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Insulin Signaling in Early Neural Crest Specification

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Dedication

I dedicate this work to my parents, Danielle Berger and Tom Shelar. I could not have asked for stronger, more devoted parents, and this work is a testament to the sacrifices they have made for my sake. My Mom made sure I had everything I needed to succeed, and my Dad worked tirelessly to ensure that I had every opportunity that he was not afforded growing up. I do not know a more inspirational pair than these two, and everything I do is to show them how much I appreciate their love and encouragement, and to make them as proud of me as I am of them.

ABSTRACT OF THE DISSERTATION

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The field of neural crest (NC) development continues to expand as new knowledge of this early developmental, multipotent, highly migratory cell population comes to light. However, as we begin to learn more about the derivatives of NC cells, it becomes evident that we must define the collection of growth factors, their concentration, and the precise timing of each to determine what allows for successful differentiation and function of NC derivatives. NC specification has recently been found to occur at the blastula stage, earlier than previously thought, and thus investigation into signaling contributions at this stage must be done. Beyond known players Wnt, FGF, and BMP, a role for insulin has not been elucidated at this or any stage, despite mounting evidence of a connection to NC development, as insulin has been implicated in connection with various neurocristopathies and it is a component of all published NC induction assays in chick and human studies. Therefore, defining the role of insulin in NC formation and its mechanism is imperative to understanding NC development. Using a robust human NC induction model and blastula stage chick embryos, we report that insulin is required for NC marker expression independently from growth and metabolism. Immunofluorescence of NC markers in chick NC specification assays and our hNC induction model, in the presence and absence of insulin, as well as in the presence of small molecule inhibitors and siRNA (human only), revealed reduced

expression of definitive NC markers in instances where insulin signaling was impeded. More specifically, temporal insulin signaling modification at blastula stage in chick and timepoint zero of human NC induction from pluripotent stem cells, indicates an early role for insulin. Inhibition of IR and/or IGFR via small molecule inhibition and RNAi knockdown resulted in reduced NC markers expression without triggering significant effects in cell number, indicating utilization of both receptors or a hybrid IR/IGFR. Furthermore, we show evidence that these signals are propagated via Pi3K and PDK1, and that NC specification is extremely sensitive to changes in the function of downstream effector FoxO1, a transcription factor modulated by insulin signaling.

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Chapter 1: Introduction – Reviewing the Relationship Between Insulin and Neural Crest

Abstract

Neural Crest (NC) cells are a transient, developmental cell population unique to vertebrates that exhibit phenomenal migratory capabilities and contribute to various cell populations in the craniofacial region and peripheral nervous system (PNS), and many other derivatives all over the adult body. Their derivatives include smooth muscle, osteoblasts, chondroblasts, melanocytes, odontoblasts, neurons and glia of the PNS, and many other vital structures. These far-reaching capabilities implicate NC in many congenital disorders, known as neurocristopathies, such as cleft lip/palate, Hirschprung's Disease, Waardenburg Syndrome, and Treacher-Collins Syndrome. In order to elucidate the pathology of these disorders, it is imperative that we define the recipe of contributing signals that initiate the specification and differentiation of NC. Often overlooked for its instructive roles, insulin has proven to be required in NC development studies, and diabetic pregnancies are associated with a higher prevalence of neurocristopathies. However, insulin's influence on NC has been widely viewed as purely metabolic, mainly due to its major impact on glucose metabolism, thus chaining its perceived involvement to pro-survival cues and glucose metabolism. Furthermore, there is a need to clarify the mechanism responsible for propagating insulin's contributions beyond regulation of glucose metabolism. This review discusses the role of various growth factors known to be associated with the earliest stages of NC development, focusing on the insulin signaling pathway, and exploring the connection between insulin and NC specification and formation beyond a metabolic dependency.

Keywords: Neural Crest, Insulin Signaling, FGF, Wnt, Specification, Pi3K, MAPK, FoxO1

1. Introduction

Since the first report of NC cells in 1868 by Wilhelm His (His 1868), studies into the specification, differentiation, contributions, and general function of NC has continued expanding. A transient cell population, unique to vertebrates, that arises during the earliest stages of gestation, the NC exhibits highly migratory capabilities and an almost unmatched differentiation potential shown by its wide range of terminal derivatives. Well-known for its major contributions to craniofacial structures, NC cells can generate osteoblasts, chondroblasts, melanocytes, smooth muscle, adipocytes, and odontoblasts. NC cells are also responsible for forming a majority of the peripheral nervous system throughout the body, showing the ability to form neurons and glia, including the ganglia responsible for enteric peristalsis. Due to this far reaching involvement in human development, disruptions in any part of NC formation can have severe consequences, which are classified as neurocristopathies (Watt & Trainor 2013). This class of diseases ranges from mild to severe to fatal. Many neurocristopathies result in craniofacial malformations, with the most well-known being cleft lip/palate, but disruption of the neural crest can also manifest as other life altering diseases such as Waardenburg Syndrome, Hirschprung's Disease, and melanomas. Early NC formation relies on delicate interaction of multiple signaling factors, all of which play an important role in proper induction and later function of derivatives. Thus, in order to properly understand NC formation, differentiation, and the pathology of the associated diseases, we must investigate early NC formation and differentiation and explicitly define the signaling milieu during the earliest stages NC development.

Attempts to dissect the dynamics behind NC formation have resulted in two models: 1. The Classic Induction model - NC arises from the ectodermal lineage; 2. The Early Induction model - NC is induced from epiblast cells. The first model has had much support considering NC is first visible at the border between the neural and non-neural ectoderm. It has long been thought that a

convergence of signals, namely Wnt, FGF, BMP (Garcia-Castro et al. 2002, Linker et al. 2004, Patthey et al. 2008/2009, Stuhlmiller and Garcia-Castro 2012a, Yardley and Garcia-Castro 2012), and other known NC contributary signals, from juxtaposed neural and non-neural ectoderm and underlying mesoderm convert these border cells into the multipotent NC cells. The first detectable NC markers appear during late gastrulation, thus putting the formation of NC during or after formation of the three classic germ layers. However, a recent publication by Prasad et al. 2019b, shows clear evidence of a restricted region at the blastula stage that is capable of forming NC without signaling input from surrounding cells. Additionally, these restricted regions do not display markers of neural or mesodermal tissues, suggesting that at stage Eyal-Giladi XII the prospective NC region have acquired the necessary signals required to form NC, and do not appear to be inhibited by a lack of inductive interaction. The implications of this result are two-fold: 1. It shows evidence that specification of NC in this model occurs without exogenous signaling after the blastula stage; 2. The other germ layers have not yet formed at the blastula stage, thus NC formation is neither a result of, nor dependent upon the formation of surrounding germ cells, but rather a chronologically equivalent population that develops in parallel with them. This study does not definitively eliminate the possibility of classic NC induction, but it does provide irrefutable support for NC specification before gastrulation. In accordance with the philosophy of Conrad Waddington's epigenetic landscape (Waddington 1957), the mere fact that NC cells maintain the capacity to form derivatives of mesodermal and ectodermal lineages suggests that they do not arise from just the mesoderm or ectoderm after they themselves have been restricted, but rather NC cells establish a distinct differentiation path of their own before formation of these other populations. While an ectodermal origin of NC clashes with Waddington's epigenetic landscape and currently accepted sequential segregation of potential, an earlier origin preceding ectodermal and mesodermal differentiation seems a more parsimonious model.

