understanding its role in embryogenesis and shed light on how an imbalance in EPHB3 signaling during early NCC formation can lead to diseases. [358,359]

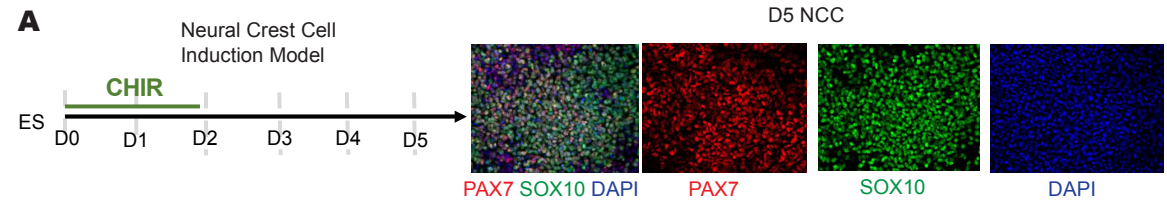
Results

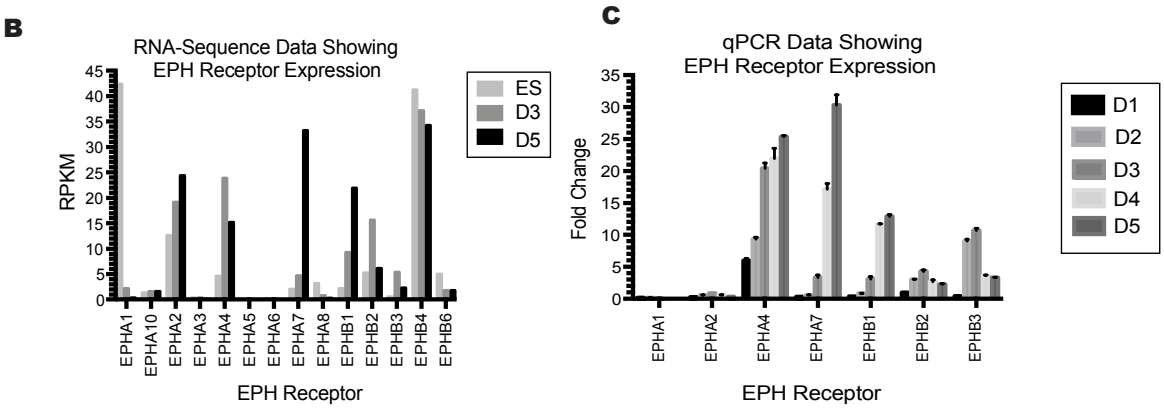
To determine whether EPHB receptors play a role in NCC induction, we set out to determine which EPHB receptors are most upregulated in early NCC induction. These experiments were carried out using a model of hNCC development based on induction of hESC, that is based on canonical WNT/ß-catenin activation via GSK3 inhibition for the first 48 hours of induction in a 5-day protocol (Figure 1a). [360, 361] This system provides with the opportunity to do transcriptional profiling, because it allows for an unlimited synchronized population of NCC.

Using RNA sequencing I was able to analyze the transcription profile and using this profile, I have identified expression of EPHA and EPHB in hESC, in day 3 and day 5 NCC. There are nine EPHA receptors and five EPHB receptors. [362] To determine whether EPH receptors play a role in NCC induction, I set out to determine which EPH receptors are most upregulated in early NCC induction.

First, I used the RNA sequence data to look at the 14 EPH receptors present in human cells. The data shows that *EPHA3, EPHA5*, and *EPHA6* are present at negligible level, and *EPHA10* levels are low relative to stronger candidate receptors, although not negligible. *EPHA1, EPHB4,* and *EPHB6* are highest in ESC. *EPHA2, EPHA7, EPHB1* are highest at day 5. Lastly, the RNA sequence data shows that *EPHA4, EPHB2* and *EPHB3* are highest at day 3, making them the targets of interest (Figure 1b). Next I ran qPCR on *EPHA4, EPHB2* and *EPHB3* to confirm and provide a higher resolution analysis of the results from the RNA sequence data. I found that the trends from qPCR were generally consistent with those of the RNA sequence data. From the qPCR data I found that *EPHA4* expression went up gradually though the NCC induction, and both *EPHB2* and *EPHB3* expression peaked by day 3. EPHB3 expression demonstrates a higher fold change from day 1 to day 2 of NCC induction, and a higher fold change between day 1 and day 2 (Figure 1c), making *EPHB3* the target of interest, because I am most interested in exploring the effects of EPH receptors in early NCC formation. To verify that this trend holds true not only on a transcriptional level, but on a translational level as well, I went on to carry out immunofluorescent microscopy for *EPHB3*, in ESC, in day 1 NCC, day 3 NCC and day 5 NCC. The data shows that *EPHB3* was weakly expressed in ESC (Figure 1D).

I determined that *EPHB3* is expressed in day 1, and day 3 NC cells; however, the expression of *EPHB3* is almost nonexistent by day 5. Together this data suggests that *EPHB3* may play an important role in early NCC induction and that it should be studied further.


A
Neural Crest Cell
Induction Model
CHIR
ES
D0
D1
D2
D3
D4
D5
D5 NCC
PAX7 SOX10 DAPI
PAX7
SOX10
DAPI


B
RNA-Sequence Data Showing
EPH Receptor Expression
RPKM
ES
D3
D5
EPHA1
EPHA10
EPHA2
EPHA3
EPHA4
EPHA5
EPHA6
EPHA7
EPHA8
EPHB1
EPHB2
EPHB3
EPHB4
EPHB6
EPH Receptor
C
qPCR Data Showing
EPH Receptor Expression
Fold Change
D1
D2
D3
D4
D5
EPHA1
EPHA2
EPHA4
EPHA7
EPHB1
EPHB2
EPHB3
EPH Receptor

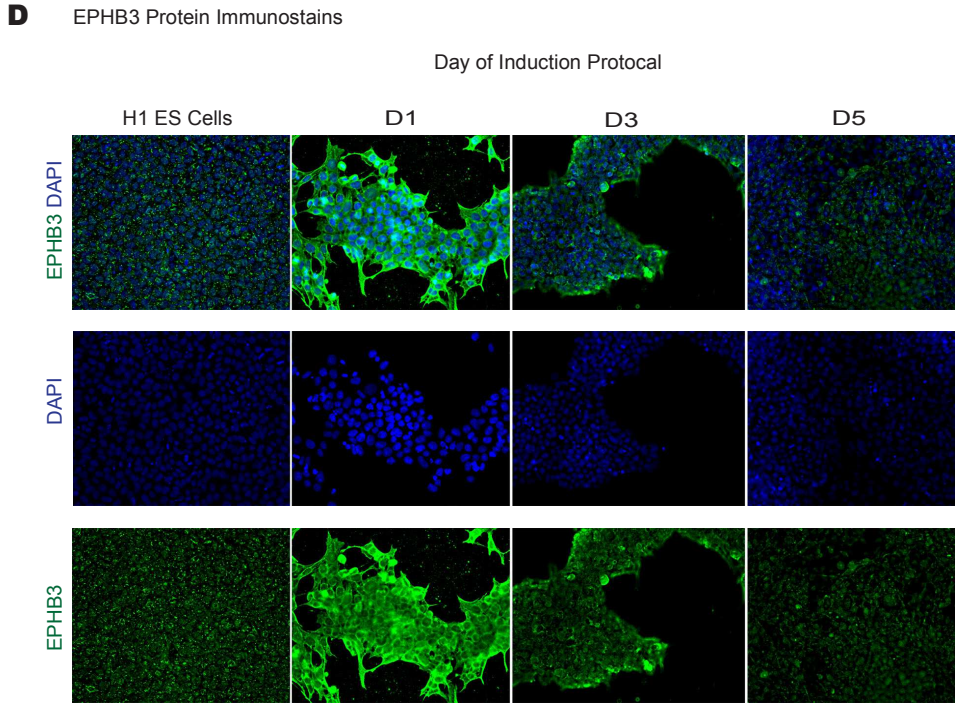

D
EPHB3 Protein Immunostains
Day of Induction Protocal
H1 ES Cells
D1
D3
D5
EPHB3 DAPI
DAPI
EPHB3

Figure 1: Expression of EPH During NCC Induction

D) Schematic depicting 5-day neural crest induction protocol using 2-day exposure to the small molecule CHIR99021. A merged image is shown of PAX7 (red), SOX10 (green) and nuclear stain DAPI (blue).
E) RNA sequence data showing the expression profile of EPHA and EPHB, measured in RPKM in ES, 3 day NCC and 5 day NCC.
F) QPCR data depicting daily RNA fold changes in NCC relative to ES cells for selected EPHA and EPHB receptors.
G) Immunofluorescence microscopy demonstrating expression of EPHB3 (green) and DAPI (blue). These images are 3x3 stitched 20x images. Immunofluorescent microscopic data showing detectable EPHB3 at different stages of hNCC induction.

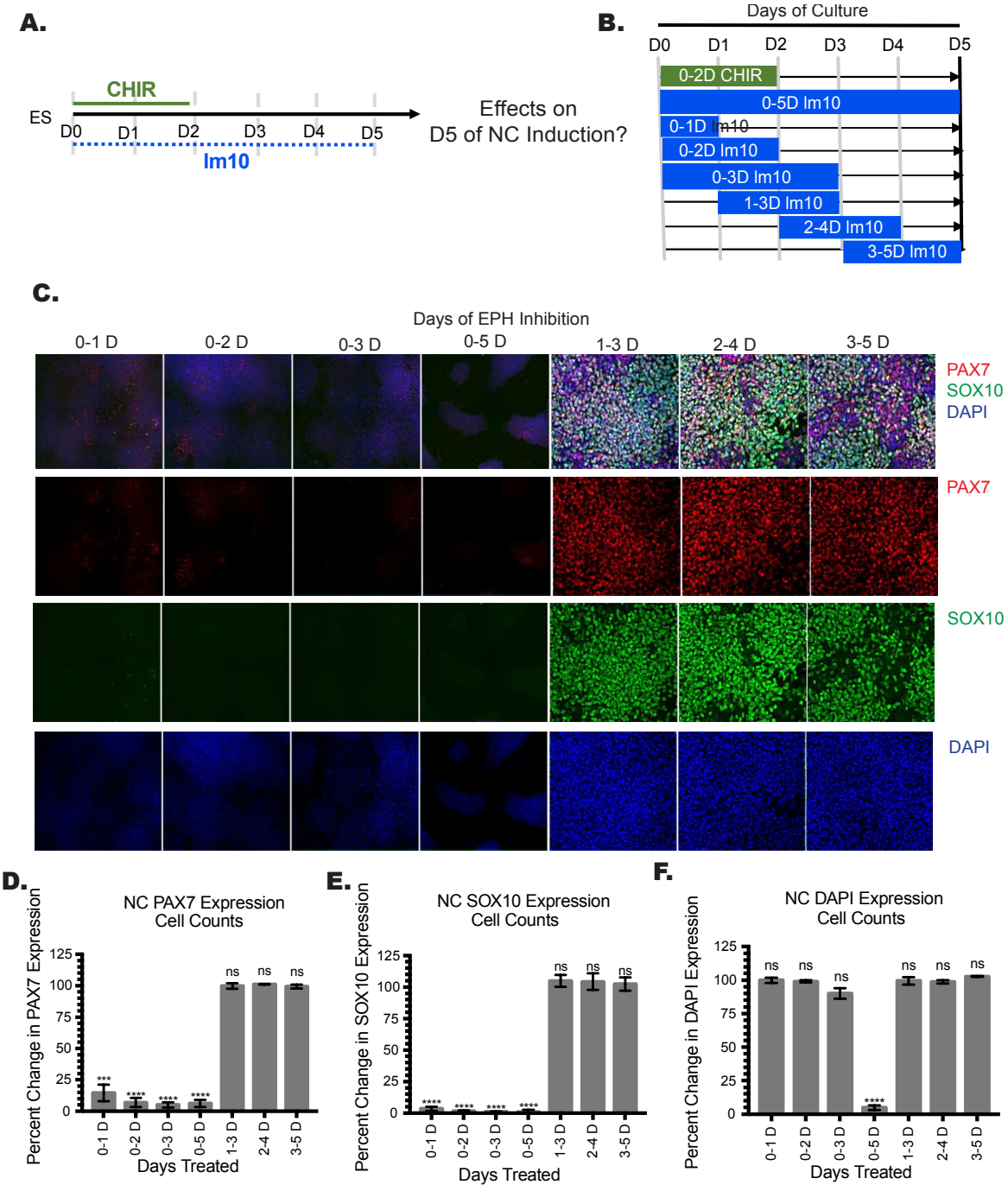

A.
CHIR
ES
D0 D1 D2 D3 D4 D5
Im10
Effects on
D5 of NC Induction?
B.
Days of Culture
D0 D1 D2 D3 D4 D5
0-2D CHIR
0-5D Im10
0-1D Im10
0-2D Im10
0-3D Im10
1-3D Im10
2-4D Im10
3-5D Im10
C.
Days of EPH Inhibition
0-1 D
0-2 D
0-3 D
0-5 D
1-3 D
2-4 D
3-5 D
PAX7
SOX10
DAPI
PAX7
SOX10
DAPI
D.
NC PAX7 Expression
Cell Counts
Percent Change in PAX7 Expression
Days Treated
E.
NC SOX10 Expression
Cell Counts
Percent Change in SOX10 Expression
Days Treated
F.
NC DAPI Expression
Cell Counts
Percent Change in DAPI Expression
Days Treated

Figure 2: NCC Induction is Inhibited by a General Inhibitor

A) Schematic depicting the addition of the general EPH inhibitor for the entirety of the 5-day neural crest induction protocol.
B) Diagram illustrating the time points at which the inhibitor is added: 0-5 days, 0-1 days, 0-2 days, 0-3 days, 1-3 days, 2-4 days, and 3-5 days.
C) Immunofluorescent images showing expression of neural crest markers SOX10 and PAX7. Cells were treated with lm10 for varying time points, as explained in part B of the figure. Image is a 2x2 stitched 20x image. Immunofluorescent microscopic data showing detectable *EPHB3* at different stages of hNC induction.
D) Cell count data showing the expression of PAX7 in NCC treated with lm10 for the above stated time points. The percent expression is measured relative to non-treated NC cells, carried out under the same conditions minus the inhibitor.
E) Cell counts demonstrating changes in expression of SOX10 treated with lm10 for different time points.
F) DAPI in cells treated with general EPH inhibitor for varying spans of time is shown relative to the control condition is shown.

First, I went on to determine if inhibiting EPH receptors in general would affect NCC formation. To do this I used a general inhibitor, Lm10, that inhibits all EPH receptors, with the exception of *EPHA7*. To carry out these experiments I used the NCC induction protocol depicted in Figure 1A, in which WNT is activated for the first 2 days. I began by adding in the general inhibitor, Lm10, for the entirety of the NCC induction protocol (Figure 2B). At the end of day 5 I found that PAX7 (red), and SOX10 (green) expression are both abrogated, but there is also a decrease in cell survival as can be seen by diminished levels of the nuclear stain, DAPI (Figure 2C) but at lower concentrations of Lm10 we did not see a significant decrease in PAX7 and SOX10 (data not shown).

Next I moved onto resolving what the key timeframe for EPH inhibition is to prevent NCC induction, and if it would be possible without affecting cell survival and proliferation. The time points I studied were initially 0-5, and then 0-1, 0-2,0-3, 1-3, 2-4 and 3-5 days (Figure 2B). The 0-1, 0-2, and 0-3-day time points were selected so that I could determine what is the key time in which EPH had to be inhibited to prevent NCC induction. Inhibition from days 2-4 was chosen because I wanted to determine if inhibition after the initial 48 hours, which is the time that we have found is essential for WNT activation would still lead to abrogation in NCC induction, as day 3 is when EPHB3 expression peaks. Then I looked at inhibition of EPH receptors from days 3-5 to determine if inhibiting EPH at the end of the induction protocol would prevent NCC formation. From this I found that EPHB3 is most critical within the first 24 hours. If the the EPHB3 inhibitor is not

added until day 1, day 2, or day 3, PAX7 and SOX10 expression are unaffected determined via immunofluorescence (Figure 2c). The data shows that when the inhibitor is added in from days 0-1, 0-2 or 0-3 PAX7 and SOX10 expression is diminished without effecting cell proliferation and survival, as shown by DAPI. When Lm10 is added for the entirety of the protocol, PAX7, SOX10, and DAPI are all effected, implying that survival and proliferation is affected. When Lm10 is added in for 1-3, 2-4, or 3-5 days, PAX7 and SOX10 expression is unaffected, and DAPI stain counts are also unaffected.

To verify the results visualized through the immunofluorescent stains, I did cell counts for PAX7, SOX10, and DAPI in the treated and untreated NCC and analyzed the treated cells relative to the untreated cells. To calculate the changes in PAX7, SOX10 and DAPI I used a Nikon Elements analysis software. From this I found a statistically significant reduction in both PAX7 expression and SOX10 expression when EPH inhibition is initiated from the first day of the induction as determined through immunofluorescent microscopy (Figure 2D, E, and F respectively). Using cell counts I found that when the inhibitor was added PAX7 results were as follow: 0-1 day (14.63%), 0-2 day (6.99%), 0-3 day (5.06%), 0-5 day (6.22%), 1-3 day (99.75%), 2-4 day (99.75%), and 3-5 day (99.29%) relative to the percent of the untreated control (Figure 2D). Through cell counts I found that SOX10 results were as follow: 0-1 day (3.37%), 0-2 day (1.58%), 0-3 day (0.87%), 0-5 day (0.82%), 1-3 day (104.99%), 2-4 day (104.32%), and 3-5 day (102.31%) relative to the percent of the untreated control

(Figure 2E). To confirm that the reduction in NCC was not a result of a reduction in cell number, I did cell counts on DAPI and found that with the exception of 0-5-day treatment, and determined that there was no statistically significant reduction in cell counts (Figure 2F). DAPI results were as follow: 0-1 day (99.97%), 0-2 day (99.08%), 0-3 day (90.00%), 0-5 day (4.82%), 1-3 day (99.60%), 2-4 day (98.76%), and 3-5 day (102.74%) relative to the percent of the untreated control (Figure 2F). With these results, going forth, I determined that EPH inhibition during the first day of induction is key to inhibiting NCC induction.