Regardless of the precise timing of NC specification or which induction model is more accurate, NC formation and development is dependent on signaling from various sources. There are a multitude of papers that delineate roles for the major players Wnt, FGF, and BMP, in multiple models and at different times of development. However, the blastula stage has been largely overlooked until recent evidence has shown specification at this early stage (Buitrago-Delgado et al. 2015, Prasad et al. 2019b). Interestingly, one commonality between all NC specification assays or induction protocols in chick and human, is the presence of insulin in the media (CHICK: Bottenstein et al. 1976, Tessier-Lavigne et al. 1988, Basler et al. 1993, Dickinson et al. 1995, Garcia-Castro et al. 2002, Patthey et al. 2008/2009, Stuhlmiller and Garcia-Castro 2012b, Prasad et al. 2019b) (HUMAN: Pomp et al. 2005, Lee et al. 2007/2010, Menendez et al. 2011, Fukuta et al. 2014, Zeltner et al. 2014, Leung et al. 2016, Hackland 2017/2019, Gomez et al. 2019a/b, Prasad et al. 2020 submitted). This is even seen in specification experiments conducted in rabbit (Betters et al. 2019), as well as xeno-free human and mouse NC induction (Aihara et al. 2010, Hackland 2017/2019) and other mouse PSC models (Gouti et al. 2014, Turner et al. 2014). Either when using supplements such as N2, B27, KSR, or other components, insulin is consistently present in additives to basal media that is categorized as neutral and naïve, theoretically allowing NC differentiation to continue uninhibited and uninfluenced by inductive or inhibitory cues. While insulin and IGF have been shown to been relevant in early neural development (Pera et al. 2003), an instructive role for insulin in early NC specification, or any other stage for that matter, has not yet been elucidated. Human studies further provide compelling evidence that insulin alters NC development, with increased prevalence of neurocristopathy in cases of insulin signaling dysregulation during pregnancy, (discussed in detail later in this review). Additionally, *in vivo* studies lead to unviable embryos in many cases, making it more difficult to tease apart the instructive contribution from the metabolic and pro-survival role of insulin in NC. The following

sections discuss early NC development and neurocristopathies with a focus on insulin and the various known contributing growth factors involved, their intracellular mechanisms, and how a potential instructive role for insulin fits into the "neural" fold.

2. Neural Crest Development

A. What is Neural Crest?

In 1868 Wilhelm His described the NC as a population of cells that form at the neural plate border which, as the neural tube forms and closes, rise up to become the neural fold before delaminating upon closure of the neural tube (His 1868). As they delaminate, the NC cells migrate laterally, dorsally, and ventrally to the locations of their terminal derivatives. As previously mentioned, one of their most notable contributions are within craniofacial structure. They are responsible for a majority of the bone and cartilage in the face, including the oral palate and skull, odontoblasts, and the melanocytes that contribute to cranial skin. They are also involved in formation of otic placodes and hair follicles. In the rest of the body they produce a large portion of the peripheral nervous system, including dorsal root ganglia and enteric nerves in the gut, and they also contribute to the secondary heart field, which forms the aortic valves. This mix of mesodermal and ectodermal derivatives establishes NC as a unique population of cells in that their differentiation capacity and derivative contribution is highly potent and involves terminal fates thought to be specific to only mesoderm or ectoderm (Kalcheim 2006, Murdoch et al. 2012).

B. Timing of Neural Crest Specification: Classic vs Early

Evidence exists to support each of the two views of NC induction. More specifically, NC cells either abide by classic induction, in which they arise from the ectodermal layer post-gastrulation, or they abide by early induction, suggesting they are specified at the blastula stage

before gastrulation. Supporting studies often consist of modifications of inductive NC signals at various stages of development, before subsequent analysis of the effect on NC markers and development of NC derivatives to assess the impact and involvement of each signal. There are vastly more studies that look into the effects of inductive signals during gastrulation and later, as the evidence of NC specification prior to gastrulation has only recently come to light. However, it does not discredit or eliminate the classic induction model, as early specification likely relies on further guidance at later stages to ensure complete and successful differentiation of NC into each of its derivatives. Some of the most important and informative experiments exploring the NC induction program are those that isolate certain regions of the NC during early formation and either incubate them in the absence of environmental signaling (Wilson et al. 2000/2001, Basch et al. 2006, Patthey et al. 2008/2009, Linker et al. 2009, Stuhlmiller et al. 2011, Betters et al. 2018, Prasad et al. 2019b) or graft them ectopically to explore their responsiveness, or lackthereof, to new surrounding signals (Le Douarin et al. 2004, Griswold and Lwigale 2012, Rothstein et al. 2018).

The question of specification versus commitment aims to define the fine line between a train that is moving but headed toward a railroad switch (ie. specified cells) or a train full speed ahead on a single track (ie. committed cells). Specification indicates the ability of a cell, one which has received instructive input, to differentiate down a specific lineage in a neutral and naïve environment but retains full capability of switching to a different lineage upon exposure to inductive signals. Commitment implies an inability to switch lineages, as the differentiation process is locked in and new signals are unable to change the course of differentiation. For example, certain mesodermal progenitor cells are unable to convert to neural tissue when grafted into a region where neural signals are expressed, and even overexpression of Sox2, a neural marker, is unable to coerce this mesodermal tissue to become neural (Wymeersch et al. 2016). Thus, these cells are committed and locked into their current differentiation program. Specification assays that isolate and grow

explanted tissue have proven to be an elegant and reliable technique to determine specification, as they allow the cells to differentiate solely based on information received prior to explantation.