To confirm that the concentrations of inhibitor I used did not have an effect on ESC, and hence the effects I was seeing from the general EPH inhibition, and not because the pluripotency of the ESC was compromised, I treated the ESC with the inhibitors for 0-1 day and 0-5 days (Figure 2A), they were fixed and immunostained at day 5 for SOX2 and OCT4, both of which are pluripotency markers. Through the use of immunofluorescence, I found that LM10 at the concentration I used it in the induction to see inhibition of NCC, did not affect OCT4, SOX2, nor DAPI levels (Supplemental Figure 2B). Next I did cell counts using the immunofluorescent images, and found that there are no statistically significant changes between ESC treated with LM10 and those not treated with LM10 in OCT4 expression for cells treated for 0-1 days (101.89%) and 0-5 days (100.96%) (Supplemental Figure 2C), nor for SOX2 expression in cells ESC treated for 0-1 days (101.37%) and 0-5 days (100.66%) (Supplemental Figure 2D). Additionally, there is no statistically significant change in DAPI counts when

cells are treated with LM10 for 0-1 days (101.55%) and 0-5 days (101.05) (Supplemental Figure 2E). Overall the data suggests that the concentration used to abrogate NCC induction does not affect ESC marker expression.

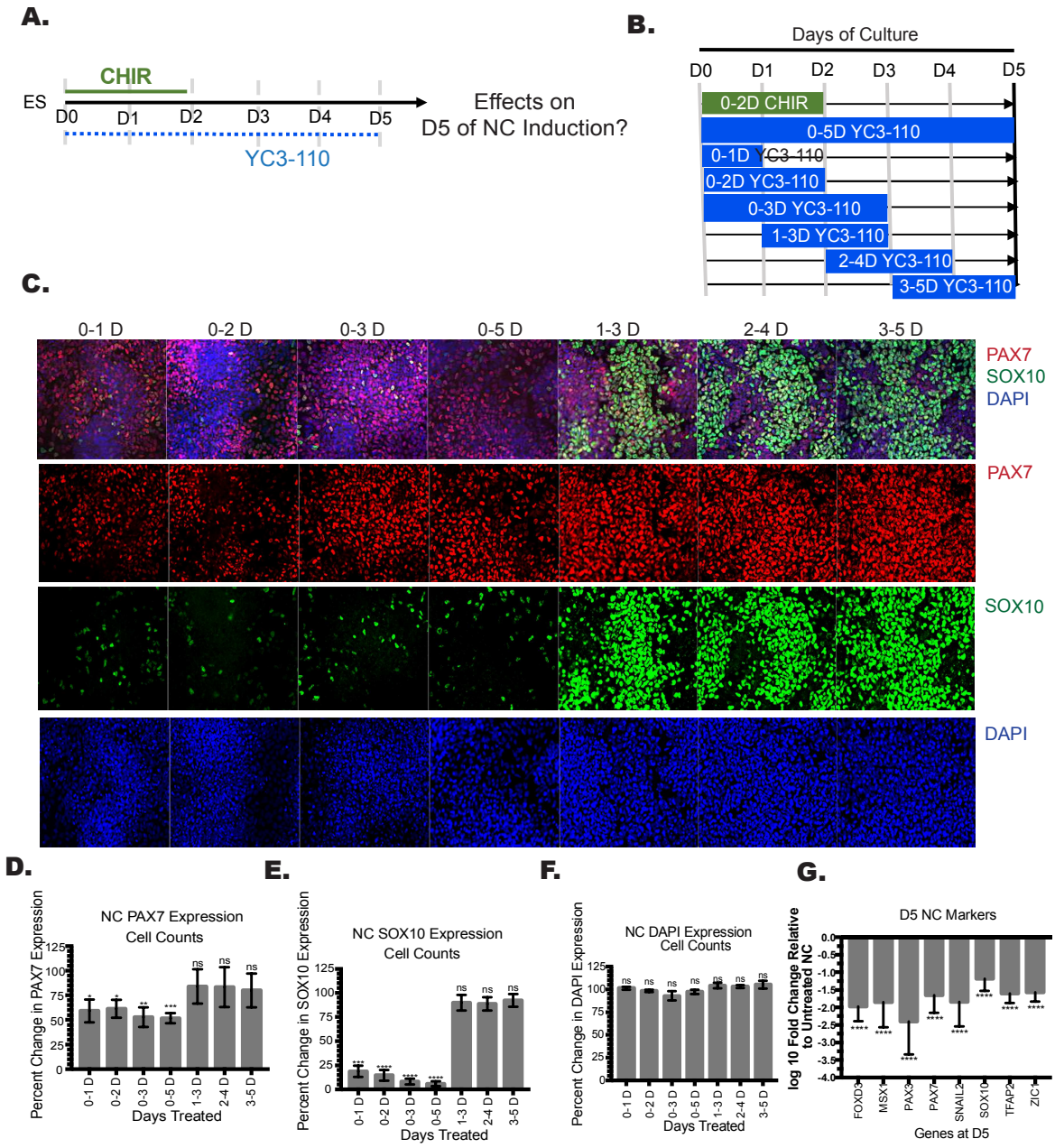

A.
CHIR
ES
D0
D1
D2
D3
D4
D5
YC3-110
Effects on
D5 of NC Induction?
B.
Days of Culture
D0
D1
D2
D3
D4
D5
0-2D CHIR
0-5D YC3-110
0-1D YC3-110
0-2D YC3-110
0-3D YC3-110
1-3D YC3-110
2-4D YC3-110
3-5D YC3-110
C.
0-1 D
0-2 D
0-3 D
0-5 D
1-3 D
2-4 D
3-5 D
PAX7
SOX10
DAPI
PAX7
SOX10
DAPI
D.
NC PAX7 Expression
Cell Counts
Percent Change in PAX7 Expression
Days Treated
E.
NC SOX10 Expression
Cell Counts
Percent Change in SOX10 Expression
Days Treated
F.
NC DAPI Expression
Cell Counts
Percent Change in DAPI Expression
Days Treated
G.
D5 NC Markers
log 10 Fold Change Relative to Untreated NC
FOXD3
MSX1
PAX3
PAX7
SNAIL2
SOX10
TFAP2
ZIC1
Genes at D5

Figure 3: YC3-110 EPHB3 Inhibitor (1uM)

A) A schematic showing the induction protocol and the treatment of cells for 5 days with the EPHB3 inhibitor, YC3-110.
B) A diagram showing the time points that the cells were treated with the specific EPHB3 inhibitor, YC3-110, for the time points as follows: 0-1days, 0-2 days, 0-3 days, 0-5 days, 1-3 days, 2-4 days, and 3-5 days.
C) Immunofluorescence microscopy showing detection of neural crest markers PAX7 (red) and SOX10 (green), and cells are marked with the nuclear stain DAPI (blue). Immunofluorescent microscopic data showing detectable EPHB3 at different stages of hNC induction.
D) Cell counts showing the proportion of cells positive for NCC marker PAX7 in NC treated with YC3-110 for varying durations. The relative percent expression is calculated in comparison to NCC not treated with YC3-110.
E) Cell counts showing the proportion of cells positive for NCC marker SOX10 in NC cells treated with EPHB3 inhibitor for different time points, relative to untreated NCC.
F) DAPI expression in cells treated with EPHB3 inhibitor for varying spans of time is shown relative to untreated NCC.
G) qPCR data presenting changes in neural crest markers (*FOXD3, MSX1, PAX3, PAX7, SNAIL2, SOX10, TFAP2,* and *ZIC1*) at day 5 of collection, YC3-110 was used for the first 24 hours of induction.

After determining that EPH receptor inhibition lead to diminished NCC induction, taken together with the findings that *EPHB3* RNA is highly expressed early in NCC induction, and is translated to protein, I focused my attention on EPHB3. I started by using a specific inhibitor for EPHB3,[363] YC3-110 first for the entirety of the protocol (Figure 3A), and then went on to find a more specified window in which EPHB3 is necessary for NCC formation, and looked at inhibition for 0-1 day, 0-2 day, 0-3 day, 1-3 day, 2-4 day and 3-5 day (Figure 3B) . Immunofluorescence shows that when YC3-110 is added from day 0, data shows inhibition of NCC; however, when it is added from days 1, 2, or 3 this effect is not seen(Figure 3C). When cells are treated with YC3-110 for 0-1 day, 0-2 day,0-3 day, and 0-5 days, *SOX10* expression is severely diminished, and there is a reduction in PAX7 as well. When looking at 1-3 day, 2-4 day and 3-5 day treatments neither a decrease in PAX7 nor SOX10 is seen, as determined based on the presence of PAX7 (red), and SOX10 (green) visualized in the panels. Additionally, there is no apparent change in DAPI (blue), which suggests that there is no change in cell survival nor proliferation, relative to the untreated condition. Next to confirm the visualized information and to get a more detailed analysis of the cell counts a Nikon Elements analysis software was used to do cell counts. Data is presented as percent relative to the control for PAX7, SOX10 and DAPI. Cell count data confirms the expression seen from immunofluorescence. The reduction in PAX7 and SOX10 is statistically significant when cells are treated from day 0 of the induction protocol (Figure

3D,3E). Using cell counts I found that when the inhibitor was added PAX7 results are as follow: 0-1 day (59.28%), 0-2 day (61.35%), 0-3 day (52.75%), 0-5 day (51.85%), 1-3 day (83.98%), 2-4 day (83.33%), and 3-5 day (80.01%) relative to the percent of the untreated control (Figure 3D). Through cells I found that SOX10 results are as follow: 0-1 day (18.80%), 0-2 day (14.68%), 0-3 day (8.15%), 0-5 day (5.94%), 1-3 day (89.79%), 2-4 day (88.68%), and 3-5 day (92.21%) relative to the percent of the untreated control (Figure 3E). Both in the case of PAX7 and SOX10 a statistically significant change is seen only for 0-1 day, 0-2 day, 0-3 day, and 0-5 day. The DAPI cell counts confirm that the reduction in NCC induction is not due to a decrease in cell count (Figure 3F). DAPI results are as follow: 0-1 day (101.42%), 0-2 day (98.43%), 0-3 day (92.90%), 0-5 day (97.33%), 1-3 day (104.21%), 2-4 day (103.21%), and 3-5 day (105.24%) relative to the percent of the untreated control (Figure 3F). There are no statistically significant changes in DAPI in the experimental condition relevant to the control conditions.

Next to see if the changes I am seeing at the protein level using NCC markers PAX7 and SOX10 are consistent at the transcript level I checked for the presence of NCC markers which include: *FOXD3*, *MSX1*, *PAX3*, *PAX7*, *SNAI2*, *SOX10*, *TFAP2*, and *ZIC1*. From here I found a statistically significant decrease in all of the tested markers at D5 in the 0-1 D treated condition, the fold changes of the treated conditions relative to the control conditions expressed as log10 are

as follows*: FOXD3* (-1.97), *MSX1* (-1.85), *PAX3* (-2.40), *PAX7* (-1.65), *SNAI2* (-1.84), *SOX10* (-1.18), *TFAP2* (-1.60), and *ZIC1* (-1.57) (Figure 3G).

To confirm the results, I found from the YC3-110 inhibitor, I used a second chemical inhibitor Q7CA.[364] Q7CA, a EPHB3 inhibitor, was used to test the same time points of EPHB3 inhibition as YC3-110 was. First I used it for the duration of the induction protocol, from 0 to 5 days (Supplemental Figure 1A), next I used it to check for different time points of EPHB3 inhibition: 0-1 day, 0-2 day, 0-3 day, 1-3 day, 2-4 day and 3-5 day (Supplemental Figure 1B), and found the same trend as YC3-110. Immunofluorescence demonstrates that the EPHB3 inhibitor must be added from day 0, to get inhibition of NCC formation, when it is added starting at day 1, 2, or 3, NCC induction is not diminished to the same degree (Supplemental Figure 1C). Next, cell counts were done to determine that there is a statistically significant percent decrease in PAX7 and SOX10 (Supplemental Figure 1D and 1E). In terms of PAX7, using cell counts show that when the inhibitor is used from 0-1 day (80.56%), 0-2 day (77.37%), 0-3 day (55.92%), 0-5 day (55.42%), 1-3 day (63.65%), 2-4 day (80.24%), and 3-5 day (99.08%) relative to the percent of the untreated control (Supplemental Figure 1D). Through cell counts the data shows that SOX10 results are as follow: 0-1 day (42.34%), 0-2 day (34.83%), 0-3 day (26.3%), 0-5 day (23.78%), 1-3 day (47.13%), 2-4 day (68.83%), and 3-5 day (98.3%) relative to the percent of the untreated control (Supplemental Figure 1E). Both in the case of PAX7 and SOX10 statistically significant changes are seen only for 0-1 day, 0-2 day, 0-3

day, and 0-5 day. The DAPI cell counts confirm that the reduction in NCC induction is not due to a decrease in cell counts. DAPI results are as follow: 0-1 day (103.18%), 0-2 day (101.40%), 0-3 day (96.27%), 0-5 day (101.06%), 1-3 day (99.36%), 2-4 day (95.47%), and 3-5 day (102.09%) relative to the percent of the untreated control (Supplemental Figure 1F).

To validate that the concentration of YC3-110 being used do not have an effect on ESC pluripotency I treated the ESC with YC3-110 for 0-1 day and 0-5 days, the cells were then fixed and analyzed through the use of immunofluorescent microscopy, at day 5 for SOX2 and OCT4. The results, via immunofluorescence and cell counts suggest that YC3-110 does not affect OCT4, SOX2, nor DAPI levels (Supplemental Figure 2B). I then went onto do cell counts using the immunofluorescent images, and no statistically significant changes between ESC treated with YC3-110 and those not treated with YC3-110 are found in OCT4 for cells treated for 0-1 days (101.89%) and 0-5 days (100.96%) (Supplemental Figure 2C), nor for SOX2 0-1 days (99.71%) and 0-5 days (102.87%) (Supplemental Figure 2C). Also, there was no statistically significant change in DAPI counts 0-1 days (99.79%) and 0-5 days (103.15%) (Supplemental Figure 2E). Overall the data suggests that the concentration used to abrogate NCC induction does not affect ESC marker expression. Next I carried out qPCR to check for pluripotency markers: *OCT4*, *SOX2*, *NANOG*, *KLF4*.[365] From qPCR the data on a log10 scale the data shows that *OCT4* (0.02), *SOX2* (0.08), *NANOG* (0.11), and *KLF4* (0.01), I found that *NANOG* has a minor statistically significant

change, whereas the other pluripotency markers are unaffected, suggesting that overall there is no effect on pluripotency markers (Supplemental Figure 2F). Data collected from qPCR done on ESC following treatment with YC3-110 suggest that overall treatment using the drug at the concentration the experiments were carried out at in NCC does not have a significant impact on ESC.

Lastly, I treated ES cells with Q7CA and the effects are similar to those from LM10 and YC3-110 for 0-1 day and 0-5 day treatments, they are fixed and analyzed through immunofluorescent microscopy at D5 for SOX2 and OCT4, and by using immunofluorescence I was able to determine that neither OCT4, SOX2, nor DAPI levels are effected (Supplemental Figure 2A). Using cells counts I found that there is no statistically significant changes between ESC treated with Q7CA and not treated with Q7CA, in OCT4 for cells treated for 0-1 days (104.82%) and 0-5 days (97.80%) (Supplemental Figure 2 B), nor for SOX2 0-1 days (105.76%) and 0-5 days (97.80%) (Supplemental Figure 2C). Additionally, there is no statistically significant change in DAPI counts 0-1 days (105.34%) and 0-5 days (97.73%) (Supplemental Figure 2D). Overall the data suggests that the concentration of Q7CA used to diminish NCC induction does not affect the tested ESC markers.

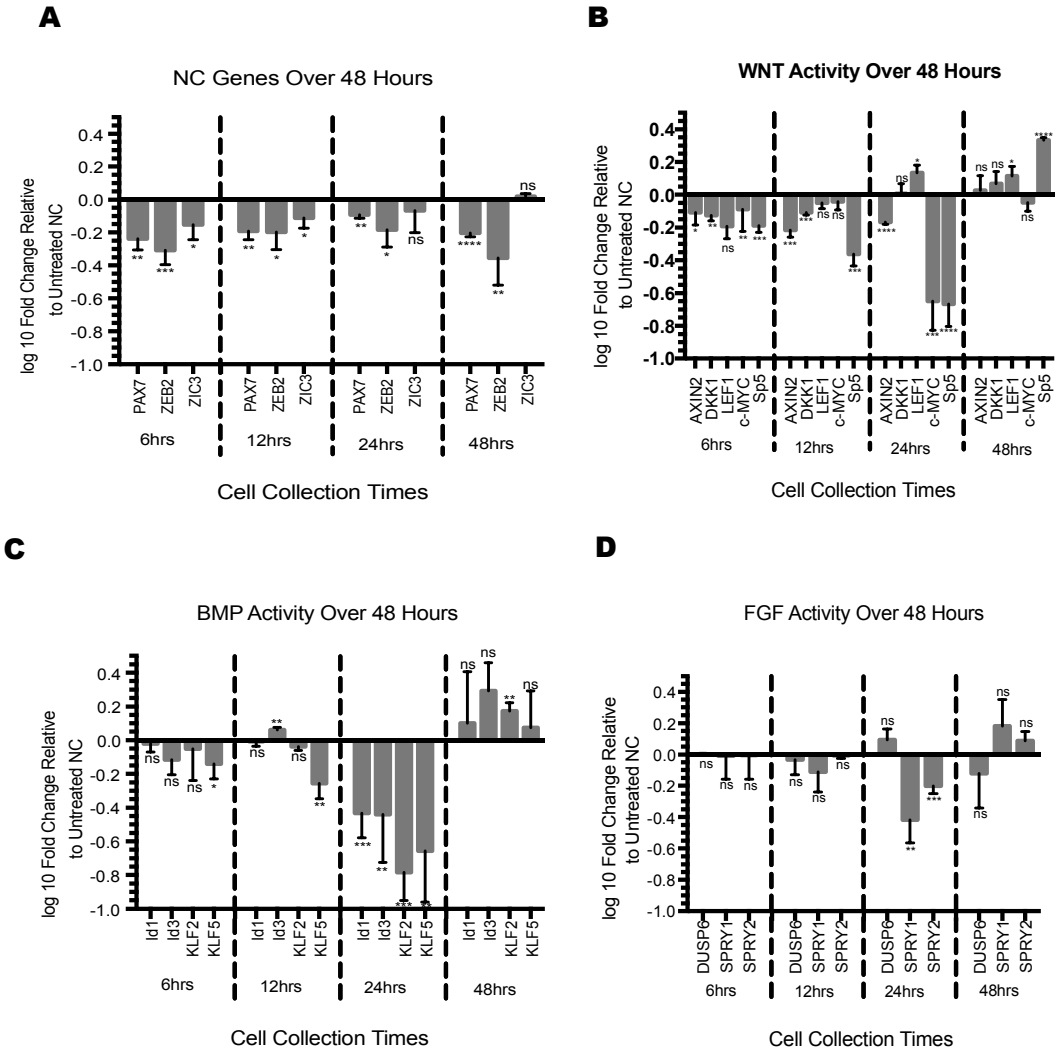

A
NC Genes Over 48 Hours
log 10 Fold Change Relative to Untreated NC
PAX7
ZEB2
ZIC3
6hrs
12hrs
24hrs
48hrs
Cell Collection Times
B
WNT Activity Over 48 Hours
log 10 Fold Change Relative to Untreated NC
AXIN2
DKK1
LEF1
c-MYC
Sp5
6hrs
12hrs
24hrs
48hrs
Cell Collection Times
C
BMP Activity Over 48 Hours
log 10 Fold Change Relative to Untreated NC
Id1
Id3
KLF2
KLF5
6hrs
12hrs
24hrs
48hrs
Cell Collection Times
D
FGF Activity Over 48 Hours
log 10 Fold Change Relative to Untreated NC
DUSP6
SPRY1
SPRY2
6hrs
12hrs
24hrs
48hrs
Cell Collection Times

Figure 4: YC3-110 Inhibitor (1uM): Early Neural Crest Genes and WNT, BMP and FGF Activity

A) QPCR data showing changes in early neural crest markers (*PAX7, ZEB2*, and *ZIC3*) at 6 hours, 12 hours, 24 hours, and 48 hours of collection respectively, all cells were treated with YC3-110 for the first 24 hours of the induction protocol.
B) QPCR data depicting changes in WNT targets (*AXIN2, SP5, LEF1*, and c-*MYC*) at 6 hours, 12 hours, one day and two day of collection, respectively. All cells were treated with YC3-110 for the first 24 hours.
C) QPCR data presenting variations in FGF targets (*DUSP6, SPRY1*, and *SPRY2*) at 6 hours, 12 hours, one day and two days, all cells were treated with YC3-110 for the duration of the culture.
D) QPCR data showing alterations in BMP targets (*Id1, Id3, Klf2*, and *Klf5*) at 6 hours, 12 hours, one day and two days, cells were treated for 24 hours with the YC3-110 molecule.