Whole embryo studies have been paramount in determining the timing of NC marker expression in endogenous environments, the relationship of one marker to another, spatial profiles, and relevance of expression patterns between models studies (Khudyakov and Bronner-Fraser 2009, Yardley et al. 2012, Geary and Labonne 2018, Betters et al. 2018). Studies of unperturbed whole embryos have also vastly improved our understanding of the source of signaling gradients that lead to formation of a NC "habitable zone" at the neural plate border, and how modifications of the intensity of signaling gradients affects that zone (Stuhlmiller and Garcia-Castro 2012b, Milet and Monsoro-Burq 2012, Mayor and Theveneau 2013). These gradients are developed partially by the FGF, Wnt, BMP chronology discussed early. This starts with high FGF at Henson's node, which is the rostral most tip of the primitive streak. As the primitive streak elongates anteriorly, it leaves behind lower FGF levels, giving way to Wnt and eventually BMP. Wnt and FGF have shown to have axial gradients in which lower levels give rise to cranial NC and higher levels give rise to trunk crest (Kieicker and Niehrs 2001, Rogers and Schier 2011, Mayor and Theveneau 2013), whereas BMP has high lateral expression which is inhibited medially and seen at intermediate levels at the NPB, with a spike in BMP4 at the NPB (Tribulo et al. 2003, Yardley and Garcia-Castro 2012). While these studies provide a map to determine spatiotemporal expression profiles and growth factor locations, they are insufficient to draw conclusions on timing of specification. To emphasize the timing of specification versus commitment, it is imperative that the prospective NC region be isolated, to some degree, to determine the amount of instruction it has already received at that stage of development, even if that means complete dissociation of cells and assessing NC potential of single cells (Prasad et al. 2019b).

C. Definitive Neural Crest Markers

Analysis of NC development is heavily reliant on the classification and expression of NC markers, including definitive and promiscuous NC markers such as Pax3/7, Sox10, TFAP2, Snai1, Snai2, FoxD3, and Msx1, amongst others. The multitude of markers associated with this multipotent cell population can be broken down into three chronological groups: 1. Prospective NC (pNC); 2. Neural Plate Border (NPB); 3. Neural Crest (NC). Studies into the roles and timing of these markers are covered in multiple model organisms, including human, chick, quail, zebrafish, xenopus, rabbit, mouse, rat, and even axolotl.

The pNC refers to the region in blastula stage embryos that will eventually develop into the NC, but at that point has no morphological definition or protein expression exclusively connected to NC. Fate mapping studies in early avian embryos have defined distinct regions that contribute to the NPB as the neural folds begin to take shape (Hatada & Stern 1994, Ezin et al. 2009, Krispin et al. 2010, Prasad et al. 2019b). Specifically, Prasad et al. 2019b shows clearly that labeled cells in the intermediate region of the equatorial plane of blastula stage embryos contribute to the population of cells that inhabit the NPB. As the NPB forms bilateral furrows, which become the neural folds, these labeled cells exhibit Pax7 expression and are now definitive NC. Furthermore, Prasad et al. 2020 (submitted) shows a human NC model (Gomez et al. 2019a) exhibiting spikes in transcript levels of Pax3, Pax7, Myb, Zic3, and GBX2 at six hours post induction, providing the earliest evidence of NC gene expression, and offering a group of candidate genes that could be used to assess the exact location of pNC *in vivo*. In the chick fate-mapping, relevant to pNC, more medial cells contribute to the neural plate and more lateral cells are found mostly in the non-neural ectoderm, but also contribute to other regions, as well, which is similarly seen in other explant studies (Patthey et al. 2008). This heterogeneity of prospective cell geography in the early blastula is fascinatingly complex but expected (Hatada and Stern 1994).

Nonetheless, the best way to establish the potential of a certain region is to isolate it, thus locking in its potentially specified state and preventing further paracrine signaling from surrounding cells. Growth of pNC explants from blastula and gastrula stage embryos in naïve neurobasal media has shown the ability to successfully express various NC markers (Basch et al. 2006, Betters et al. 2018, Prasad et al. 2019b). Pax7 has proven to be one of the earliest and most reliably definitive NC markers across various models (Basch et al 2006, Garcia-Castro et al. 2006, Murdoch et al. 2012). Additionally, fate mapping studies using mouse models and cre-driven Pax7 or Wnt1 tracked the contribution of their respective genes in NC derivative formation (Danielian et al. 1998, Murdoch et al. 2012, Debacche et al. 2018). Specifically, Pax7 expressing NC cells and their progeny were found in various tissues including septal cartilage, trigeminal ganglia, scalp, tooth buds, cardiovascular tissue, and sensory organs (Murdoch et al. 2012). However, Pax7's paralogue, Pax3, is also present in most model organisms but in some (ie. xenopus) is more indicative of NC than Pax7 (Monsoro-Burq et al. 2005). Although, in xenopus it has been found that Msx1 is expressed prior to Pax3 and Msx1 appears to be slightly more promiscuous, and thus less restricted to the neural plate border. Nevertheless, NC markers are not visible until mid-late gastrulation, so establishment of which markers are visible early allows for more accurate and sensitive analysis.

After pNC comes the NPB, which has been identified with expression of combinations of Pax3/7, Id2/3, Msx1/2, Snai1, Dlx3/5, Zic1/3, Gbx2, Sp5, and TFAP2**α** (Milet and Monsoro-Burq 2012, Prasad 2012, Prasad et al. 2019a). However, a majority of these markers are not completely restricted to NC regions. *In situ hybridization* showing the spatiotemporal expression profile of these markers in chick helps visualize the promiscuity of some of these NPB specifiers (Khudyakov and Bronner-Fraser 2009). For example, TFAP2**α** is first seen at HH 4 in the rostral NPB regions, but its expression extends laterally into the non-neural ectoderm. This expression pattern is

maintained as development progresses, as TFAP2**α** can be seen extending caudally along the developing neural folds but continuing its lateral expression gradient. *DLX3* shares a similar promiscuous profile, however, its expression extends medially into the neural ectoderm. Contrarily, Pax7 only appears at the NPB and does so throughout the entirety of the neural fold, and more significantly, it is only seen in the dorsal most region of the neural folds. This spatiotemporal expression profile supports the notion that it is the earliest and most reliable definitive NC marker (Basch et al. 2006). A more concise list of definitive NPB specifiers includes Pax3/7, Zic1, and Msx1 (Simoes-Costa and Bronner 2015), however, in some instances Pax3 or Pax7 alone may be enough to define NC, as they are highly restricted to NC cells and are expressed slightly before the others.

As the expression of the above markers expands and the NC cells develop further, another set of markers begin to show up. These markers are pre-migratory and migratory NC markers, including Sox9/10, FoxD3, Ets1, and Snai2. Some of the early markers begin to diminish but many of them are co-expressed with these pre-migratory markers. These later markers indicate progression towards delamination and migration of developing NC. As the neural tube fuses, the population of NC cells at the dorsal most region of the neural tube undergoes EMT, in which they delaminate and migrate laterally, anteriorly, and posteriorly (Theveneau and Mayor 2012). This transition sees conversion of expression of E-cad to N-cad (Scarpa et al. 2015), amongst other markers, as well as the maintained expression of migratory NC markers, and some NPB specifiers.

Many labs study the phenomenon of NC migration, from the innate ability of these cells to reach the location of their specified derivatives to the organization within which they populate these areas (Mclennan & Kulesa 2007, Theveneau and Mayor 2012, Bronner and Simoes-Costa 2016, Szabo and Mayor 2018). Ets-1 and Hnk-1 are well-known migratory markers that are associated with NC, but their expression is far from limited to NC cells, and they alone are not sufficient to

determine a cell is both NC and migratory. It is believed that the population of NC cells at any one axial location are completely heterogenous and multipotent prior to delamination (Baggiolini et al. 2015), and within this population there exists cells primed as "trailblazers" (Mclennon et al. 2015, Kulesa and Mclennon 2015), which are capable of guiding other NC cells to their fated destination. However, this distinction appears to be more relevant to trunk than cranial NC. Studies have shown that when a trunk trailblazer is rendered non-functional, the train of NC migration is halted while a NC cell from the back of the line becomes hypermotile, surpassing all of its counterparts and establishing itself as a new trailblazer, which carries on leading the NC migratory highway (Richardson et al. 2016). However, this same study shows a much less organized model for cranial NC, suggesting continuous rearrangement of the leaders and followers. Nonetheless, whether a NC cells is a trunk trailblazer, trunk follower, or cranial NC part of the grouped egression from the dorsal NT, these cells exhibit unique expression profile mixes between NC markers, migratory markers, and other, which speaks to the heterogeneity of the NC population, as well as the robust co-expression of markers than can be seen at later stages.

For a more in-depth and thorough analysis of all NC markers and their spatiotemporal expression profiles and contributions in various model systems, please refer to Prasad et al. 2019a.

D. Instructive Signals contributing to NC Formation

At each step of NC formation, from specification to differentiation, there is a unique and specific recipe and order of growth factors that must contribute instructive signals to accomplish proper NC development. Consistently, Wnt, FGF, and BMP come up as the most involved and required signaling pathways with regards to NC formation, however, they are not the only ones. Notch/Delta, Retinoic Acid, Sonic Hedgehog, and TGF**β** show a definitive involvement in the process, although do not seem to be as integral. From a chronology standpoint, FGF and Wnt

signals dominate the earlier stages, with FGF acting as somewhat of a BMP inhibitor (Wilson et al. 2000, Monsoro-burq et al. 2003, Pera et al. 2003). As Wnt begins to increase it acts to reduce FGF influence on the cells, and as FGF begins to diminish at the later part of early NC development, BMP expression and signaling increases (Wilson et al. 2001, Steventon et al. 2009, Patthey et al. 2009, Hackland et al. 2017/2019).

FGF has major implications in various aspects of development. With a wide range of ligands and multiple cell membrane receptors, it is involved as early as embryonic preimplantation and contributes to organogenesis and limb development, as well (Yuan et al. 1995, Wilson et al. 2000, Stuhlmiller and Garcia-Castro 2012b, Ornitz and Itoh 2015). Its universal involvement in development stresses the importance and sensitivity of the developing embryo to the concentration of its signal for patterning. In NC this is exemplified by medial shifts in NC marker expression when prospective NC from gastrula stage chicken embryos is exposed to an FGFR inhibitor (Stuhlmiller and Garcia-Castro 2012a). Furthermore, beads coated in FGF4 and implanted into non-neural ectoderm of developing chick embryos lead to ectopic expression of Pax7, which was co-expressed with Brachyury, a mesodermal marker (Yardley and Garcia-Castro 2012). Taken together, these results indicate a clear optimal range of FGF signaling within what appears to be a mediolateral gradient that is required for successful NC formation. However, there is a clear distinction between FGF requirements in cranial and trunk NC, as is evidenced by the control of axial identity (determined by levels of HOX gene expression) by FGF in a human trunk NC induction model (Hackland et al. 2019). This antero-posterior gradient in trunk highlights the distinct classes of NC and the implications of fluctuations in the levels required for significant growth factors.

Arguably more studied in regard to NC, but just as delicately involved, is the Wnt signaling pathway. It is present at early blastula stages (Wilson et al. 2001), where the groundwork for

distinction of the germ layers (and NC, if you are of the opinion that it is the fourth germ layer) is laid out, and has been proven to be necessary and sufficient for NC induction in chick (Garcia-Castro et al. 2002). Like FGF, Wnt has shown a clear ability to dictate NC development when administered in specific ranges. Specifically, *in vitro* human models show that lower concentrations lead to cranial NC (Leung et al. 2016, Hackland et al. 2017, Gomez et al. 2018), and higher concentrations robustly produce trunk NC (Gomez et al. 2019, Hackland et al. 2019).

The requirement of early Wnt for NC is two-fold: 1. Early Wnt promotes continued expression of NC markers in appropriate regions whereas late Wnt lacks the ability to stimulate NC formation 2. Proper NC formation relies on early Wnt to set the stage for BMP. Many human induction protocols utilize small molecules to regulate BMP activity (Lee et al. 2007/2010, Menendez et al. 2011, Hackland et al. 2017/2019). It has been proven that BMP is unable to form NC on its own (Garcia-Castro et al. 2002, Patthey et al. 2009), however, it can induce NC ectopically (Selleck et al. 1998), demonstrating it is just as vital to the production of functional NC as Wnt and FGF. A spike in BMP4 expression is observed in neural folds and eventually the dorsal neural tube, which interestingly can be reproduced in the non-neural ectoderm with ectopic FGF4 exposure, and this is paired with ectopic Pax7 and Wnt8c expression (Yardley and Garcia-Castro 2012). While insufficient by itself, fluctuations in BMP expression during NC development can greatly impact the success of NC formation. Top-down inhibition (TDI) of BMP (Hackland et al. 2017/2019) has clear success in improving reproducibility in NC induction protocols, speaking to the volatility of BMP, as well as the sensitivity of NC to BMP, while other models inhibit BMP and/or TGF$\boldsymbol{\beta}$ in their protocols (Lee et al. 2007/2010, Menendez et al. 2011).