After determining that the first 24 hours EPHB3 activity is crucial for NCC induction, I went on to further analyze the 0-1 day treated YC3-110 samples via qPCR. To investigate why there was a reduction in NCC induction I went on to determine whether any of the early NCC markers are affected, I looked at *PAX7, ZEB2,* and *ZIC3.*[366,367368] At 6 hours I found that on a log10 scale *PAX7*(-0.24), *ZEB2*(-0.31) and ZIC3(-0.15) are statistically significantly downregulated. At 12 hours all three are downregulated as well, *PAX7*(-0.19), *ZEB2*(-0.20) and *ZIC3*(-0.11) are statistically significantly downregulated. At day 1 all three are downregulated, *PAX7*(0.19*), ZEB2*(-0.20) and *ZIC3*(-0.11) are statistically significantly downregulated. At day 1, day 1 data shows that *PAX7*(-0.20) and *ZEB2*(-0.35) are downregulated statistically significantly, but *ZIC3*(0.02) has no statistically significant change.

Studies done in *Xenopus,* chicken, mice, and zebrafish embryos, and hNCC based on hESC provide evidence that WNT, FGF, and BMP are critical in cranial NCC formation.[369]During the formation of the neural plate border, WNT and FGF interactions are crucial.[370,371,372] Then during neurulation, BMP and WNT are needed for activation and maintenance of transcription factors that are crucial for the specification of NCC.[373,374,375]

It has been shown that WNT activation is necessary for NC cell induction. Taking this into consideration I ran qPCR for direct targets of WNT, which include *AXIN2, SP5, DKK1, LEF1*, and *c-MYC.*[376,377] at 6 hours, 12 hours, one day and two days. At 6 hours I saw a statistically significant decrease measured in log10

fold change in *AXIN2* (-0.11), *DKK1*(-0.13), *c-MYC* (-0.09.), *SP5* (-0.19); however, *LEF1* (-0.2) shows a statistically significant increase relative to *LEF1* in the control condition. At 12 hours there is a statistically significant decrease in *AXIN2* (-0.21), *DKK1*(-0.11), *SP5* (-0.36); however, *c-MYC* (-0.04), and *LEF1* (-0.05) shows a statistically significant increase relative to *LEF1* in the control condition. At 24 hours there is a statistically significant decrease in *AXIN2* (-0.17), *c-MYC* (-0.65), *SP5*(-0.67), a statistically significant increase in *LEF1*(0.13), and no statistically significant change in *DKK1*(0.01). At 2 days there is a statistically significant increase measured in fold change in S*P5* (0.33), a statistically significant increase in *LEF1*(0.12), and no statistically significant change in *SP5*(0.33), *DKK1*(0.07) nor *c-MYC* (-0.05) (Figure 4B).

WNT, BMP and FGF have been shown to play a role in determining the NCC fate.[378,379,380,381,382,383] Using samples collected at 6 hours, 12 hours, one day and two days from the group treated YC3-110, we went on to assess whether BMP and FGF were. First, I looked at targets of BMP, which include *Id1, Id3, Klf2* and *Klf5*.[384] At 6 hours there is a statistically significant decrease *Klf5*(-0.14), however there is no statistically significant change in *Id1* (-0.02), *Id3*(-0.12), nor *Klf2*(-0.05) (Figure 4C). At 24 hours there is no statistically significant change in *Id1*(-0.01), *Klf2*(-0.04), there is a statistically significant increase in *Id3*(0.06), and a statistically significant decrease in *Klf5* (-0.26).

At 48 hours there is a statistically significant decrease in *Id1*(-0.43), *Id3*(-0.44), *Klf2*(-0.78) and Klf5(-0.66). At 2 days there is a statistically significant increase in Klf2(0.17), but no statistically significant increase in *Id1*(0.10), *Id3*(0.29) nor *KLf5*(0.07).

Lastly, I looked at the targets of FGF: *DUSP6, SPRY1*, and *SPRY2*. [385,386] Here I found that there is no statistically significant change in *DUSP6*(0.00), *SPRY1* (-0.01), nor *SPRY2* (-0.00) at 6 hours. At 12 hours that there is no statistically significant change in *DUSP6*(-0.03), *SPRY1* (-0.11), and *SPRY2* (-0.00). At 24 hours that there is no statistically significant change in *DUSP6*(0.09), there is a statistically significant decrease in *SPRY1* (-0.42), and *SPRY2* (-0.20). At 2 days there is no statistically significant change in *DUSP6*(-0.12), *SPRY1* (0.18), nor *SPRY2* (0.08) (Figure 4D). These results suggest that that EPHB3 inhibition leads to diminished NCC induction because WNT, BMP and FGF are all effected.

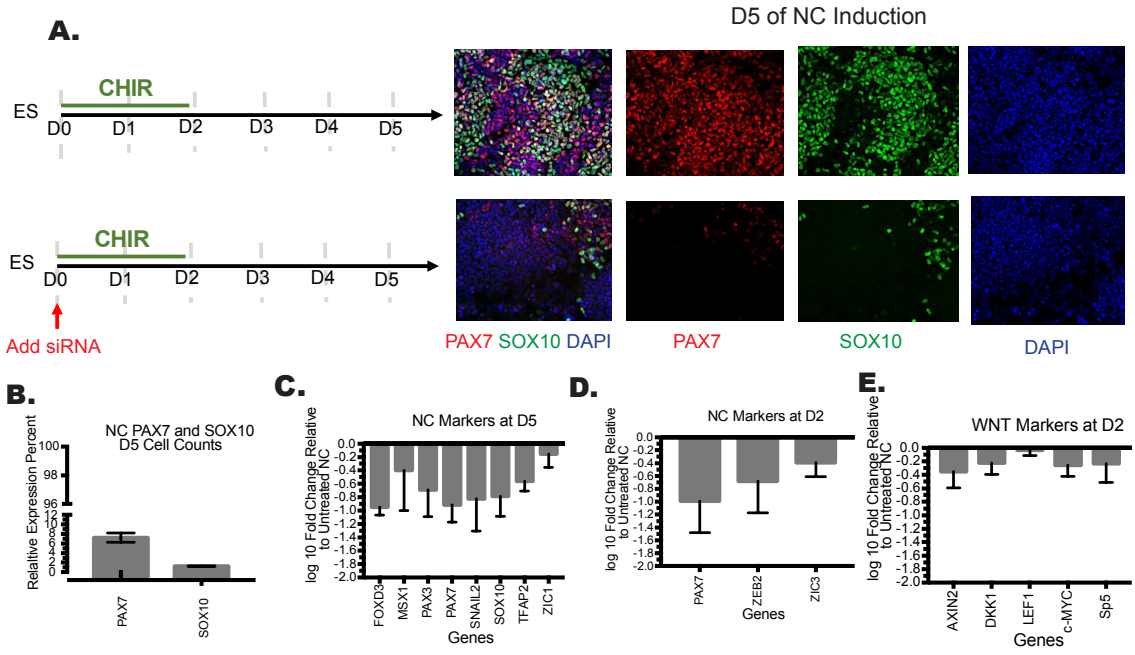


Figure 5: NCC Induction is Inhibited by siRNA Knockdown of EPHB3

A) A diagram showing a NCC induction with and without the addition of siRNA. The first row shows a control induction done a scrambled siRNA, the second row shows a NC induction carried out with the addition of siRNA to knockdown EPHB3. Immunofluorescent images showing expression of neural crest markers *PAX7*(red), *SOX10*(green) and nuclear stain DAPI(blue).

B) Cell count data showing the expression of PAX7 and SOX10 expression in cells treated with siRNA, relative to a control NCCs.

C) QPCR data presenting changes in fold change of neural crest markers (*FOXD3, MSX1, PAX3, PAX7, SNAIL2, SOX10, TFAP2,* and *ZIC1*). Cells were collected at day 5 of the induction.

D) QPCR data depicting changes in WNT targets (*AXIN2, SP5, LEF1*, and *c-MYC*) at day 2 of collection. Gene fold is calculated relative to untreated cells collected at D2 of the NCC protocol.

E) QPCR data showing changes in early neural crest markers (*PAX7, ZEB2*, and *ZIC3*) at day 2 of collection. The gene fold is determined relative to D2 collection untreated NCC.

Next I used siRNA to look at the effects of *EPHB3* knockdown. Through the use of immunofluorescence, I was able to find that that PAX7 and SOX10 expression in the treated condition is diminished relative to the control condition (Figure 5A) on cells collected at D5 of the NCC induction. Next using the Nikon elements software, I was able perform cell counts to quantify the presence of PAX7 and SOX10 positive cells and the data shows that they are reduced to 8.8% and 2.9%, respectively (Figure 5B), the DAPI cell counts are unchanged (data not shown). On samples collected at D5, through qPCR analysis the data shows that *FOXD3* (-0.86), *MSX1*(-0.26), *PAX3*(-0.40), *PAX7* (-0.73), *SNAI2* (-0.48), *TFAP2* (-0.66) and *ZIC1*(-0.13) are decreased (Figure 5C). Using siRNA, a reduction in the early NCC cell markers *PAX7* (-0.65) *ZEB2* (-0.33), and *ZIC3* (-0.24) (Figure 5D) in the qPCR data on samples collected at day 2 is seen. At day 2, there is a reduction in *AXIN2*(-0.18), *DKK1*(-0.09), *LEF1*(0.02) and *c-MYC* (-0.13), and SP5 (-0.3) (Figure 4E). Overall, through the use of siRNA to knockdown EPHB3 I found that NCC formation is inhibited. When either small molecules, or siRNA are used against EPHB3, it leads to a decrease in NCC formation, if the inhibition was started from day 0.

Discussion

Neural crest cells (NCC) give rise to many derivatives of the body which include the peripheral nervous system, pigment cells, and tissues of the craniofacial skeleton, such as bone, cartilage and connective tissue. Due to the wide range of NCC contributions, abnormalities in their formation, migration, differentiation

and/or maintenance can lead to many pathologies, which are broadly referred to as neurocristopathies. A deeper understanding of the signaling involved in NCC formation would provide new insights, which would contribute to therapies that address these pathologies.

Studies carried out in mouse, zebrafish, *Xenopus* and chick embryos, have addressed the signaling contributions responsible for NCC formation, contributions of the BMP, FGF and WNT pathways have been elucidated.[387,388] Significant effort has been put into mapping out the gene regulatory network necessary for the formation of vertebrate NCC, still much more remains to be uncovered. Much progress has been made in understanding the role of NCC in model organisms, but human embryo studies lag considerably behind, due to technical and ethical limitations, human embryonic stem cell based models offer an appealing alternative to advance our understanding of human NCC development. Cell surface receptors such as EPH receptors have been shown to play an important role during migration. The role of EPH receptors has been studied in the context of axon guidance, plasticity in neuronal connections, at sites of nervous system injury, in glucose homeostasis and diabetes, bone maintenance and bone remodeling diseases amongst others,[389] but not in early NCC induction.

To date, this is the first study, to the extent of my knowledge that studies EPH in the context of early hNCC formation from hESC. Here I describe, for the first time, studies addressing the role of EPH receptors during early NCC

development, focusing on the early stages of their formation. I report that EPH receptors and their EFN ligands are dynamically expressed during hNCC formation. I found that EPH function is critical for the early steps in hNCC formation. Specifically, *EPHB3* is upregulated early on in NCC formation, and its inhibition either through small molecules or through siRNA knockdown strategies prevents NCC formation.

In this study I explored the role of EPH receptors, while focusing on EPHB3 in the NCC induction process. Here, I have shown that that EPHB3 plays an essential role in NCC induction within the first 24 hours. Additionally, this data suggests that aberrations in EPHB3 disrupt WNT, BMP and FGF signaling in early NCC induction. Taken together this data introduces a new player to the balance of early NCC induction, EPHB3.

EPHB3 has the highest expression change during early NCC induction so I went on to do a time course treatment and found that it is essential to have EPH activation starting from D0 of induction, if EPHB3 the inhibition is initiated after the D1 (defined as at the 24-hour mark), significant NCC inhibition will not be seen. Next I went onto determine whether this is in conjunction with WNT, BMP, and FGF or if it occurs independent. These signaling molecules lead to up-regulation of transcription factors in early NCC precursors, included in the list of these transcription factors is *PAX3, PAX7, ZIC1* and *MSX1/2*.

This initiates markers that specify NCC, such as *SOX10, FOXD3*, and *AP2*.[390] Taking this information into consideration I decided to look at targets of WNT, BMP and FGF and found that the WNT, BMP, and FGF pathways are affected by EPHB3 inhibition.

In general, when an EFN binds to an EPH, receptor ligomerization results in autophosphorylation on many of the cytoplasmic domains, which includes the juxta-membrane region, the kinase domain and the SAM domain.[391] To determine whether inhibiting EPH would inhibit NCC induction, I started by using a general EPH inhibitor, LM10. 20% of the human kinases possess a small gatekeeper residue, a threonine reside (Thr693)[392] which includes all EPH, with the exception of EPHA7. Lm10 is a quinoxaline-based inhibitor, it inhibits phosphorylation of the tyrosine kinases, which prevents EPH activation, amongst others.[393] It is possible that that inhibition of other RTK might be contributing to this effect.

Data shows that when the general inhibitor is used starting from day 0, NCC formation is inhibited. When LM10 is used for the entirety of the NCC cell induction protocol, there is a significant decrease in the presence of DAPI in addition to a decrease in PAX7, and SOX10 which means that cell survival and/or proliferation are negatively influenced. LM10 has been shown to bind strongly with tyrosine kinases that have threonine as the gatekeeper, these targets include EPHA/B, but also include KIT, LCK, SRC, YES, DDR1, BRAF, VEGF-A and ABL1/2.

This means that the proliferation and survival could be affected by a disruption in one or multiple of these pathways alongside the dysregulation of the EPH.

Next, we went on to use a specific inhibitor for EPHB3,[394] YC3-110. There is a cysteine in the hinge region of the EPHB3 kinase domain, which is unique to EPHB3 and not a commonality amongst other human kinases, this feature is shared only with two other kinases, LKB1 and PINK1 in the human genome. YC3-110 is an electrophilic quinazoline which targets the cysteine in the hinge region of the EPHB3 kinase domain. However, the cysteine residue in LKB1 and PINK1 is predicted to point away from the active site, making the cysteine region much less accessible than the cysteine of the EPHB3, which makes the potency of the YC3-110 highly selective for EPHB3. Using YC3-110 we are able to find that the specific inhibition of EPHB3 leads to diminished NCC induction. Unlike LM10 the use of YC3-110 for the duration of the NCC induction protocol does not lead to a decrease in cell count. However, I found that the first 24 hours are the most crucial for EPHB3 activity. If the inhibitor is not added at day 0, we do not see a significant decrease in NCC markers, supporting the idea that the first 24 hours are critical for NCC specification, suggesting that NCC induction is most susceptible to abrogation in the early stages. If the inhibitor is added at day 0 there us a decrease in the proportion of SOX10 positive cells.

Next we used a second EPHB3 inhibitor to verify that EPHB3 is necessary for NCC formation. Q7CA is an electrophilic quinazoline that can cross the plasma membrane and reach the cytoplasmic kinase domain of EPHB3 in live cells and

covalently modify C717. Q7CA irreversibly inhibits EPHB3 by covalently modifying C717. The results are similar to what is found with the use of YC3-110. The inhibition of PAX7 and SOX10 is stronger with the use of Q7CA relative to YC3-110. The caveat being Q7CA also inhibits EGFR, so the results could be in part due to the inhibition of EGFR. The same trends are seen through the use of Q7CA as are seen with YC3-110.

The effects of blocking EPHB3 activity through the use of small molecule inhibitors is confirmed through the use of siRNA to knockdown EPHB3. The use of siRNA validates specificity, siRNA begins to affect RNA immediately, but effects on the protein are not seen until later. The trends seen using siRNA confirm the importance of EPHB3 in NCC induction.

Cells treated with YC3-110 for 0-1 days and collected at day 5 were used for further analysis. At day 5 I found that a wide range of NCC genes are expressed in normal NCC induction. The early NCC genes: PAX7, PAX3, MSX1 and TFAP2A are expressed. [395,, 396,,397,,398] Additionally, PAX7, TFAP2A and SOX10 are traditionally expressed in NCC derived from human embryonic stem cells.[399] NCC genes, *ZIC1, FOXD3,* and *SNAI2* are expressed.[400] Taking this information together with the results where I saw that all NCC gene expression is significantly reduced by day 5 suggesting that, EPHB3 is crucial for early NCC induction. Looking at early NCC genes *PAX7, ZEB3*, and *ZIC3*[401] I found that using an EPHB3 inhibitor, *PAX7* expression is decreased, but *ZEB2* and *ZIC3* expression were not. *PAX7* is one of the earliest NCC markers, and is expressed at the

border of the developing neural plate.[402,403]. *ZIC3* and *ZEB2* expression peaks at the pre-neural border stage;[404,405] however, by day1 they are not affected. This suggests that the EPHB3 inhibitor has perturbed NCC induction early during the process in a manner that affects *PAX7*.

WNT activity in hESC leads to differentiation, and is crucial in initiating NCC development from hESC.[406] *SP5* and *AXIN2* are direct targets of ß-catenin.[407,408] *C-MYC* expression is regulated by ß-catenin.[409] We see that there is a decrease in *AXIN2, SP5* and *c-MYC* which suggests that WNT/ß-catenin signaling is decreased. *GBX2* is directly activated by WNT/β-catenin signaling, the results show that there is a decrease in GBX2, which indicates a decrease in WNT/ß-catenin signaling.[410] WNT inhibitor Dickkopfl1(*DKK1)* is a target of ß-catenin/TCF mediated transcription, the results that show that there is no significant change in *DKK1* due to the inhibition of EPHB3, suggesting that although the targets of WNT/B-catenin are decreased it is not due to the Wnt antagonist *DKK1.*[411] When WNT signaling is inactive, *LEF1* is bound to Groucho-related co-repressors, in this form it negatively regulates the expression of WNT signaling genes.[412] *LEF1* is a downstream mediator of the WNT/β-catenin signaling pathway, additionally it can modulate gene transcription independently of WNT.[413] The results suggest that EPHB3 leads to dysregulation of WNT/ß-catenin activity which leads to a decrease in NCC induction.

Numerous studies have shown that WNT signaling is needed for NCC induction, but the exact details explaining how WNT signaling works to activate NCC transcription factors is still largely unknown. Studies have started to address this gap in knowledge. In chick it has been shown that *AXUD1*, a transcription factor, works downstream of WNT and directly regulates *FOXD3* expression, through interactions with *MSX1* and *PAX7*, which are neural plate specifiers.[414] A study in *Xenopus* has shown that DKK2, a wnt antagonist, works through the LRP5/6 receptor and activates ß-catenin which is needed for NCC specification.[415] Another study in *Xenopus* has demonstrated that *hes3*, a transcription factor, negatively regulates WNT signaling.[416]

BMP activity has been shown to be essential for NCC induction and maintenance. *Id* and *Klf* genes have been implicated in the self-renewal of cells, and studies have shown that they are direct targets of BMP.[417,418,419] To this effect, I looked at the expression of *Id1, Id3, Klf2*, and *Klf5* in cells that underwent the NCC induction protocol, these cells were subjected to YC3-110 for the first day of induction and then they were collected at day 5. I found dysregulation of these BMP targets, most significantly at 24 hours into the induction protocol, which suggests that disruption of EPHB3 signaling in early NCC development leads to dysregulation of NCC formation.

Mammalian systems have been used to investigate the role of BMP signaling in NCC induction. In inducible knockout mice, which were derived by Wnt1-cre or Pax3-Cre it has been shown that a knockout of the BMP receptors Alk1/3/5 or Tgfbr2 leads to defects in regions derived from NCCs, such as cardiac, pharyngeal and the craniofacial region.[420,421] At early stages (E8.5), neural plate border genes (*Msx1/2, AP2a, Pax3*, and *Sox9*) are not affected, but by E9.5 their expression is downregulated. Additionally, a NCC model originating from embryonic stem cells via WNT signaling activation has shown that BMP regulation is necessary for NCC induction.[422] Taking the data together from the different model systems, it can be deduced that first WNT and FGF signaling must be activated for initial induction of NCC, and then WNT and BMP are needed for the progression of NCC induction.[423]

Studies show the significance FGF plays in NCC induction. *DUSP6,*[424] *SPRY1* and *SPRY2*[425] are direct targets of FGF. I studied the expression of *DUSP6, SPRY1* and *SPRY2* in day 5 cells that had undergone the NCC induction protocol in conjunction with the EPHB3 inhibitor and found that FGF activity was dysregulated at day 1 and day 2, more strongly at day 1. These results suggest that inhibition of EPHB3 leads to dysregulation of the FGF pathway.

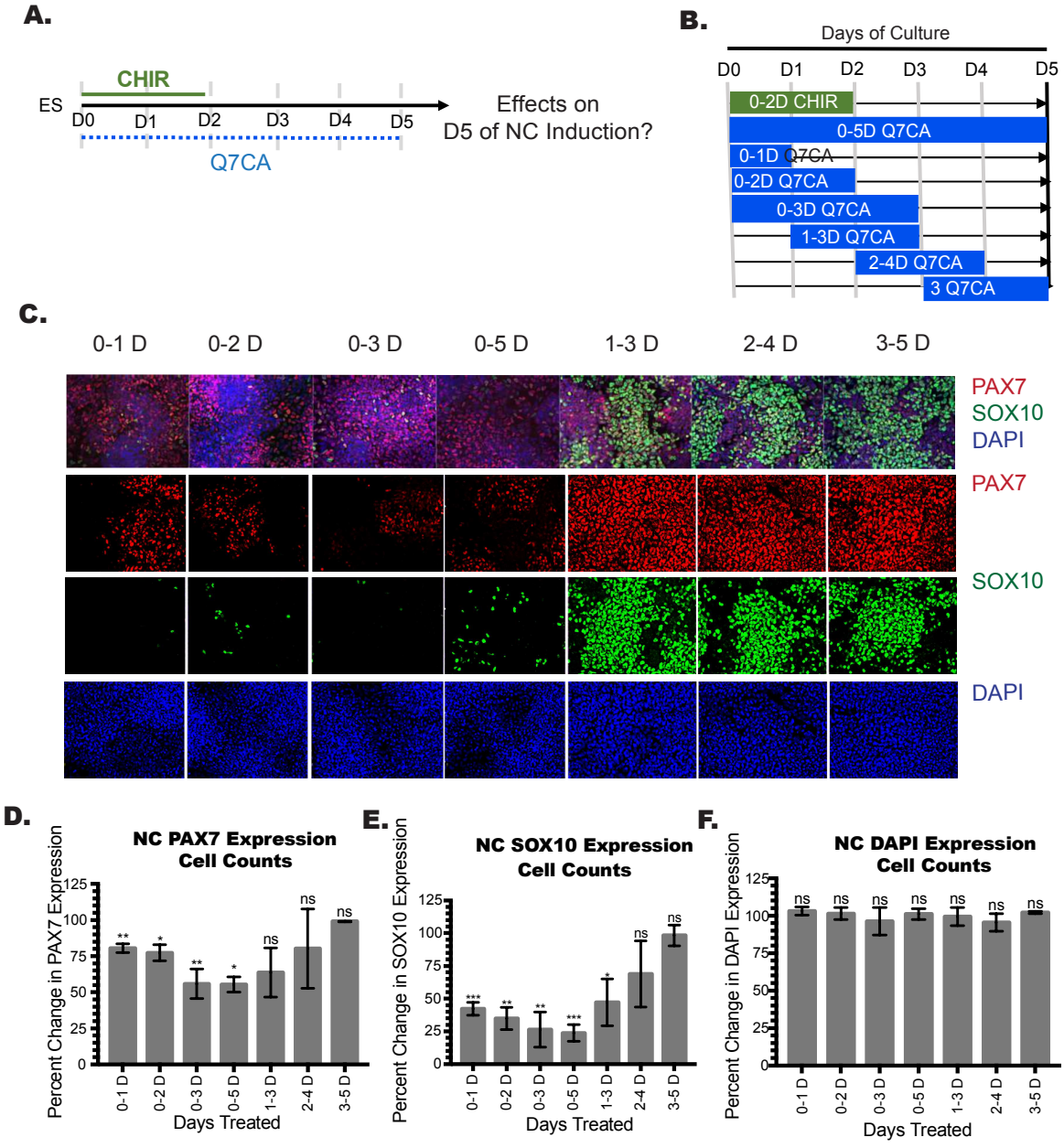
Studies using chicken embryos have shown that FGF signaling is essential for NCC induction. When FGF signaling is inhibited, in the gastrula epiblast, specifically in the neural plate border region, expression of the NCC marks *Pax7*

and *Snai2* ais inhibited. When FGF signaling is inhibited after gastrulation, *Pax7* and *Snai2* are not inhibited. Studies suggest that FGF/MAPK plays a direct role in the formation of the cells at the neural plate border, and provides evidence that ERK signaling is needed during NCC formation.

In a study done in rabbit, explants that were taken from gastrula rabbit embryos at the prospective NCC region, and it was found that they were unable to express Pax7 and Sox10, which are NCC markers, when an FGF inhibitor was added.[426] Another study in which FGF signaling was inhibited in a hESC derived NCC model, NCC induction was inhibited, despite the presence of Wnt signaling.[427] The precise role of FGF signaling in zebrafish and mouse embryos remains to be established.

Overall, the data suggests that, EPHB3 receptors display dynamic expression during early human NCC formation. Inhibition of EPHB3 via siRNA or with the use of a small molecule specific inhibitor abrogates NCC formation. The sensitivity to EPHB3 function seems to be highest during the first day of NCC formation. The inhibition of EPHB3 in this model leads to a reduction of WNT signaling output and BMP and FGF dysregulation. However, we cannot be certain that the pathway is being dysregulated as an effect of EPHB3 inhibition, further studies would need to be conducted to determine the mechanism by which this occurs. Understanding this may lead to a more efficient NCC induction, which would allow for the mass production of NCC needed to make

NCC derivatives for implantable tissues. A better understanding of the role of EPH in NCC induction may result in improved gene therapies, and the construction and integration of implantable tissues, such as the oral palate.


A.
CHIR
ES
D0
D1
D2
D3
D4
D5
Q7CA
Effects on
D5 of NC Induction?
B.
Days of Culture
D0
D1
D2
D3
D4
D5
0-2D CHIR
0-5D Q7CA
0-1D Q7CA
0-2D Q7CA
0-3D Q7CA
1-3D Q7CA
2-4D Q7CA
3 Q7CA
C.
0-1 D
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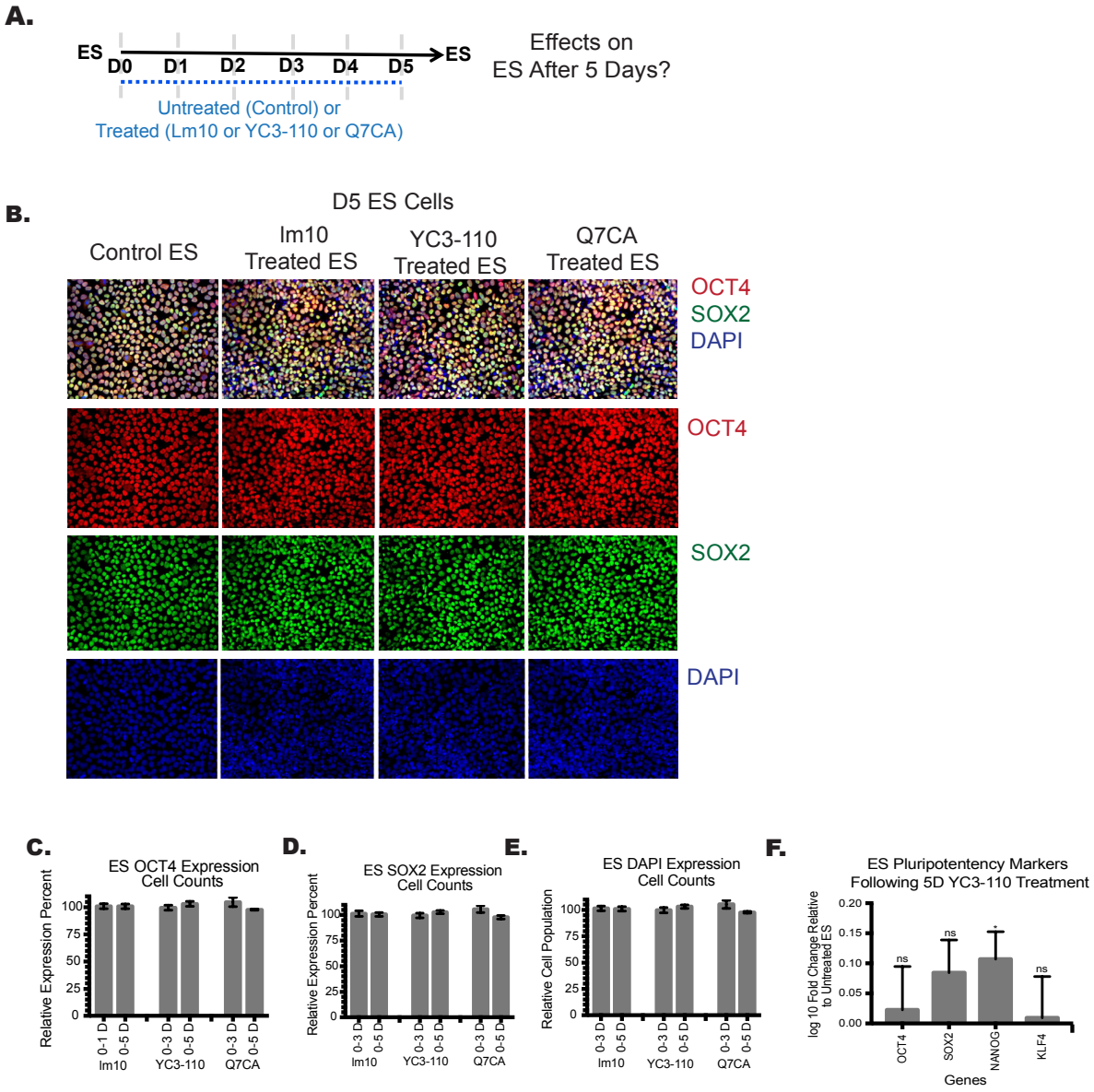
0-3 D
0-5 D
1-3 D
2-4 D
3-5 D
PAX7
SOX10
DAPI
PAX7
SOX10
DAPI
D.
NC PAX7 Expression
Cell Counts
Percent Change in PAX7 Expression
Days Treated
E.
NC SOX10 Expression
Cell Counts
Percent Change in SOX10 Expression
Days Treated
F.
NC DAPI Expression
Cell Counts
Percent Change in DAPI Expression
Days Treated

Supplemental Figure 1: NCC Induction is Inhibited by EPHB3 Inhibitor (Q7CA used at 500nm)

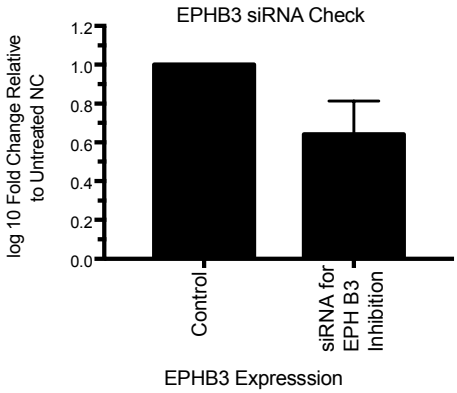
A) A diagram showing the induction protocol and the treatment of cells for 5 days with the EPHB3 inhibitor, Q7CA.
B) An illustration drawing out the time points that the cells were treated with the specific EPHB3 inhibitor, YC3-110, for the time points as follows: 0-1 days, 0-2 days, 0-3 days, 0-5 days, 1-3 days, 2-4 days, and 3-5 days.
C) Immunofluorescence microscopy showing expression of neural crest markers PAX7(red) and SOX10(green), and cells are marked with the nuclear stain DAPI(blue). The images are 2x2 stitched images taken at 20x. Immunofluorescent microscopic data showing detectable EPHB3 at different stages of hNC induction.
D) Cell counts demonstrate expression of NCC marker PAX7 in NCC treated with the EPHB3 inhibitor 2 for different durations of time. The relative percent expression is determined in comparison with NCC not treated with the inhibitor. All cells were collected at D5
E) Cell counts were used to calculate changes in expression of SOX10 in NC cells treated with Q7CA for different time points, relative to untreated NCC, all samples were collected at D5.
F) DAPI was used to count the number of cells in the Q7CA treated conditioned and was compared to the DAPI cell counts in untreated NCC. Cell were fixed and cells were counted at D5 of culture.


A.
ES
D0
D1
D2
D3
D4
D5
ES
Effects on
ES After 5 Days?
Untreated (Control) or
Treated (Lm10 or YC3-110 or Q7CA)
B.
D5 ES Cells
Control ES
lm10
Treated ES
YC3-110
Treated ES
Q7CA
Treated ES
OCT4
SOX2
DAPI
OCT4
SOX2
DAPI
C.
ES OCT4 Expression
Cell Counts
Relative Expression Percent
100
75
50
25
0
0-1 D
0-5 D
0-3 D
0-5 D
0-3 D
0-5 D
lm10
YC3-110
Q7CA
D.
ES SOX2 Expression
Cell Counts
Relative Expression Percent
E.
ES DAPI Expression
Cell Counts
Relative Cell Population
F.
ES Pluripotentency Markers
Following 5D YC3-110 Treatment
log 10 Fold Change Relative
to Untreated ES
0.20
0.15
0.10
0.05
0.00
ns
ns
*
ns
OCT4
SOX2
NANOG
KLF4
Genes

Supplemental Figure 2: EPHB3 Inhibiton Does Not Effect ESC Pluripotency

A) A schematic showing ES cells for 5 days either untreated or treated with Lm10, Q7CA, or YC3-110.
B) Immunofluorescence microscopy showing expression of pluripotency markers OCT4 (red) and SOX2 (green), and the nuclear stain, DAPI(blue). The panels depict untreated or control ES, ES treated with lm10, YC3-110, and Q7CA. Cells were collected at D5 and were treated with the respective inhibitors for the entirety of the culture. The first panel shows 2x2 merged image stitched images taken at 20x, and the second row shows just OCT4 expression, the third shows SOX2 expression and the fourth shows DAPI.
C) Cell counts show OCT 4 expression in cells treated with the lm10, YC3-110, and Q7CA, respectively. The expression percent is calculated relative to untreated ES cells collected at D5 of ES maintenance culture.
D) Cell counts percentages calculated relative to untreated ES cells show *SOX2* expression in cells treated with the lm10, YC3-110, and Q7CA, respectively.
E) Cell counts are used to determine the relative cell populations in cells where the lm10, YC3-110, and Q7CA were used. Relative cell populations are determined in respect to untreated ES cells collected at D5.
F) QPCR data demonstrates fold changes in pluripotency markers *(OCT4, SOX2, NANOG*, and *KLF4*), in cells treated with YC3-110, fold change is determined relative to expression of the markers in untreated cells. All cells were collected at D5.

A.



Supplemental Figure 3: EPHB3 RNA Expression is Inhibited by siRNA Targeting EPHB3

A) QPCR data demonstrates fold changes in the expression of EPHB3 transcript levels in cells treated with siRNA to knockdown EPHB3 and in control cells, collected at day 2.