Altogether, studies into the main contributing signals responsible for NC formation indicate that FGF expression is responsible for the upregulation of Wnt, which has been shown to increase expression of BMPs, but propagation of BMP signals is negatively regulated by FGF-

mediated MAPK signaling and positively regulated by Wnt, and thus is more relevant as FGF subsides. It also explains why BMP expression and signaling may be so unpredictable in *in vitro* studies, as it is so regulated by both Wnt and FGF. Yet, this whole program may be subject to regulation by glycolysis, or, more likely, the molecule that drives this process: Insulin.

3. Insulin Signaling in Early Development and Neural Crest

A. Why Investigate Insulin?

As studies examining the timing of NC formation continue to point to NC specification occurring at the blastula stage, it opens up further questions into which signaling pathways are involved, as this is unknown territory for NC. This consideration must look at both the presence of growth factors during this stage and subsequently the associated congenital disorders found as a result of complications involving each one *in utero*. Another major consideration is the consistency with which this growth factor may be found in successful experiments investigating the growth and development of a particular cell type. One such growth factor that has been somewhat overlooked in NC and checks each of these boxes is insulin. It has long been regarded as essential for growth and survival, especially considering its vital role in glucose metabolism, but elucidating an instructive role beyond this in early NC specification has proved elusive.

B. Sources of Insulin

Overall, insulin is crucial to glucose metabolism, maintaining homeostasis, production of pro-survival and anti-apoptotic cues, as well as signaling for proliferation and differentiation (Siddle 2011, Guo 2014, Boucher 2014). Insulin is formed after processing of immature protein known as pre-proinsulin, which undergoes cleavage to become proinsulin, which is later cleaved further into insulin, leaving behind the cleaved C-peptide (Liu et al. 2018). This cleavage can occur

after proinsulin is transferred to the embryo, as evidence of proinsulin is seen in chicken embryos as early as E0.5 (similar to HH3/4 in chick) (Alarcon et al. 1998, Hernandez-Sanchez et al. 2006). In the earliest stages of development, insulin is maternally sourced (Heyner et al. 1989, Ruiz-Palacios et al. 2017), which is unsurprising considering most insulin production occurs in the yet-to develop pancreas. However, during neurulation, proinsulin is proposed to be expressed in the embryo (Alarcon et al. 1998), whereas pancreatic buds initiate by E2.5 (similar to HH17), which coincides spatiotemporally with the first instance of IGF production in the embryo. Regardless, insulin's presence has been shown to improve a fertilized embryo's ability to implant itself in the uterine walls (Rao et al. 1990) and its inclusion in medium for *in vitro* fertilization has increased the survival rate of the treated embryos, resulting in higher probability of pregnancy and even higher incidence of twins compared to control medium (Fawzy et al. 2017).

C. Insulin Receptors and Ligand Specificity

Insulin signaling is initiated by an interaction between the insulin ligand and a receptor. The insulin receptor (IR) is a transmembrane protein that forms as a homo- or hetero-dimer between its two isoforms: IR-A and IR-B (Belfiore et al. 2009, Boucher 2014,). These two isoforms are the result of alternative splicing, but interestingly the IR-A isoform is more highly expressed in neonatal tissue and adult brain tissue, whereas IR-B is more commonly found in adult differentiated tissue, including the adult liver (Belfiore et al. 2009). The insulin ligand, however, is capable of binding more than just these two isoforms, as it has shown the ability to interact with other receptors, as well. Insulin-like growth factor (IGF) receptors (IGFRs) and the IR-related receptor (IRR) are also capable of interacting with insulin, as are hybrid IR/IGFR receptors (HRs), which are present in the early embryo (Bailyes et al. 1997, Hernandez-Sanchez et al. 2006, Benyoucef et al. 2007, Zhang et al. 2007, Belfiore et al. 2009, Siddle 2011).

HRs have proved to be capable of binding to insulin, as well as the IGF ligands and proinsulin, regardless of the isoforms of each monomer in the heterodimeric receptor (Garcia De-Lacoba et al. 1999, Benyoucef et al. 2007). Furthermore, the presence of typical IR homodimers in early developmental stages is dramatically lower than that of HRs, although this trend inverts as development continues (Garcia De-Lacoba et al. 1999). Additionally, these HRs showed a greater affinity for insulin and proinsulin at earlier stages, compared to IGF ligands. While the ability of each of these ligands to bind to and signal via homodimers of the opposite receptor (ie. insulin binding to IGFR) is greatly reduced compared to is native receptor (Benyoucef et al. 2007), their presence and functionality in early development suggests a distinct role for each one.

Before detailing the intracellular components of the insulin signaling pathway and its effectors, it is important to mention the ability of both IR and IGFR to function as a transcription factor on their own (Sarfstein et al. 2012, Batista et al. 2019, Hancock et al. 2019). Immunogold EM to visualize both isoforms of IR in the nucleus, nuclear fractionation Western Blotting, Co-Immunoprecipitation (CoIP), and Chromatin Immunoprecipitation (ChIP) sequencing revealed IR's interaction with RNA Polymerase II inside the nucleus of mouse livers, human liver samples, and human cells lines. Interestingly, nuclearization of activated IR and IR signaling via Pi3K are not mutually exclusive, which is noteworthy considering these two methods of transcriptional activation do not target the same genes. Furthermore, in models of obesity and diabetes the levels of nuclear IR are reduced accordingly, supporting the notion that initiation of IR translocation is dependent on insulin interaction.

D. Intracellular Propagation of Insulin Signaling

The promiscuity between the aforementioned ligands and receptors is made possible due to the sequence similarity between these proteins, but also by the equal promiscuity of the

intracellular signaling pathways that are utilized by each (Ward et al. 2013). While insulin and IGF ligands bind more preferentially to their native receptor (IR and IGFR, respectively) they remain capable of binding to the other receptor. IR, IGFR, and HRs utilize Pi3K and MAPK intracellular signaling cascades to propagate their signal, but insulin is not the only growth factor that exploits these branches, as FGF, PDGF, VEGF, EGF, and others also signal via these pathways.