Table S1. List of Primers

Transcript	Forward	Reverse
PAX7	AACGACAGAACCCGACTATGTTC	CGGCGTTGGGTGGAAA
ZEB2	CCAAGAGAGGAAGAGGAAGATGAAA	TCTTCCTTCATTTCTTCTGGACCAT
ZIC3	TCTGCAAAGTGTGCGACAAGT	TGACCCTTGAGATTCATGAACCT
AXIN2	CGGGAGCCACACCCTTCT	TGGACACCTGCCAGTTTCTTT
SP5	CTTCGGGTGTCCATGCCTC	GTGCGGTCCTGGAGAAAGG
DKK1	GCACCTTGGATGGGTATTCCA	GCACAACACAATCCTGAGGC
LEF1	ATGTCCAGGTTTTCCCATCAT	CTGAGGTGTTACAATAGCTGG
MYC	GCCACGTCTCCACACATCAG	TCTTGGCAGCAGGATAGTCCTT
FOXD3	TCATCACCATGGCCATCCT	GGAAGCGGTTGCTGATGAAC
MSX1	AAGTTCCGCCAGAAGCAGTA	GCGGTTCTGGAACCATATCT
MSX2	CCAGGTCAAAATCTGGTTCC	GCATAGGTTTTGCAGCCATT
PAX3	GAACCCGGGCATGTTCAG	ACGGCACGGTGTTTCGA
PAX7	AACGACAGAACCCGACTATGTTC	CGGCGTTGGGTGGAAA
SNAIL2	GATCCTCAGCTCAGGAGCATACA	GGAGTATCCGGAAAGAGGAGA
SOX10	GAGGCTGCTGAACGAAAGTGA	GCGGCCTTCCCGTTCT
TFAP2	GAGAGTAGCTCCACTTGGGTG	GTCGTGACGGTCCTCGC
ZIC1	CAAAACTTTCAGCACCATGCA	TCCCAGAAGCAGATGTGATTA

Materials/Methods

2.1 hESC Maintance

HESCs are maintained in mTESR from Stem Cell Technologies (component #85851 and # SCBW6732) on Matrigel (Reference # 354277). Cells were passed using Versene from Gibco (Reference # 15040-66).

2.2 Neural Crest Induction

HESCs are dissociated into single cells using Accutase (Life Technologies), the cells are then counted and plated onto vessels coated with Matrigel (Corning) at a density of 20,000cells/cm2 in media containing DMEM/F12 (Thermo Fisher Scientific no. 175020048), supplemented with 1X serum-free B27 supplement (Invitrogen, Cat. No. 17504- 044), 1X Glutamax (Thermo fisher scientific, Cat. No. 35050061), 0.5% BSA (Sigma, A7979), a more detailed protocol can be found in the publication by Gomez, et al. In all conditions the GSK3 inhibitor, CHIR(TOCRIS) and Rock inhibitor were added for 48 hours.

2.4 Drug Treatments

The general EPH inhibitor LM-10 (graciously provided by Dr. Christina Nevado from the University of Zurich), YC3-110 and Q7CA were provided by Dr. Chao Zhang from the University of Southern California. LM-10(500nM), YC3-110(1uM) and Q7CA(500nM) were all resuspended in DMSO, the corresponding amounts of DMSO was used in the control conditions. Concentrations ranging from 10nM to 2uM, used concentrations were selected based on a consistency of DAPI counts between control and treated conditions, while PAX7 and SOX10

expression was diminished in the experimental conditions. At lower concentration of the inhibitor I did not see a significant decrease in PAX7 and SOX10 (data not shown).

2.5 siRNA

SiRNAs were purchased from Dharmacon (sp) and Lipofectamine RNAiMax reagent was purchased from Invitrogen. Reverse transfection technique was used, siRNA and Lipofectamine were added into the wells of 96-wells, before cells were added. A final concentration of 1.2 pMole of siRNA and 0.05μL of Lipofectamine was used per well. siRNA was diluted to 6 pMole in 100μL OptiMEM, and Lipofectamine was diluted to 0.5μL in 100μL of OptiMEM. H1 cells were accutased and the hNC induction protocol was carried out as described in Gomez et al. Prior to plating the cells the matrigel used to coat the wells was aspirated, and 10μL of Lipofectamine and 20μL of siRNA were added to each well. The Lipofectamine/siRNA mixture was incubated at room temperature for 10-20 minutes, and then the cells were plated. Cells were mixed at 8.9k cells/100μL and plated in 90μL (8k cells/well) with CHIR and Rock Inhibitor concentrations for 120μL (1.2x normal), which brought the Rock Inhibitor and CHIR levels to the normal protocal levels. Scrambled siRNA and Cy3-tagged siRNA were used as a control to check transfection efficiency.

2.6 Immunoflorescence

Cells were fixed with 4% paraformaldehyde for 10 minutes, after which they were permeablized with 0.4% Triton X-100 for 10 minutes. Cells were then blocked using 10% fetal bovine serum (FBS) with 0.05% Tween 20 for 1 hour at room temperature. Primary antibody incubations were done overnight at 4C (Supplemental Figure 1), followed by secondary which was done at room temperature for an hour. Immunostained cells were imaged using a Nikon Eclipse Ti microscope, the images were processed using Adobe Photoshop, and cell counts were carried out using Nikon Elements software. The software detects intensity levels of the immunostained cells which are then compared to a negative control which sets the threshold of what is counted as a immunostain positive cell. Antibodies used: Mouse anti- SOX10 (Santa Cruz Biotechnology, SC271163), 1:200. Mouse anti-PAX7 (1:10), Mouse anti- EPHB3 (Santa Cruz, sc-5141139) 1:150

2.7 Quantitative PCR

Total RNA was extracted using TRIzol reagent from Life Technologies (# 15596026). cDNA was generated from 500ng of RNA using the High Capacity cDNA Reverse Transcription kit (number RR014B) from Clonetech. SYBR Green from Applied Biosystems was used in the reactions, which were done on a One Step Real Time PCR Detection System from Applied Biosystems qPCR. Three biological replicates were used for drug treated samples, two biological replicates were assessed for siRNA treated samples.

Chapter 3: Tissue Engineered Organoid Model for Oral Palate Formation and Fusion

Abstract

Neural crest cells (NCC) contribute to many derivatives in the body, ranging from cells of the craniofacial region, such as cartilage and bone, to neurons of the peripheral nervous system[428]. Abnormalities in either the development or migration of NCC can result in a wide range of diseases. These are known as neurocristopathies and include cleft lip and cleft palate[429]. Oral clefts occur in 1 in every 700 live births [430]. An adequate model is needed to study the effects of gene mutations and toxins on human palate development, one that is preferably constituted of cells from the correct ontogeny. A model from a recent report utilizes mesenchymal cells from the umbilical cord and primary epidermal keratinocytes from juvenile donors,[431] rather than NCC as in the developing embryo. The pioneer study does not use the correct ontogeny for the origin of cell derivatives. To study oral palate formation and development, including causes of cleft palate we have developed a surrogate model of the embryonic human oral palate. Unique to our system, each cell type is generated from hESC via developmentally relevant pathways: osteocytes via a NCC intermediate, and epithelial cells from hESC via ectoderm. Here we report a model to study human palate development based on cells derived from biologically relevant ontogeny.

Introduction

A majority of the derivatives that make up the craniofacial region, such as the skeleton and the connective tissue in vertebrates arise from a multipotent cell population, known as cranial NCC.[432] When there is a dysregulation in the formation or migration of NCC a wide range of diseases and malformations known as neurocristopathies arise.[433] For proper formation of these structures it is essential that there is strict temporal and spatial control on a genetic, molecular and cellular level for the three-dimensional structures of the face.[434] A primary reason for this might be that there are many aspects that go into creating these craniofacial structures, it is a highly intricate process and the machinery that controls NCC formation, migration and differentiation requires strict control. The cranial NCC arise during embryogenesis in the dorsal neural tube and then migrate to the frontal process, after which they migrate to the first, second, third, and fourth pharyngeal arches.[435,436]

NCCs must interact with the ectoderm, neuroectoderm, and the endoderm in *vivo* for the craniofacial region to appropriately develop.[437,438,439] Any aberrations in this process can lead to craniofacial malformations. A feasible and efficient way to study the effects of NCC ontogeny in craniofacial develop is to use an in *vitro* model of human craniofacial development, which allows for isolated studies of the effects of NCC disease etiologies in a technically efficient, cheap and most importantly ethical manner.

NCC migration occurs primarily by communicating with surrounding tissues that offer instructive cues for migration.[440] Both attractive and repulsive signals are used in NCC migration events, the exact nature of these migration cues is not fully known. Both FGF2 and FGF8 have been shown to play a role in NCC migration. Additionally, ephrin ligands (EFN) and ephrin receptors(EPH) play a role in NCC migration. EPH are a subfamily of receptor tyrosine kinases, and their roles in cell activity include modulation of integrin, NCC formation and migration.[441,442,443,444]

During embryonic development the face of vertebrates is composed of the frontonasal prominence (FNP), mandibular prominence (MNP), maxillary prominence(MXP), and the paired lateral nasal (LNP) prominence. The FNP is responsible for the formation of the primary palate, the philtrum, the upper lip, the forehead and the nose.[445] NCC interact with the epithelia, the facial ectoderm and the forebrain to give rise to the FNP.[446] Typically, the LNP and the FNP will fuse with the MXP.[447] The MNP and the MXP come from the first pharyngeal arch. The MNP is responsible for the formation of the lower jaw and yhe MXP is responsible for the formation of the upper jaw. Development of the MXP and MNP relies on interaction of NCC, the surface ectoderm, pharyngeal endoderm and mesoderm.[448,449]

Later on two outgrowths from the MXP form the secondary palate. The MXPs flank the tongue, and they rise horizontally and arrange themselves over the tongue, grow, connect, adhere and fuse to form palatal shelves.[450,451,452] Cleft

palate, a neurocristopathy can occur when there are hinderances in the formation of the secondary palate as the palatal shelves have a NCC derived mesenchyme core.[453] This core is covered with an epithelial layer of ectodermal origin.[454] Between the two layers there is a basement membrane. Epithelial cells are enriched with extracellular matrix (ECM) proteins such as fibronectin, collagen, laminin, hyaluronic acid and integrins.[455] When the palatal shelves undergo fusion the NCC derived mesenchyme has gone down an osteogenic lineage, which separates the nasal cavity from the roof of the mouth. Upon fusion, the medial edge epithelium (MEE) is reduced to a single layer of basal cells.[456] At this point the palatal shelve in humans is about 150-200 microns thick.[457] When the palatal shelves come into contact during the fusion process the MEE of the two palatal shelves form the midline epithelial seam (MES) upon contact, and the MES is removed when fusion occurs.[458] If the mesenchyme is not removed and the mesenchyme derived osteoblasts do not merge, then cleft palate occurs. For proper palatogenesis many signaling events must be tightly regulated, these signaling events are largely mediated by epithelial-mesenchymal interactions. These signaling molecules include sonic hedgehog (SHH), bone morphogenic proteins (BMP), and growth factors such as fibroblast growth factor (FGF) and TGF.[459] FGF10 and SHH regulation is necessary for the outgrowth of the palatal shelves. MEE differentiation prior to fusion is largely regulated by FGF10 in conjunction with its downstream partners Jag2 and Irf6.[460] EFN have been shown to be involved in the adhesion of MEE of the two palatal shelves. Prior to

adhesion and fusion EFNB2 is highly expressed in the MEE.[461] It has been shown that TGFB3 plays an essential role in the fusion events.[462,463]

When regulatory factors are improperly regulated, palatal fusion can be negatively affected. Both epidermal growth factor(EGF), and epidermal growth factor receptor (EGFR), are present in the mesenchyme and the MEE right before adhesion and fusion of palatal shelves in humans and rodents.[464,465,466] EGF and EGFR are necessary for ECM production, proliferation and growth of the mesenchyme and the epithelium.[467] Right before the point of fusion EGFR levels goes down and the cells terminate their proliferative activities.[468,469] If EGF levels are high, then fusion events will be delayed. A study has shown that when TGFB3 levels are higher than EGF levels, fusion commences and when EGF levels are higher than TGFB3 levels fusion of the palatal shelves is delayed.[470] Cleft palate occurs as a result of dysregulation of any of these processes, or by any other aberrations in the fusion events. Many genetic and environmental factors can lead to cleft palate.[471,472] However, not all factors that lead to cleft palate have yet been explored. Typically, animal models or animal organ cultures are used in *vitro* to study these defects. Maintaining animal colonies are expensive, and take a long time, and the studies are not done in human due to technical and ethical complications. An alternative is the use of 3D cultures, especially if they come from the proper precursor cells, in this case NCC cells for the osteoblasts, and ectoderm for the epithelial cells.

I have engineered an oral palate model, that uses the correct components which allow for adequate morphogenesis. This model consists of 2 cell types: epithelial cells, and osteoblasts. I am able to individually differentiate these cell types and put them together as spheres resembling the palatal shelves to mimic palatal fusion. Unique to my system, each cell type is generated from hESC via developmentally relevant pathways: osteoblasts via a NCC intermediate, and epithelial cells from hESC. Upon differentiation of the desired cell fates, I put them together in 3D organoid type cultures, that I have termed palate spheres, which will allow us for the first time to investigate environmental and genetic contributions associated with palate development throughout its morphogenesis.

Results

The cranial neural crest is responsible for the formation of many head derivatives, such as the connective tissue, cartilage and bone.[473] At the start of development NCC segmentation and migration are mainly conserved between species. Further on in development there are many differences, as vertebrate facial morphologies are highly diverse. To obtain the intricate structures of the craniofacial region, there is a much interplay between genes, signaling pathways and extrinsic environmental signals. This necessitates an in *vitro* human embryonic organoid model for oral palate development.[474] The spatiotemporal patterning must be strictly regulated during craniofacial development. Unlike axial and appendicular and skeletal bone, bones of the craniofacial region are derived from NCC. Craniofacial bones go through intramembranous calcification, rather

than endochondral ossification.[475,476] To study oral palate formation and development, including causes of cleft palate I have developed a surrogate model of the embryonic human oral palate.

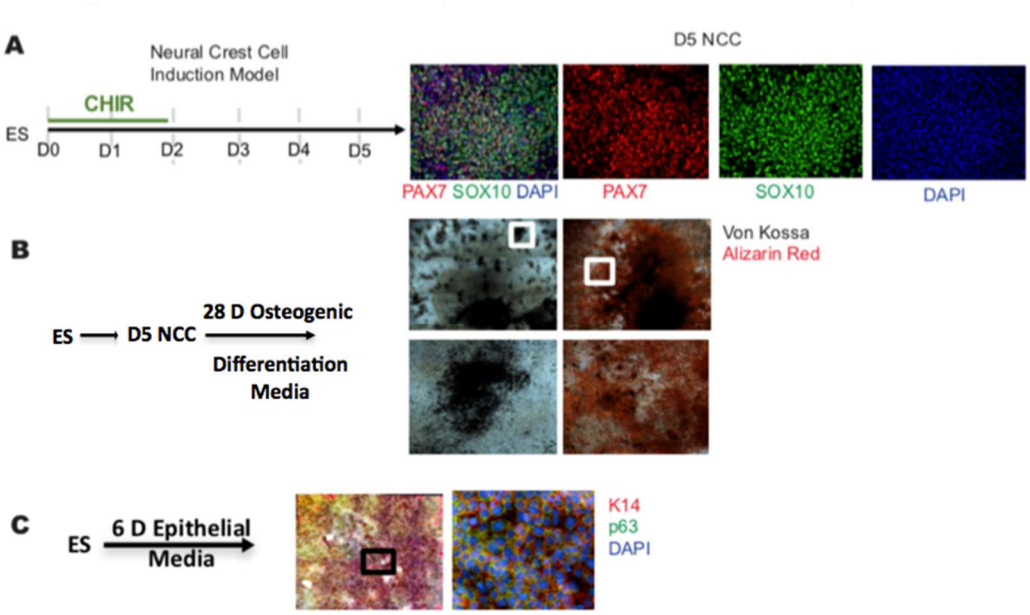


Figure 1: Oral Palate Formation Components in 2D: NCC, Osteoblasts and Epithelial Cells

A) Schematic depicting 5D neural crest induction protocol using the 2D CHIR.A merged image is shown of PAX7(red), SOX10 (green) and DAPI (blue).
B) A diagram showing the osteoblast differentiation process in a 2D culture. ES cells are first induced into NCCs, which are then maintained in osteoblast media for 28 days, upon which they are fixed and stained for the presence of Von Kossa (black) and Alizarin Red (red) shown by these bright field images.
C) The epithelial differentiation protocol is portrayed here in which ES cells are treated with epithelial media for 6 days and are marked by the presence of K14(red) and p63(green).

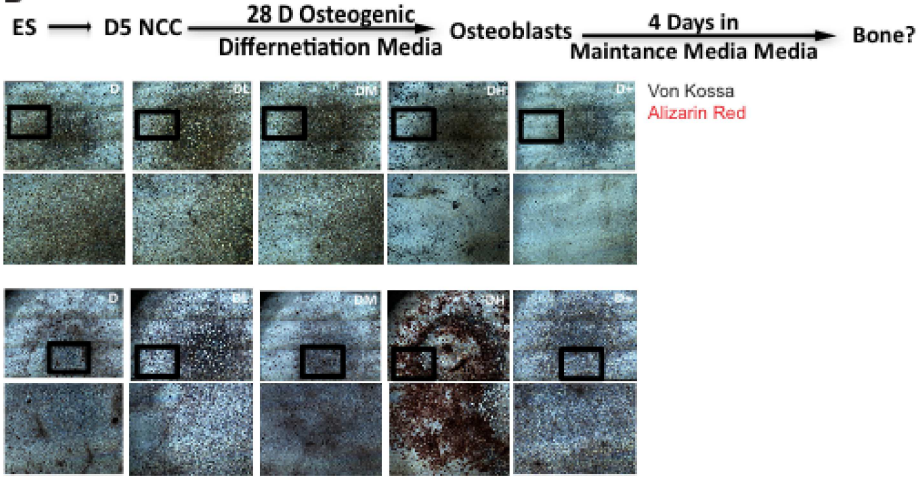
I have engineered 3D spherical structures that contain an osteoblast layer at the center that is surrounded coated with epithelial cells. Each one of these cell types maintains their marker expression in both 2D and 3D co-cultures. As is shown here first NCC are formed from hESC by using CHIR for 2 days, at day 5 I see that they express the NCC markers Pax7 and SOX10 (Figure 1A). Next I demonstrate that these day 5 NCC can be differentiated into osteoblasts that can mineralize, as is shown by the expression of Von Kossa and Alizarin Red (Figure 1B). I also show that I can differentiate hESC into epithelial cells that are marked by K14 and p63 (Figure 1C).

A Maintance Media Conditions Tested On Osteoblasts and Epithelial Cells Indiviudally

D: DMEM
DL: DMEM+AA(5uM)+BGP(0.25mM)
DM: DMEM+AA(10uM)+BGP(0.5mM)
DH: DMEM+AA(10uM)+BGP(2uM)
D+: Mixed Media (1:10 Osteogenic Differentiation Media/1:10 epiblast media)

AA: ascorbic acid
BGP: beta-glycerophosphate

B



C

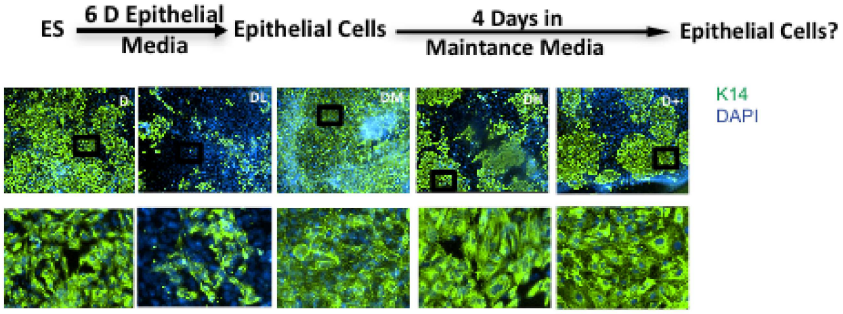


Figure 2: Determining a Maintenance Media for the Osteoblast and Epithelial Co-Cultures

A) Lists the different maintenance media combinations that were tested to maintain the epithelial and osteoblast cell types.
B) These images show the effects on osteoblast mineralization after treatment with the different maintenance medias for 4 days. The top row shows Von Kossa stains of 3x3 stitched images taken at 10x, the second row shows zoomed in regions of the respective images. The third row shows Alizarin Red stains of 3x3 stitched images taken at 10x, the fourth row shows zoomed in regions of the respective images.
C) These images show the effects on epithelial cells after treatment with the different maintenance medias for 4 days, the top row shows K14 and DAPI, they are 3x3 images. The second row shows zoomed in regions of the conditions respectively.

Next I went onto determine which media combinations could best support both types of cells. Through literature it was found that low AA and BGP are important to maintain osteoblasts, and epithelial cells are highly susceptible to differentiating into other cell types. So media with low levels of AA and BGP where tested, and a media including high levels of epithelial media were tested. From this it was found that media made up of DMEM containing 10uM of AA and 2uM BGP worked well to maintain both epithelial cells and mineralization on osteoblasts (Figure 2A and 2B).

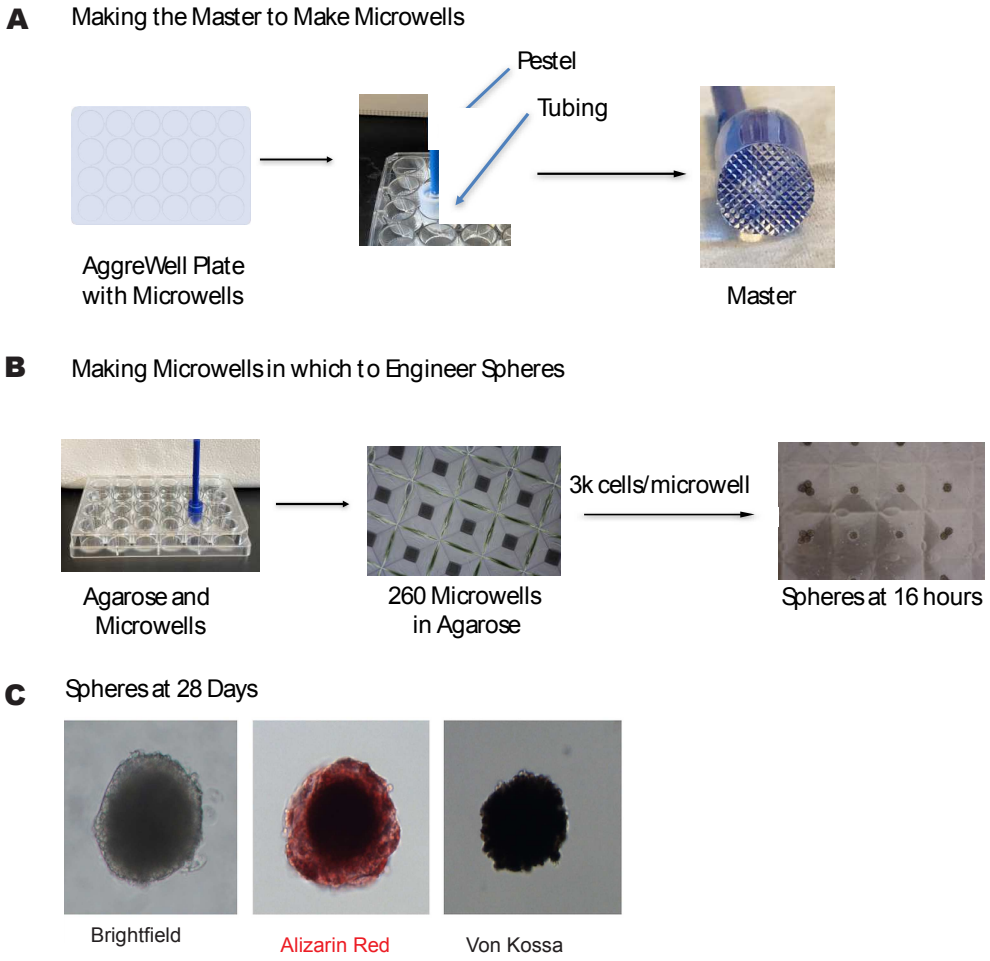


Figure 3: Making Microwells to Make Spheres, and Engineering OsteoSpheres

A) The illustration shows a 24 well AggreWell plate with microwells. Tubing was used to line the well, and then PDMS was poured in, and a pestel was used as a handle to make a master for stamping the microwells into 2% agarose.
B) Diagram shows that the microwell master was used to make 260 microwells in the 2% agarose. Then 3k cells were put in per well, making that a total of 780,000 cells in 250ul of osteo differentiation media. An image of how the cells had aggregated into spheres is shown at 16 hours.
C) The image to the left shows a brightfield image of an osteosphere. The image in the middle shows positive expression of alizarin red, and the image on the left shows positive expression of von Kossa.

To make uniform and reproducible microwells, a master of a 24 well AggreWell was made using tubing to line the walls of the Aggrewell plates to get a master mold that would be easy to stamp into agarose plates (Figure3A). Next these master molds were used to make 260 microwells in agarose. Agarose was poured into the Aggrewells and then the master was placed into the agarose and allowed to dry for about 5 minutes at room temperature (Figure 3B). At 28 days the spheres were collected and they were found to be alizarin red and von kossa positive (Figure 3C), which shows that the osteospheres are mineralized.

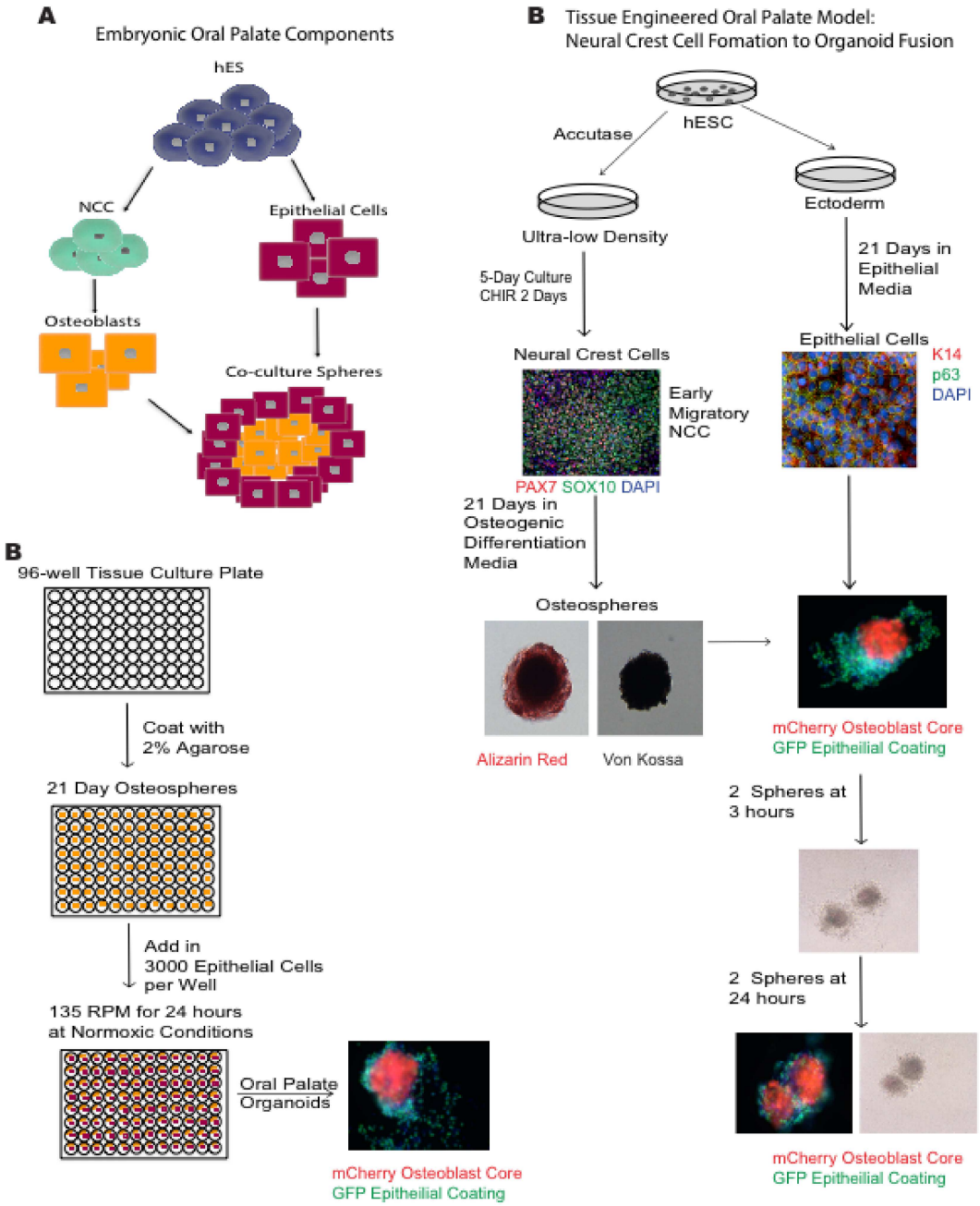

A
Embryonic Oral Palate Components
hES
NCC
Epithelial Cells
Osteoblasts
Co-culture Spheres
B
96-well Tissue Culture Plate
Coat with
2% Agarose
21 Day Osteospheres
Add in
3000 Epithelial Cells
per Well
135 RPM for 24 hours
at Normoxic Conditions
Oral Palate
Organoids
mCherry Osteoblast Core
GFP Epitheilial Coating
B
Tissue Engineered Oral Palate Model:
Neural Crest Cell Fomation to Organoid Fusion
hESC
Accutase
Ultra-low Density
Ectoderm
5-Day Culture
CHIR 2 Days
21 Days in
Epithelial
Media
Neural Crest Cells
Early
Migratory
NCC
PAX7 SOX10 DAPI
Epithelial Cells
K14
p63
DAPI
21 Days in
Osteogenic
Differentiation
Media
Osteospheres
Alizarin Red
Von Kossa
mCherry Osteoblast Core
GFP Epitheilial Coating
2 Spheres at
3 hours
2 Spheres at
24 hours
mCherry Osteoblast Core
GFP Epitheilial Coating

Figure 4: Engineering Oral Palate Spheres: Osteoblast Center with an Epithelial Coating

A) This figure shows an overview of the derivatives used in the oral palate model. It outlines the intermediates and derivatives, and their path from hESC. The oral palate is composed of a mesenchyme layer at the core, which is derived mainly from NCC, and it is surrounded by oral epithelium. Here we have an internal osteoblast layer (orange) (derived from NCC), surrounded by epithelial cells (red).

B) Osteoblast spheres are moved into new 96 well plate coated with agarose, epithelial cells are added, and allowed to shake under culture conditions, after 24 hours the osteospheres are found to be coated with epithelial cells, creating a two cell type co-culture.

C) This diagram gives an overview for the process of oral palate organoid sphere formation. For NCC induction hESCs are dissociated and plated at a density of 20,000 cells/cm2 in NCC and NCC cells are collected at 120 hours. Osteoblasts spheres are made by maintain 3000cells/per well for 21 days, after which they are collected and stained or alizarin red and von kossa to test for mineralization. After 21 days the osteospheres are coated with epithelial cells, and then the co-culture spheres are placed into agarose coated 96 well plates and allowed to fuse. Fusion is seen after 24 hours.

There are two main components that go into the embryonic oral palatal shelves, there are the NCC derived osteoblasts that make up the core of the palatal spheres and they are coated with hESC derived epithelial cells (Figure 4A). Next, tissue culture 96 well plates were coated with agarose and the osteospheres were added into the wells and then 3000 epithelial cells per well were added in and they were put into an incubator at normoxic conditions and maintained for 24 hours at 135 RPM, which resulted in an epithelial coating of the osteospheres (Figure 4B). Lastly, I showed that the palatal sphere organoids are able to fuse. Here I show an overview of the process of that is involved in making the different components of the palatal sphere organoids and the contact that occurs within 3 hours and the fusion event that occurs within 24 hours of contact (Figure 4C).

Discussion

NCC generate several head components, and thus are critical to craniofacial pathologies, including cleft lip/palate. I have engineered 3D spherical structures that contain an osteoblast layer at the center and is coated with epithelial cells. Each one of these cell types maintains their marker expression in both 2D and 3D co-cultures. 3D models are ideal because 2D models are less biologically relevant, as they have no diffusion gradients, less relevant gene behavior, less predictive drug screens, 3D models have a similar morphology and polarity and similar gene expression and gradients to those of human *in vivo* systems.[477] This model is human and even though the human and mouse genome are similar, not everything is conserved.

A majority of what we currently know about craniofacial development and abnormalities is from genetic mutations that we can observe after birth, but not during embryogenesis, and from genetically engineered mice. A lot of our knowledge comes from studies done in humans and animal models that have been exposes to teratogens and other environmental factors. Understanding epigenetics in the formation of oral palate formation are key in understanding why craniofacial abnormalities occur and what can be done to address this issues.[478] Using a 3D engineered model of oral palate differentiation we can study these abnormalities and their causes ranging from early stages when NCC formation first takes place ranging to the point where the oral palate is formed. This model allows us to study normal basic components in the development, allowing us to explore the different stages at which dysregulation can occur that leads to abnormalities in oral palate development and fusion. It opens the door to exploration of these abnormalities from the time that NCC arise to the time that the palatal shelves rise and there is fusion. It will be used to study the effects of gene mutations, or environmental toxins that lead to disease states.

3D models are also more efficient in testing concentration gradients and for personalized medicine. They can be used to screen a wide variety of drugs before you see how the patient will react to the certain drugs and can be used to predict which drugs will be effective. Additionally, they are a good intermediate between 2D cultures and 3D cultures, it is much more effective to do large drug screens on organ models than it is to do them in *vivo* animal model systems, they

provide a cost effective alternative.[479] A 3D is more lifelike, but not a replacement for an animal model. Additionally, 3D cultures may help prevent overgrowth of one cell type over the other, as in 2D cultures soft cell types end up overtaking hard cell forming cell types.

Materials and Methods

3.1 hESC Maintance

HESCs are maintained in mTESR which is purchased from Stem Cell Technologies (component #85851 and # SCBW6732), they are grown on Matrigel (Reference # 354277). For maintance cells were passed using Versene from Gibco (Reference # 15040-66).

3.2 Neural Crest Induction

Accutase (life Technologies) is used to dissociate hESCs into single cells, the cells are then counted and plated into wells coated with Matrigel (Corning) at a density of 20,000cells/cm2 in media containing DMEM/F12 (Thermo Fisher Scientific no. 175020048), the media for the neural crest cell induction is supplemented with 1X serum-free B27 supplement (Invitrogen, Cat. No. 17504-044), 1X Glutamax (Thermo fisher scientific, Cat. No. 35050061), 0.5% BSA (Sigma, A7979). The neural crest cell induction protocol with more specifics can be found in the publication by Gomez, et al. In all conditions the GSK3 inhibitor, CHIR(TOCRIS) and Rock inhibitor were added for 48 hours. All results are from cells collected at 120 hours (day 5 of the induction protocol).

3.3 Osteoblast Differentiation

For the osteoblast differentiation day 5 neural crest cells were accutased, counted and plated in an osteoblast media adapted from Lee, Gabsang, et al480. The media composition is 8.4mM β-glycerol phosphate, 0.1 μM dexamethasone and 200 μM AA in α-MEM medium containing 10% FBS. In 2D cultures the cells were plated at 100,000cells per/cm2 and they were maintained for 28 days maximum and then they were collected and stained with von Kossa or Alizarin red.

3.4 Epithelial Cell Differentiation

For epithelial differentiation ES cells were passed onto matrigel following the normal cell passaging protocol, they were grown until they were 90% confluent, upon which the mTeSR was taken out and cells were rinsed with PBS containing Mg/Ca, and then epithelial differentiation media was as described in Metallo, Christian M., et al,[481] the media is composed of 1x NEAA, 1xN2, 1um RA and 25ng/ml BMP4, and was changed ever 24 hours for 6 days. At the end of day 6, cells were either collected to be immunostained with p63 and K14 they were accutased and used to coat osteospheres.

3.5 Determining a Maintenance Media

After making each one of the cell types and doing immunostains to determine that they were differentiated into the desired cell type, a media that would allow for each respective cell type to be maintained were added in. Going through literature it was determined that in any co-culture media ascorbic acid(AA) and/or beta glycerophospate (BGP) were used. Several maintenance medias were tested.

D: DMEM Only
DL: DMEM+AA (5uM)+ BGP(0.25mM)
DM: DMEM+AA (10uM)+ BGP (0.5mM)
DH: DMEM+ AA (10uM)+ BGP (2mM)
D+: Mixed Media (1:10 Osteo Media /1:50 Epiblast Media)

3.6 Preparing Plates to Make Osteospheres

AggreWell plates from stem cell technologies were used to make an inverted master mold out of PDMS, which could then be used as a mold with agarose to recreate the wells of the AggreWell to carry out the experiments in a cost effective manner. Pipe tubing was used to line the inside of the AggreWell wells, which were then filled with PDMS mixed at a 1:10 ratio of curing agent to base respectively. A pestal was used as a handle to remove and later use the molds as a stamp. Then 2% agarose is added into 24 well plates, and the master is used to recreate the microwells.

3.7 Making and Collecting Osteospheres

In 3D cultures the cells were plated at 3,000 cells per microwell, so assuming 260 microwells per 24-well 780,000 NC cells were added in with 300ul of osteoblast differentiation media were added to each well. Spheres were collected at different time points to analyze them for mineralization, using von Kossa and Alizarin Red. The ones used for the spheres were collected at 21 days. Before deciding that 3000k was the best size for the osteospheres, densities of 500 cells/microwell, 1000 cells/microwell, 3000 cells/microwell, 5000cells/microwell.

3.8 Coating Osteospheres with Epiblasts to Construct Oral Palate Spheres

After formation osteoblas**t** spheres are moved from the AggreWell molds into 96 well plates, one sphere per well and the epithelial cells are then added. After 48 hours, they are moved to new 96 well plates and 3000 epithelial cells are added per well, and incubated at 135 RPM under culture conditions for 24 hours.