The MAPK pathway is a signal amplification cascade that is initiated after intracellular transphosphorylation of the receptor tyrosine kinase, and in the cases of insulin and FGF, the Erk1/2 MAPK pathway is activated (Siddle 2011, Boucher et al. 2014, Ornitz and Itoh 2015). Activation of the insulin receptor substrate (IRS) leads to recruitment of Grb2 to the intracellular receptor domains, initiating activation of the first substrate Ras, which then phosphorylates Raf, leading to activation of Mek, and finally phosphorylation of Erk1/2. The effectors of this branch are the Elk1 protein and eIF4E, of which the former enters the nucleus and is involved in proliferation, mitogenic transcription, and differentiation, while the ladder aids in regulation of protein synthesis (Siddle 2011, Boucher 2014, Dinsmore and Soriano 2018).

The Pi3K branch is much more complex, with more steps and greater division into sub-branches. Here, IRS activates Pi3K, which converts PIP2 into PIP3, leading to activation of PDK1, which may or may not be bypassed to phosphorylate Akt. From here, the complexity of the Pi3K branch expands, with Akt being responsible for phosphorylation of various enzymes. This regulation includes initiation of Glut4 translocation to the membrane to support glucose metabolism, activation of mTOR, activation of PKB, activation of SREBP1, activation of S6K, inhibition of GSK-3**β**, and inhibition of FoxO1, and more. With this collection of substrates and effectors, insulin is capable of directing different steps in glucose metabolism, lipogenesis, proliferation, and differentiation. Akt also inhibits pro-apoptotic signal BAD, thus promoting survival of the cell (Boucher 2014, Haeusler et al. 2018, Dinsmore and Soriano 2018).

As alluded to earlier, the MAPK and Pi3K branches are also utilized by FGF, PDGF, EGF, and more growth factors. While EGF has shown to be somewhat involved in NC development (Garcez et al. 2009), FGF and insulin appear to be the most heavily involved growth factors that signal via the MAPK and Pi3K pathways during the stages in which NC is specified. This shared use of MAPK and Pi3K muddies the distinction between FGF and insulin signaling in NC studies that only focus on the intracellular mechanisms. However, the common theme in published papers suggests that FGF utilizes the MAPK branch more heavily than Pi3K in early NC development (Stuhlmiller and Garcia-Castro 2012b, Haeusler et al. 2018, Xu et al. 2018, Dinsmore and Soriano 2018). Studies in chick using early stage specification assays show increased phospho-Erk1/2 activation mediated by FGF (Stuhlmiller and Garcia-Castro 2012a), whereas FGF inhibition in xenopus leads to reductions in Erk activity (Geary and Labonne 2018). There is evidence of FGF utilizing the Akt pathway to drive NC formation in zebrafish by way of a small molecule inhibitor (CAPE) which is said to specifically inhibit FGF-mediated Akt activity (Ciarlo et al. 2017). CAPE administration affected Crestin and Sox10 (premigratory NC markers in zebrafish), as well as Pax7a and Dlx2a (early NC markers), but RNA-seq data suggests a stronger effect on late markers compared to early markers. However, this study also shows FGF demonstrating preferential activation of Erk over Akt. Conversely, insulin administration led to higher levels of pAkt levels, feeding further into the narrative of insulin preferentially signaling via Pi3K and FGF favoring MAPK, while supporting the notion that Akt signaling is essential to NC formation. Despite the plethora of FGF ligands capable of activating MAPK/Pi3K via FGFRs in various ways (Ornitz and Itoh 2015), it appears their instruction in relation to NC is primarily distinct from insulin due to the preferred intracellular signal transduction pathway.

E. Insulin and Diabetic Environments Regulate FoxO1

While some studies indicate MAPK as more vital in early stages of NC development than Pi3K, it could be argued that the modulation of Pi3K is more sensitive and the consequences are more severe. This is based on the fact that Pi3K, via Akt, regulates a wider range of transcription factors and functions than MAPK, as mentioned above, and none more important than the regulation of FoxO1 translocation (Accili and Arden 2004). In the search for clarity between the overlapping intracellular mechanisms exploited by insulin and FGF, FoxO1 stands out as a reliable reporter of insulin mediated Pi3K signaling (Gross et al. 2009, Guo 2014, Nies et al. 2016). Other growth factors have shown the ability to regulate the expression of FoxO1, and PDGF can affect its translocation (Essaghir et al. 2009), but FoxO1's intracellular function in most cell types, appears to be more intimately linked to insulin signaling via Pi3K, as opposed to FGF. This is likely the case in NC development, as well. Considering the evidence that FGF-mediated MAPK activity may include the regulation of Pi3K signaling (Ciarlo et al. 2017), this may explain reports where FGF is thought to influence FoxO1 localization. If this is in fact the source of FGF-mediated changes in FoxO1, it would more definitively and uniquely link Pi3K-mediated regulation of FoxO1 to insulin, as opposed to the purported Akt regulation by FGF-mediated MAPK, which would represent a more indirect regulation of FoxO1.

Insulin functions as a negative regulator for FoxO1, signaling via Akt which phosphorylates FoxO1 and prevents its nuclear translocation (Matsuzaki et al. 2003, Gross et al. 2009). Alternatively, in the absence of insulin, Akt does not phosphorylate FoxO1, thus FoxO1 is free to enter the nucleus where it carries out its functions as a transcription factor or cofactor for a wide range of targets (Vogt et al. 2005, Zhang et al. 2015, McClelland Descalzo and Satoorian et al. 2016). In normal conditions, FoxO1 is involved in transcription of a myriad of different genes that participate in reactive oxygen species detoxification, DNA repair, cell cycle arrest, glucose

metabolism, homeostasis, and death (Vogt et al. 2005, Carter and Brunet 2007). The specific activity will vary depending on the circumstances under which FoxO1 nuclearization is mediated, but the targets range from essential, such as p21 and p27, which are involved in cell cycle regulation (Dijkers et al. 2000, McClelland Descalzo and Satoorian et al. 2016), to more instructive like TGF**β**1 (Song et al. 2006, Conway and Kaartinen 2013, Zhang et al. 2015).

F. Insulin and FoxO1 Crosstalk with Other Pathways

This multifunctionality of FoxO1 and sensitivity to changes in the intra- and extracellular milieu speaks to a larger influence of insulin in NC, as specific environments can dictate more than just the output of insulin signaling. The context-dependent response of FoxO1 to insulin is magnified by the known interactions between this pathway and other signaling molecules, especially the aforementioned Wnt, FGF, BMP, and TGF**β**, which are known to heavily contribute to early NC development.

Pertinent to NC, it has been suggested that there is crosstalk between insulin and Wnt signaling. Glycolysis, which is regulated by insulin signaling, has been shown to influence **β**-catenin nuclearization during tail bud elongation in chick (Oginuma et al. 2017). In this study a glycolysis inhibitor is used to asses changes in **β**-catenin nuclear localization in normal versus non-glycolytic conditions, and they report a reduction in **β**-catenin nuclearization in the absence of glycolysis, thus neutralizing Wnt activity and resulting in impaired elongation of the tail bud. This potential crosstalk is particularly relevant given the reported role for Wnt/**β**-catenin in early NC development, being amongst the earliest and most important molecules known to be able to elicit NC induction (Garcia-Castro et al. 2002, Patthey et al. 2008, Stuhlmiller and Garcia-Castro 2012a/b, Mayor and Theveneau 2013, Leung et al. 2016). Additionally, the glycolysis-mediated effect on Wnt signaling reveals lower transcript levels of known Wnt targets, including Axin,

whereas neural markers Sox2 and Sox1 were upregulated, suggesting a switch to neural fate in the absence of glycolysis (Oginuma et al. 2017). In support of this reported hindrance of embryo elongation, increased exogenous insulin exposed to chick embryos during late gastrulation (HH 4) leads to elongation of the embryo (Patwardhan et al. 2004). Furthermore, only certain genes, namely mesodermal and neural related genes, showed modified expression levels, while definitive endoderm genes were unaffected, suggesting a focused effect on specific cell types which are more in line with the potential of NC.

Another report indicated signaling via IR in the absence of IGFR can increase levels of **β**-catenin stabilization (Rota and Wood 2015). Alternatively, it has been shown that IGFR inhibition decreases **β**-catenin via reduced Pi3K, and therefore, decreased phospho-GSK-3**β** (Zhang Q.Y. et al. 2015). However, a modification of GSK-3**β** downstream of Pi3K in the insulin signaling pathway does not influence the ability of GSK-3**β** to form the **β**-catenin destruction complex, as the phosphorylation site targeted in Wnt signaling is unaffected by activity at the site targeted by PKB in the Pi3K pathway (Beurel et al. 2015). Moreover, sequestration of GSK-3**β** after Pi3K branch activity is not sufficient to influence propagation of Wnt signaling, as only a small amount of GSK-3**β** is required to form the **β**-catenin destruction complex (Voskas et al. 2010). Furthermore, high glucose, or otherwise diabetic, environments can result in increased abundance of aged glycosylated end-products (AGEs), one of multiple post-translational modifications known to affect FoxO1 activity, which in turn leads to FoxO1 interacting with **β**-catenin cofactor TCF/LEF in the nucleus (Zhu et al. 2016, Doumpas et al. 2018). The consequential instability of **β**-catenin in the nucleus reduces the effectiveness of Wnt signaling, further signifying the importance of investigating crosstalk between components of the insulin signaling and Wnt/**β**-catenin pathways is vital to understanding NC formation and expression of NC markers.

While insulin regulation of Wnt may be critical to early NC formation, FGF is also required for NC specification and appears to interact with insulin. Glycolysis inhibition leads to a reduction in MAPK phosphorylation in a manner that mimics, but is not equal to, FGF/MAPK small molecule inhibitions (Oginuma et al. 2017). While this study suggests that FGF is responsible for expression of rate-limiting glycolytic enzymes, glycolytic activity is activated by insulin. Nonetheless, it is postulated that glycolysis coordinates signaling of Wnt and FGF in tail bud elongation, indicating requirement of insulin and glycolysis for proper timing of NC relevant growth factor signaling. Furthermore, increased insulin promotes expression of Noggin (Patwardhan et al. 2004), a BMP antagonist, which supports neural development and matches the high FGF, low BMP patterns in the developing neural plate. This indicates that insulin may actually play a role in regulation of all significant NC contributing signals, potentially suggesting insulin's involvement precedes, or at least coincides with early FGF, Wnt, and BMP. However, FGF has proven to mediate insulin sensitivity (Berglund et al. 2009, So et al. 2015, Oginuma et al. 2017), and crosstalk from GPCRs, which also utilize Pi3K, at IRS has been well documented (Law et al. 2016), which may indicate that FGF and insulin signaling are temporally linked and rely on one another for adequate balance for early embryonic patterning. As mentioned above, FGF signaling via MAPK has proven vital to NC induction in chick embryos, but the effects of possible interaction with the insulin pathway during NC formation have yet to be investigated. Interestingly, FGF-induced ectopic Pax7 expression was less common with the addition of naïve neurobasal supplement N2 to the FGF coated beads, which contains insulin (Yardley and Garcia-Castro 2012). This suggests that insulin, or another of the five N2 components, has antagonistic effects on FGF. It is important to note that this effect was seen in the non-neural ectoderm, and not in the epiblast, neural ectoderm, or pNC regions. Nonetheless, whether this influence on Pax7 of N2 in the presence of FGF occurs via

crosstalk or sequestration of shared intracellular branches between FGF and insulin, it provides more evidence to support the idea that FGF signaling is regulated by, or competes with, insulin.

4. Neurocristopathies Associated with Insulin and Insulin Signaling

A. The Diabetes Epidemic and Population Studies of Diabetic Pregnancies

As mentioned earlier, the unique attributes and far-reaching contributions of NC cells means any disruptions in their development can advance into catastrophic congenital disorders. Interestingly, pregnancies hindered by diabetic conditions in which insulin signaling is abnormal result in a higher incidence of neurocristopathies in the offspring (Correa et al. 2008). Specifically, diabetic pregnancies are 1.352 times more likely result in cleft/lip palate in the offspring (Spilson et al. 2001), with an 82% increase in heart defects when NC cells are exposed to high glucose (Roest et al. 2007), and women who are both diabetic and obese are 3.1 times more likely to have offspring with congenital defects, including cleft lip/palate and septal heart deformities (Moore et al. 2000). This effect is particularly alarming considering the worldwide diabetes epidemic, with the World Health Organization (WHO) reporting 422 million people (8.5% of total population) being considered diabetic in 2014, with 1.6 million deaths directly resulting from diabetes. Five years later, the International Diabetes Federation (IDF) reports that the current adult diabetic population worldwide is 9.3%, which is over 463 million people. Coincidentally, this is also similar to the proportion of diabetic individuals within the US in 2017, according to the Centers for Disease Control and Prevention (CDC). This number is trumped by the 33.9% of adults in the US that are considered pre-diabetic (2014/15 report), and the 9.2% of healthy women who develop gestational diabetes (Desisto et al. 2014, Alfadhli 2015). Furthermore, it is estimated that $295 million was spent in 2019 on diabetic health in the US, which has the third largest adult diabetic population at 31 million, surpassed only by China at 116.4 million and India at 77 million.