3.9 Fusing the Oral Palate Spheres

The oral palate spheres were fused in 96 well plates, the surface of the wells was coated to agarose to create a non-adherent u-shape bottom. The osteoblast and epiblast co-culture spheres were then left to fuse for 24 to 48 hours. After which they were imaged and collected for further analysis.

3.10 Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 minutes, after which they were permeablized with 0.4% Triton X-100 for 10 minutes. Cells were then blocked using 10% fetal bovine serum (FBS) with 0.05% Tween 20 for 1 hour at room temperature. Primary antibody incubations were done overnight at 4C (Supplemental Figure 1), followed by secondary which was done at RT for an hour. Immunostained cells were imaged using a Nikon Eclipse Ti microscope, the images were processed using Adobe Photoshop, and cell counts were done using Nikon Elements software. The software detects intensity levels of the immunostained cells which are then compared to a negative control which sets the threshold of what is counted as a immunostain positive cell. Antibodies used: Mouse anti- SOX10 (Santa Cruz Biotechnology, SC271163), 1:200. Mouse anti-PAX7 (1:10), p63 and K14.

Concluding Remarks and Future Applications

Significance and Applications

In therapeutic settings, even a small proportion of improperly regulated cells might lead to disease and tumor states.[482] Chemical factors involved in the induction and maintenance of neural crest cells (NCC) have been extensively studied;[483] however, the effects of cell-cell contacts, such as EPH/EFN interactions have yet to be studied. This is particularly important because any implanted cells that fail to differentiate into NC and maintain an ES state could have major implications when implanted into the body.

A majority of the connective and skeletal tissues in the face are derived from cranial NCC. To form these intricate three-dimensional structure all steps of NCC induction and derivative formation must be temporally and spatially accurate. If these process are not properly regulated neurocristopathies can arise.[484] As a result, genetic disorders or failures in NCC processes are often evident in the craniofacial region. A better understanding of the role of EPH in NCC induction may result in improved gene therapies, and the construction and integration of implantable tissues, such as the oral palate. This understanding may allow for the induction of NCC in the large quantities needed to make implantable tissues, and for making a fusion model of the oral palate to allow for an effective, comparatively cheap, animal free method to study oral palate fusion in *vitro*. Clefts can occur in the primary palate, referring to the alveolar process and the lip, and/or they can occur in the secondary palate that refers to the hard and/or

soft palate.[485,486] Current treatments involve surgeries beginning at 6 months old and spanning a decade or longer, and do not guarantee success.[487] The development of an in *vitro* oral palate fusion model is key, as it will allow for an isolated, xeno-free, cost effective and efficient method.

Role of EPH Receptors in Neural Crest Cells

We have found that EPHB3 receptors play an essential role in NCC differentiation. In early human NCC formation *EPHB3* displays a dynamic role, where *EPHB3* transcript levels are upregulated early in our NCC induction model relative to ESC, but they are downregulated by the end of the induction protocol. Through the use of small molecule specific inhibitors as well as *EPHB3* targeting siRNA I found that *EPHB3* activation is essential during the first 24 hours, and the sensitivity is the greatest during the first 24 hours. The inhibition of EPHB3 in this NCC model leads to reduction of WNT signaling overall, and to dysregulation of, BMP and FGF signaling, but it does not mean that they are direct targets. The direct targets are not clear, but further studies are needed to show their effects. Additionally, in *vitro* validation would be beneficial to the studies,to do this, similar steps can be carried out in the chick model. To go about this, the specific EPHB3 inhibitor should be used to establish the necessary concentration that leads to EPHB3 inhibition, and presumably NCC induction inhibition. After which a temporal analysis should be carried out to determine if EPHB3 is critical at the early stages of NCC induction. The EPHB3 inhibitor will be used to treat explants from HH stage 3, and EG XII embryos. Explants from HH stage 3 chicken

embryos will be taken from the primitive streak. (Specification of the neural crest occurs during gastrulation and requires PAX7). These explants will be cultured for 10-36 hours under control and experimental conditions, then they will be stained with an anti-PAX7 antibody to examine the presence of NCC. Additionally, EG Stage XII eggs will be used to study the blastula stage.

To further validate and study the effects of EPHB3 inhibition on NCC induction, a genetic validation using CRISPR to carry out loss of function (LOF) and gain of function (GOF) studies for EPHB3 should be carried out. The most beneficial would be an inducible knockouts (KO) in ESC, so that at different periods in time EPHB3 expression can be altered to further assess the temporal profile ranging from early induction to differentiation of osteoblasts, and through fusion events.

Co-immunoprecipitation can be used to determine which EFNs are binding to EPHB3 during NCC induction. The NCC induction will be carried out and the cells will be lysed and then treated with an EPHB3 antibody immobilized on beads. The targets will be eluted and analyzed using a sodium dodcyl sulfate-polyacylamide electrophoresis (SDS-PAGE), and then run on a western blot and immunostained with antibodies targeting EFN that are known to typically partner with EPHB3.

Potential Applications of an in *Vitro* Oral Palate Fusion Model

We have made a human embryonic stem cell derived-neural crest cell model which gives us the potential to carryout in *vitro* studies on the effects of gene mutations and toxins on human palatal development. To achieve this, we have engineered 3D spherical structures that contain an osteoblast layer at the center and are then coated with epithelial cells. Our model is unique in that the cells used to acquire the osteoblasts are derived from human embryonic stem cells (hESC) induced neural crest cells and the epithelial directly from hESC. This model could be used to study the effect of gene mutations, such as those in neural crest transcription factors, or environmental toxins that lead to palatal clefting, and as such may lead to novel diagnostic and therapeutic approaches for cleft palate.

This model can be used to carry out studies which would help to determine at which stage/s of neural crest formation to oral palate differentiation and fusion process is EphB3 necessary. Additionally, different chemical inhibitors and knockout lines can be used with this model to test for a broad range of mutations that can lead to clefting of the oral palate.

Addendum: Sensitivity of CBP-p300 Inhibition on Human Embryonic Stem Cell Maintenance and Induction to Human Neural Crest Cells

Introduction

CREB binding protein (CBP) and p300 are nuclear molecules involved in chromatin remodeling. CBP and p300 interact with transcriptional activators and repressors, and are involved in the cell differentiation, and the coordination of chromatin mediated transcription. Studies suggest that in the process of cell growth regulation, the demand for CBP/p300 may exceed the amount present. Additionally, reports suggest that mutations in the human CBP gene may lead to mental retardation, congenital malformations, and hematological malignancies. The p300 gene has been shown to play a role in leukemia and in colorectal and gastric carcinomas. CBP and p300 have been shown to play a significant role in embryogenesis, human development, cell differentiation and in tumor suppressor proteins involved in controlling the cell cycle.[488]

Human pluripotent stem cells (hPSCs) can be differentiated into a numerous cell types in *vitro.* Induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESC) have been used widely for studies involving developmental processes that would otherwise have technical or ethical implications if done in *vivo,* when refereeing to human embryogenesis.[489] Human embryonic stem cell derived cell fates make the possibility of using cells for therapeutic purposes a reality.[490]

NCC are formed at the border of the neural plate after which they migrate through the developing embryo and give rise to a vast number of derivatives.[491]

Aberrations to the differentiation and migration of NCC lead to diseases, disorders and syndromes which are known as neurocristopathies.[492]

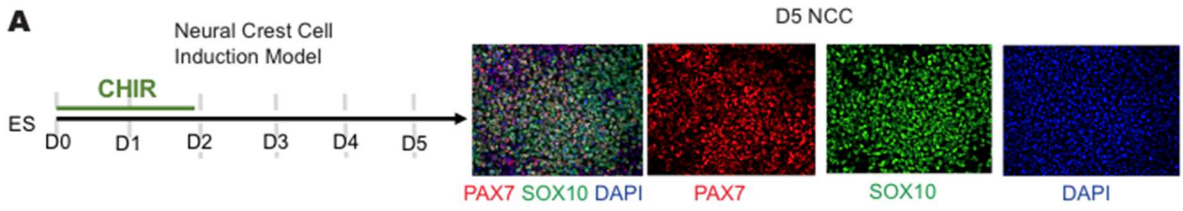
Purpose

To investigate the sensitivity and the level of CBP and p300 required in cell fate determination ranging from human embryonic stem cells (hESC) to the formation of human neural crest cells (hNCC). I differentiated NCC from hESC to determine which cell fate is the most sensitive to reductions in CBP and p300 levels.

Results

To determine whether CBP and p300 play a role in neural crest cell (NCC) fate determination, I set out to determine if by using CBP/p300 inhibitors I could inhibit the formation of NCC in *vitro*. I used hNCC model of development based on the induction of hESC, this is a 5-day protocol based on canonical WNT/ B-catenin activation via GSK3 inhibition for the first 48 hours of induction (Diagram 1).[493, 494]

Diagram 1: NCC Induction Protocol



This system was used to carry out the NCC inductions for these experiments, the inhibitors were added in from time point 0, and maintained for 2 or 5 days of culture. In addition to determining whether NCC induction would be affected by

CBP/p300 inhibition, I investigated whether hESC maintenance would be affected by CBP-p300 inhibition. To this end, I used the CBP-p300 inhibitors to see if they would have a consistent effect on NCC induction and ESC maintenance.

First, I used the inhibitor CBP-30 to determine if inhibiting CBP-p300 at varying concentrations would prevent NCC induction. To carry out these experiments I used the NCC induction protocol depicted in Diagram 1A, in which WNT is activated for the first 2 days. I began by adding in CBP-30 for the entirety of the NCC induction protocol (Figure 1A). At the end of day 5, when using 1uM of CBP-30, I found that SOX10 (green) expression was abrogated (Figure 1C) but at concentrations lower than 1uM for the concentrations of CBP-30, I did not see a significant decrease in PAX7 and/or *S*OX10 (data not shown). Using 3uM CBP-30, I found that SOX10(green) expression was completely diminished. When using 5uM and 10uM CBP-30, I found that SOX10 (green) was completely diminished, and PAX7 was severely decreased. At all concentrations, DAPI was unaffected meaning that there was no decrease in cell survival nor proliferation can be seen by maintained levels of the nuclear stain (DAPI). When CBP-30 was used in hESC to determine if maintenance of hESCs was being affected, it was found that regardless of whether 1uM, 3uM, 5uM, or 10uM of the inhibitor were used there was no decrease in OCT4 (red) nor SOX2 (green). Additionally, DAPI was unaffected suggesting that cell survival and proliferation were not affected (Figure 1 B and Figure 1D).

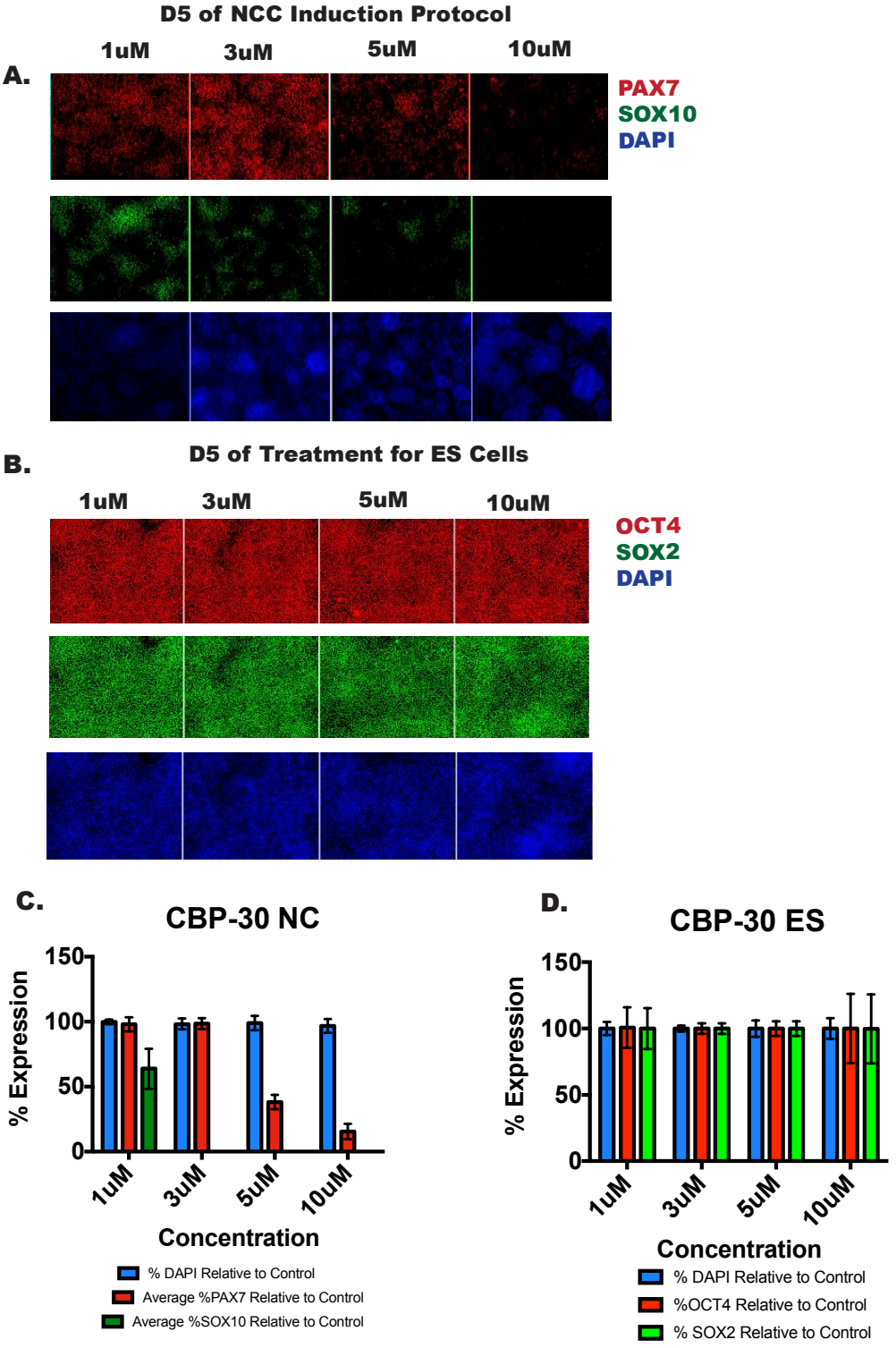

D5 of NCC Induction Protocol
A.
1uM
3uM
5uM
10uM
PAX7
SOX10
DAPI
B.
D5 of Treatment for ES Cells
1uM
3uM
5uM
10uM
OCT4
SOX2
DAPI
C.
CBP-30 NC
% Expression
150
100
50
0
1uM
3uM
5uM
10uM
Concentration
% DAPI Relative to Control
Average %PAX7 Relative to Control
Average %SOX10 Relative to Control
D.
CBP-30 ES
% Expression
150
100
50
0
1uM
3uM
5uM
10uM
Concentration
% DAPI Relative to Control
%OCT4 Relative to Control
% SOX2 Relative to Control

Figure 1: Effects of CBP-p300 Inhibitor (CBP30)

A) Immunofluorescence microscopy shows detection of NCC markers PAX7 (red) and SOX10 (green), and cell nuclous stained with DAPI (blue).
B) Immunofluorescence microscopy shows detection of ESC markers OCT4 (red) and SOX2 (green), and stained with the nuclear stain DAPI (blue).
C) Cell counts show the proportion of cells positive for NCC marker PAX7 and SOX10 in NCC treated with CBP30 for varying durations. The relative percent expression is calculated in comparison to NCC not treated with CBP30. DAPI expression in cells treated with CBP30 inhibitor for varying spans of time is shown relative to untreated NCC.
D) Cell counts show the proportion of cells positive for ESC markers SOX2 and OCT4 in ESC treated with CBP30 for different time points. Calculations are relative to untreated ESC. DAPI expression in cells treated with CBP30 inhibitor for varying spans of time is shown relative to untreated ESC.

Next, I used the inhibitor, ICBP-112 to confirm that the results that were obtained by inhibiting CBP30-p300, with the first inhibitor (CBP-30) at varying concentrations were reproducible and they would prevent NCC induction. To carry out these experiments I used the NCC induction protocol depicted in Diagram 1A, and ICBP-112, was added in for the entire 5 days of the NCC induction protocol. At the end of day 5, when using 1 uM of CBP-30, I found that PAX7 (red) and SOX10 (green) expression was abrogated (Figure 2C) but at concentrations of ICBP-112 lower than 1uM we did not see a significant decrease in PAX7 and/or SOX10 (data not shown). When using 3uM ICBP-112, I found that SOX10 (green) expression was completely diminished, PAX7 was largely unaffected. When using 5uM and 10uM ICBP-112, I found that SOX10 (green) was completely diminished, and PAX7 (red) was severely decreased. In the 1uM and 3uM conditions, DAPI was unaffected meaning that there was no decrease in cell survival nor proliferation as can be seen by diminished levels of the nuclear stain. However, when I used a concentration of 5uM or 10uM, none of the cells survived. When ICBP-112 was used in hESC to check if maintenance of hESC markers was being affected, it was found that regardless of whether 1uM, 3uM, 5uM, or 10uM of the inhibitor were used there was no decrease in OCT4 (red) nor SOX2 (green). Additionally, DAPI was unaffected suggesting that cell survival and proliferation were not affected (Figure 2 B and Figure 2D). This suggests that NCC are significantly more sensitive to CBP-p300 inhibition than hESC are.

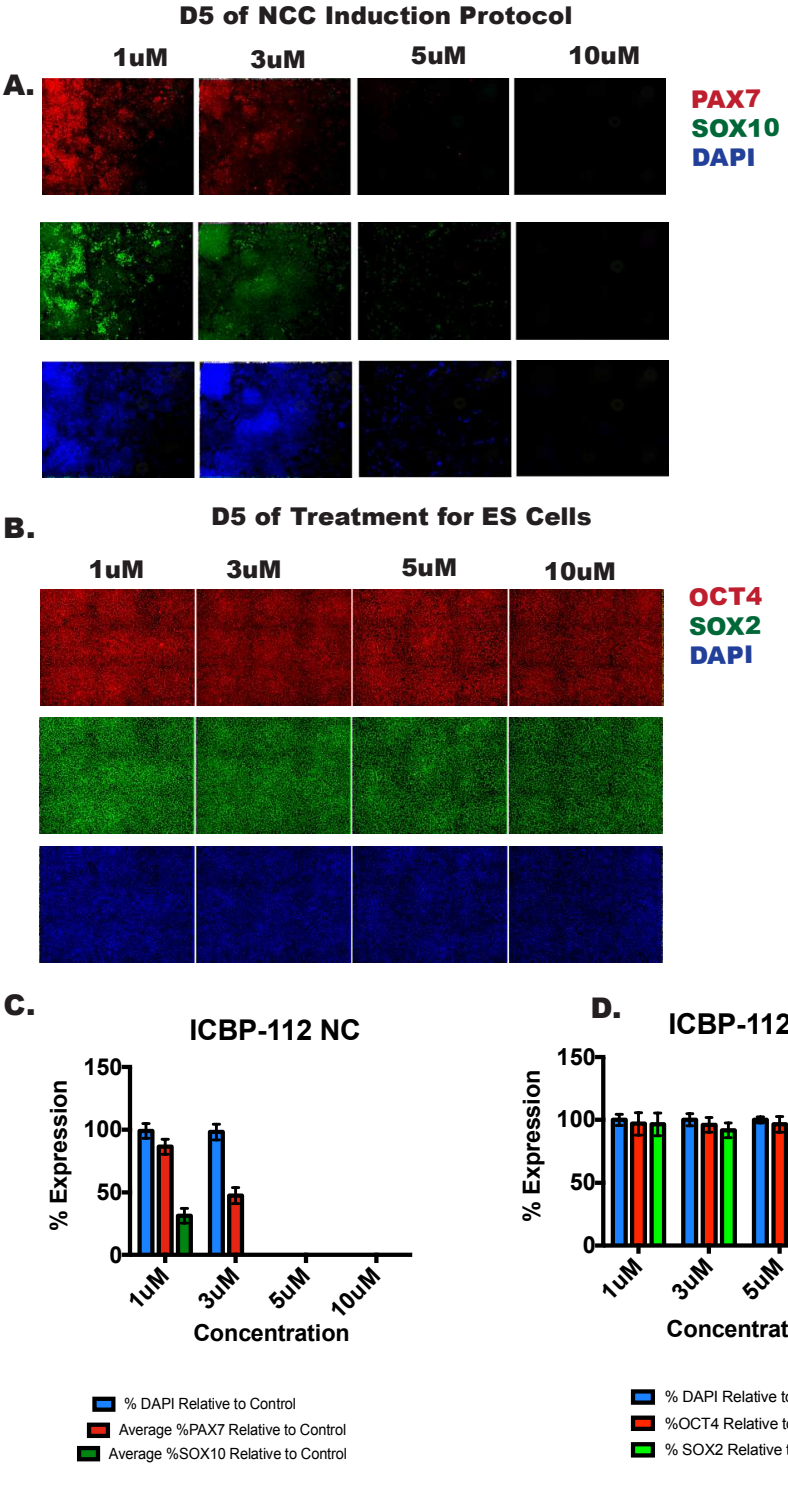

D5 of NCC Induction Protocol
1uM
3uM
5uM
10uM
A.
PAX7
SOX10
DAPI
B.
D5 of Treatment for ES Cells
1uM
3uM
5uM
10uM
OCT4
SOX2
DAPI
C.
ICBP-112 NC
% Expression
150
100
50
0
1uM
3uM
5uM
10uM
Concentration
% DAPI Relative to Control
Average %PAX7 Relative to Control
Average %SOX10 Relative to Control
D.
ICBP-112 ES
% Expression
150
100
50
0
1uM
3uM
5uM
10uM
Concentration
% DAPI Relative to Control
%OCT4 Relative to Control
% SOX2 Relative to Control

Figure 2: Effects of CBP-p300 Inhibitor (ICBP-112)

A) Immunofluorescence microscopy showing detection of NCC markers PAX7 (red) and SOX10 (green), and cells are marked with the nuclear stain DAPI (blue).
B) Immunofluorescence microscopy showing detection of ESC markers OCT4 (red) and SOX2 (green), and cells are marked with the nuclear stain DAPI (blue).
C) Cell counts showing the proportion of cells positive for NCC markers *PAX7, SOX10* in NCC treated with ICBP-112 for varying durations. The relative percent expression is calculated in comparison to NCC not treated with ICBP-112. DAPI expression in cells treated with ICBP-112 inhibitor for varying spans of time are shown relative to untreated NCC.
D) Cell counts showing the proportion of cells positive for ESC markers SOX2 and OCT4 in ESC cells treated with EPHB3 inhibitor for different time points, relative to untreated NCC. DAPI expression in cells treated with ICBP-112 for varying spans of time is shown relative to untreated NCC.

Next, I used the inhibitor US-13A to confirm the results that were obtained by inhibiting CBP30-p300, with the other two inhibitors (CBP-30 and ICBP-112) at varying concentrations would consistently prevent NCC induction. To carry out these experiments we used the NCC induction protocol depicted in Diagram 1A, and US-13A, was added in for the entire 5 days of the NCC induction protocol (Figure 3A). At the end of day 5, when using 1uM of US-13A, I found that PAX7 (red) and SOX10 (green) expression were both abrogated (Figure 2A and Figure 2C) but at lower concentrations of US-13A, lower than 1uM I did not see a significant decrease in PAX7 nor SOX10 expression (data not shown). When using 3uM US-13A, I found that PAX7 (red) and SOX10 (green) expression was decreased. When using 5uM and 10uM US-13A, I found that SOX10 (green) was completely diminished, and PAX7 was severely decreased. In the 1uM and 3uM conditions, DAPI was unaffected, suggesting that there was no decrease in cell survival as can be seen by unaffected levels of the nuclear markers. At 5uM, DAPI expression was slightly decreased, showing that cell survival and proliferation are negatively affected. However, when I used a concentration of 10uM, none of the cells survived. When US-13A was used in hESC to check if maintenance of hESC was affected, it was found that regardless of whether 1uM, 3uM, or 5uM of the inhibitor were used there was no decrease in OCT4 (red) nor SOX2 (green). Additionally, DAPI was unaffected suggesting that cell survival and proliferation were not affected. However, when cells were subjected to

10uM US-13A, there was no cell survival (Figure 3 B and Figure 3D). This suggests that not only can CBP-p300 influence cell fate, CBP-p300 also play a role in cell survival, which agrees with literature. Here the data suggests that if the inhibition of CBP-p300 is significant enough, it leads to apoptosis, meaning a certain level of CBP-p300 is necessary for cell survival and maintenance.

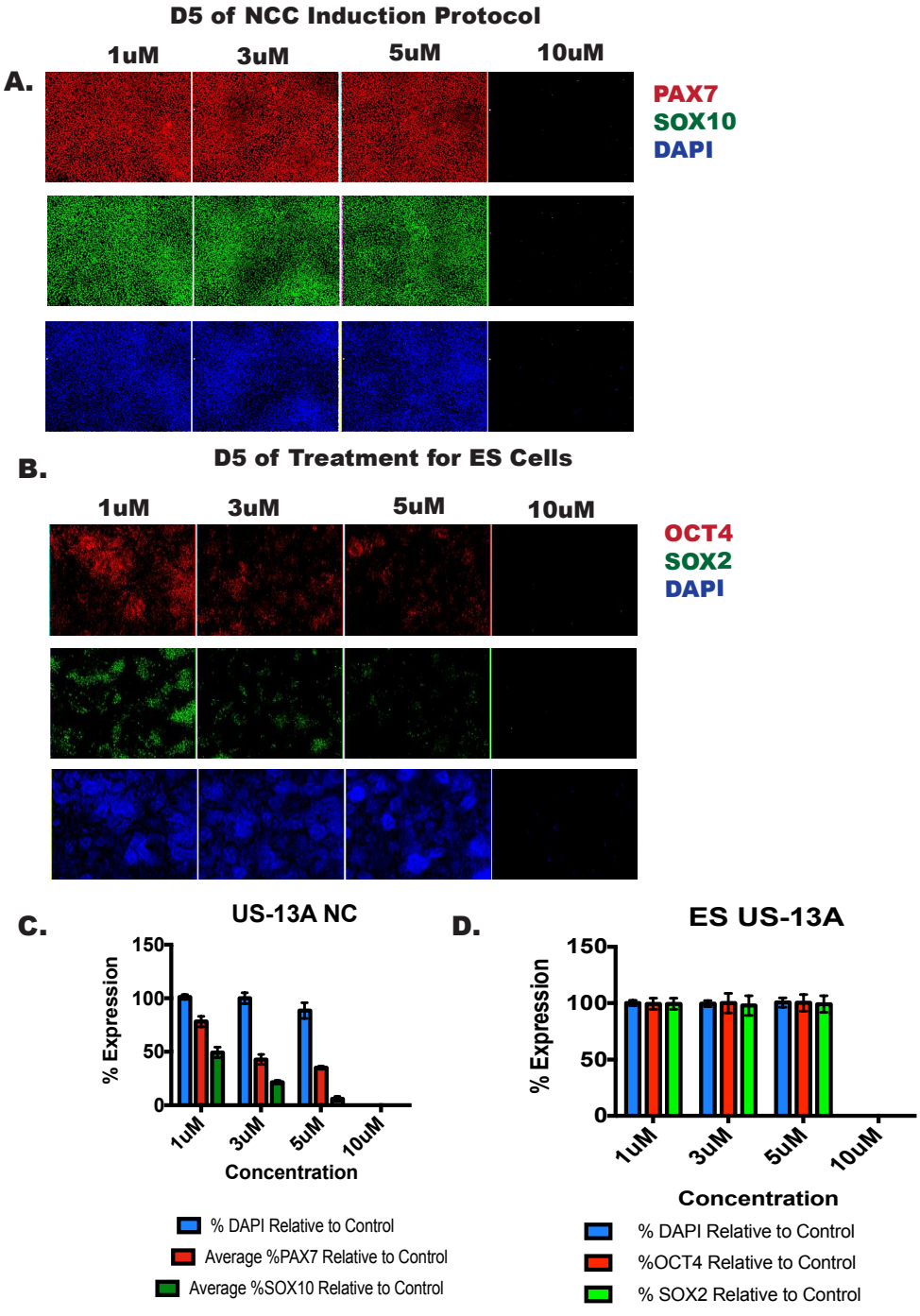

D5 of NCC Induction Protocol
A.
1uM
3uM
5uM
10uM
PAX7
SOX10
DAPI
B.
D5 of Treatment for ES Cells
1uM
3uM
5uM
10uM
OCT4
SOX2
DAPI
C.
US-13A NC
% Expression
150
100
50
0
1uM
3uM
5uM
10uM
Concentration
% DAPI Relative to Control
Average %PAX7 Relative to Control
Average %SOX10 Relative to Control
D.
ES US-13A
% Expression
150
100
50
0
1uM
3uM
5uM
10uM
Concentration
% DAPI Relative to Control
%OCT4 Relative to Control
% SOX2 Relative to Control

Figure 3: Effects of CBP-p300 Inhibitor (US13-A)

A) Immunofluorescence microscopy showing detection of NCC markers *PAX7* (red) and *SOX10* (green), and cells are marked with the nuclear stain DAPI (blue).
B) Immunofluorescence microscopy showing detection of pluripotency markers *OCT4* (red) and *SOX2* (green), plus cells are marked with the nuclear stain DAPI (blue).
C) Cell counts showing the proportion of cells positive for NCC marker *PAX7, SOX10* in NCC treated with US-13A for varying durations. The relative percent expression is calculated in comparison to NCC not treated with US-13A. DAPI expression in cells treated with US-13A inhibitor for varying spans of time is shown relative to untreated NCC.
D) Cell counts showing the proportion of cells positive for ESC marker *SOX2, OCT4* in NCC cells treated with EPHB3 inhibitor for different time points, relative to untreated ESC. DAPI expression in cells treated with US13-A inhibitor for varying spans of time is shown relative to untreated NCC.

To check whether the inhibition of CBP-p300 would lead to a decrease in NCC transcripts, and the trend would follow what was seen at the protein level, I used qPCR to look at NCC genes. When the samples were treated with CBP30 for 5 days, I found that *SOX10* expression decreased (0.5 fold), *PAX7* was slightly decreased (0.9 fold), *SNAIL2* (0.55 fold), and *FOXD3* (0.6 fold). When the cells were treated with CBP30 for 2 days, *SOX10* (0.55 fold), *PAX7* (0.75 fold), *SNAI2*(0.8 fold), *FOXD3* at 0.7 fold.

When the samples were treated with US13A for either 2 or 5 days, almost all expression of the NCC genes was abolished. When cells were treated for 2 days with US-13A, *SOX10, SNAIL2*, and *FOXD3* expression were almost at 0. *PAX7* expression was down to 0.15 fold. When the cells were treated with US13A for 5 days, no NCC genes were expressed. These results suggest that CBP- p300 are essential for NCC formation.

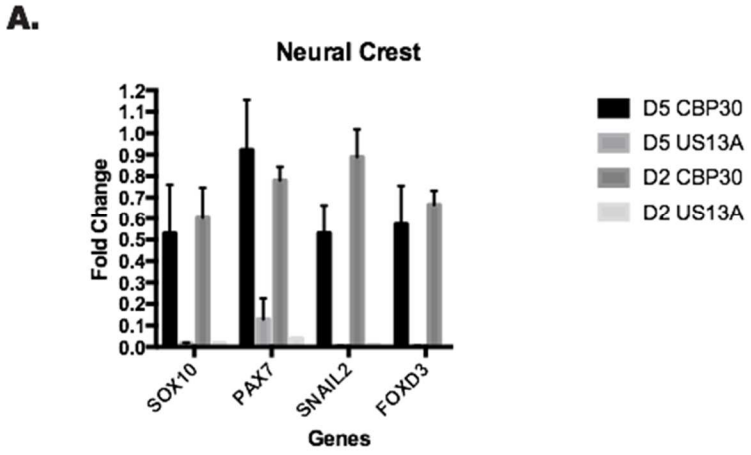


Figure 4: Inhibition of CBP-p300 Leads to Abrogation of Cell Fate Genes

A) QPCR data presenting changes in NCC (*SOX10, PAX7, SNAIL2, FOXD3*) at day 5 of collection, the inhibitors CBP30 or US-13A were maintained either for 2 days or for 5 days as is specified.

Discussion

DNA binding transcription factors that have the ability to recognize the promoter and enhancer elements in close proximity of the site for transcriptional state are necessary for gene transcription. Coactivators work to promote transcription through connecting the basal transcriptional machinery and transcription factors. CREB-binding protein (CBP), a conserved protein, and E1A-binding protein (p300), which is a paralog of CBP are responsible for recruiting basal transcription machinery to the promoters.[495] Literature suggests that CBP/p300 is involved in physiological and pathological processes, which include proliferation and apoptosis. When the transcriptional and epigenetics functions of CBP/p300 are dysregulated, cancers such as leukemia can result. It has been suggested that drugs targeting CBP/p300 function could be possibly used as anti-cancer treatments.[496]

The studies shown here suggest that CBP/p300 is essential in the differentiation of hESC to hNCC. However, the concentrations the inhibitors are used at to see an effect on NCC markers, do not affect the maintenance of hESC. When the inhibitors are used at high concentrations on the hESC, there was cell death, similar trends in cell survival were seen in differentiating NCC. These studies show that US-13A is a more potent inhibitor than CBP30, at least in terms of effecting cell survival. CBP-30 leads to cell death, in hESC at a concentration of 5uM, where a concentration of 10uM is needed to cause cell death in the cells treated with US-13A. Overall, these studies demonstrate that CBP/p300 inhibition

impacted NCC differentiation from hESC, but not hESC maintenance.

As a transcriptional regulator, CBP/p300, mediates cross-talk between signaling cascades. Many groups suggest that CBP/p300 serves as a toggle switch between numerous pathways and that these pathways antagonize each other.[497] There is competition for CBP/p300 between cells, as there isn't always enough supply to meet the demand. From this, I suggest that the amount of CBP/p300 required to induce NCC from hESC significantly outweighs what is needed to maintain stemness in hESC. This is in line with what other studies have shown, in the terms of CBP/p300 requirement for cell differentiation. Numerous studies show that CBP/p300 can lead to both positive and negative crosstalk between signaling pathways.[498] CBP/p300 plays a large role in determining whether the cells will maintain their current fate, differentiate, or apoptosis occurs.

Materials/Methods

A1.1 hESC Maintenance

HESCs are maintained in mTESR from Stem Cell Technologies (component #85851 and # SCBW6732) on Matrigel (Reference # 354277). Cells were passed using Versene from Gibco (Reference # 15040-66).

A1.2 Neural Crest Induction

HESCs are dissociated into single cells using Accutase (Life Technologies), the cells are then counted and plated onto vessels coated with Matrigel (Corning) at a density of 20,000cells/cm2 in media containing DMEM/F12 (Thermo Fisher Scientific no. 175020048), supplemented with 1X serum-free B27 supplement (Invitrogen, Cat. No. 17504- 044), 1X Glutamax (Thermo Fisher Scientific, Cat. No. 35050061), 0.5% BSA (Sigma, A7979), a more detailed protocol can be found in the publication by Gomez, et al. All results are from cells collected at 120 hours (day 5 of the induction protocol). In all conditions the GSK3 inhibitor, CHIR(TOCRIS) and Rock inhibitor were added for 48 hours.

A1.4 Drug Treatments

The CBP-p300 inhibitors (US-13A, CBP-30, ICBP-112) were graciously provided by Dr. Christina Nevado from the University of Zurich. All inhibitors were all resuspended in DMSO, the corresponding amounts of DMSO was used in the control conditions. Concentrations ranging from 10nM to 2uM, used concentrations were selected based on a consistency of DAPI counts between control and treated conditions, while PAX7 and SOX10 expression was

diminished in the experimental conditions. At lower concentration of the inhibitor we did not see a significant decrease in PAX7 and SOX10 (data not shown). The concentrations used, for which data is shown are as follows: US-13A (1uM, 3μM, 5μM, 10uM), CBP-30 (1uM, 3μM, 5μM, 10uM), ICBP-112(1uM, 3μM, 5μM, 10uM)

A1.6 Immunoflorescence

Cells were fixed with 4% paraformaldehyde for 10 minutes, after which they were permeablized with 0.4% Triton X-100 for 10 minutes. Cells were then blocked using 10% fetal bovine serum (FBS) with 0.05% Tween 20 for 1 hour at room temperature. Primary antibody incubations were done overnight at 4C (Supplemental Figure 1), followed by secondary which was done at RT for an hour. Immunostained cells were imaged using a Nikon Eclipse Ti microscope, the images were processed using Adobe Photoshop, and cell counts were done using Nikon Elements software. The software detects intensity levels of the immunostained cells which are then compared to a negative control which sets the threshold of what is counted as a immunostaining positive cell. Antibodies used: Mouse anti- SOX10 (Santa Cruz Biotechnology, SC271163), 1:200. Mouse anti-PAX7 (1:10).

A1.7 Quantitative PCR

Total RNA was extracted using TRIzol reagent from Life Technologies (# 15596026). cDNA was generated from 500ng of RNA using the High Capacity cDNA Reverse Transcription kit (number RR014B) from Clonetech. SYBR Green

from Applied Biosystems was used in the reactions, which were done on a One Step Real Time PCR Detection System from Applied Biosystems qPCR. Three biological replicates were used for drug treated samples, two biological replicates were assessed for siRNA treated samples.

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