While likelihood of congenital defects is generally higher in diabetic pregnancies, investigations into the consequences of diabetes during pregnancy, represented as fluctuations in insulin or glucose levels or mutations in the insulin signaling pathway, have revealed a higher occurrence of neurocristopathies in these offspring. One of the most well-known and most common neurocristopathies is cleft lip/palate, seen in approximately 1/800 births (Rahimov et al. 2012) and associated with a mutation in Pax7 affecting DNA binding (Leslie et al. 2015, Beaty et al. 2016), occurs more frequently in mothers with pre-gestational or gestational diabetes (Correa et al. 2008). There is evidence of variations between and predispositions within different ethnicities and socio-economic backgrounds, but the ultimate commonality is poor diet, obesity, and diabetes (Stott-Miller et al. 2010). Other common neurocristopathies that can be attributed to dysregulation of insulin are micrognathia, anencephaly, microtia, and cardiac defects, specifically in the heart tube and outflow tracts (Morgan et al. 2008, Correa et al. 2008).

B. Investigating the Connection between Insulin Dysregulation and Neurocristopathies

To further investigate the connection between insulin dysregulation and congenital defects, many mammalian diabetes models have been generated either by genetic modification or drug administration. A common molecule used is streptozotocin (STZ), which allows researchers to induce diabetes with temporal control. STZ treatment in rats prior to mating resulted in noticeable reductions in birthweight and size of offspring (Siman et al. 2000). Exposure to a single high dose of STZ at 5 days postcoitus resulted in a prolonged gestation period by two full days, but most notable was a 13% increase in maxillofacial deformities in the offspring (Garcia et al. 2015). There was also a higher incidence of intrauterine deaths, but the surviving pups exhibited reduced naso-occipital length and clefting of the lip and palate, as well as other underdeveloped craniofacial structures. Furthermore, these types of malformations have been suggested to be influenced by

hyperglycemia during the earliest stages of pregnancy (Cederberg et al. 2003). The exact effect of low insulin, high glucose, or any associated environmental change such as oxidative stress, on NC cells is up for debate. Some studies suggest it merely causes a delay in development, as seen in the aforementioned 23-day gestation in rats who had underdeveloped NC-related facial structures. Other studies reveal a hindrance of the phenomenal migratory capabilities of NC, showing either increased apoptosis or a complete inability to delaminate from the neural tube to form dorsal root ganglia, elements of the PNS, and cardiac outflow tract (Suzuki et al. 1996, Morgan et al. 2008, Chen et al. 2013). These migratory challenged NC cells also exhibit reductions in Pax3, Pax7, and migratory marker HNK-1. However, these and other studies addressing the role of insulin signaling in NC development focus on later stages of development or adult tissue (Nekrep et al. 2008, Olerud et al. 2009), leaving a gap in our knowledge in early NC development.

C. Intracellular Insulin Signaling Components and Association with Neurocristopathies

A clear connection has been established between Pi3K and MAPK mutations, NC development, and manifestation of neurocristopathies. However, the mechanisms and pathways linking insulin to NC development and associated pathologies remain largely unexplored. One of the biggest caveats of these studies is how widely used these intracellular branches are for both instructive and proliferative/survival signals, thus making LOF studies very often lethal to the developing fetus. Many of these components leave embryos unviable in double knockouts, but partial knockouts have allowed for more in depth analysis.

Signaling via MAPK is required for proper development and function of NC. In gastrula stage chick embryos, inhibition of Erk1/2 via electroporation of a plasmid which overexpresses Erk1/2 negative regulator Mkp3 (Stuhlmiller and Garcia-Castro 2012a). Interestingly, this inhibition also led to ectopic medial expression of Pax7 in the neural ectoderm. Nonetheless, the

loss of Pax7 in the NPB matched the phenotype of dominant-negative FGFR electroporations and small molecule inhibition of FGF-mediated MAPK, supporting the notion of MAPK being linked more closely to FGF signaling in early stage NC specification. Patients exhibiting craniofacial and cardiac deformities likened to DiGeorge syndrome, including cleft lip/palate, as well as PNS developmental mutations, including absent DRG, have presented with haplo-insufficiency in Erk1/2, preventing proper MAPK signaling (Newbern et al. 2008). This result was confirmed *in vivo* in conditional Erk1/2 knockouts in developing NC of transgenic mice generated with Wnt1 responsive Cre elements flanking exon 3 of Erk1/2. Furthermore, deletion of Erk1/2 resulted in absent DRG and other undeveloped structures in the PNS, including Schwann cells (Newbern et al. 2012). Conversely, Erk1/2 GOF leads to various craniofacial anomalies, such as cleft lip/palate, increased skull size, micrognathia, and others similar to Noonan syndrome (Nakamura et al. 2009). Somewhat unsurprisingly, these deformities were remedied by reducing Erk activity via small molecule inhibition. Additionally, Ets1, which is both an effector of Erk1/2 downstream of FGF, as well as a migratory marker seen in NC and other cell types, has been shown to be activated by FGF in NC (Davidson et al. 2006). Furthermore, Ets1 deficient mice presented with cardiac defects derived from NC cells, including overgrown ventricular cartilage (Gao et al. 2010). This phenotype had increased Sox9 expression in the ectopic tissue, suggesting overexpression of this NC and chondrocyte marker is regulated by Ets1, either directly or indirectly, and also speaks to the delicate mixture of NC markers and differentiation potential in this highly heterogeneous population.

The studies discussed here regarding FGF's proclivity for signal propagation via Erk1/2 display a common theme: FGF is proven or implicated to be responsible for the initiation of these signals, but insulin is not mentioned. Alternatively, in studies investigating Pi3K and the downstream components in NC development, there appears to be implications that Insulin is directing the activity of Pi3K.

Pi3K has two major domains: the p110 catalytic domain, and the p85 regulatory domain (Tups et al. 2010). Each of these subunits have various isoforms, and some are more relevant to insulin and NC development than others. Double knockout of p85 is embryonic lethal by E 12.5, with apparent facial clefting, but knockout of a single allele allowed for some viable embryos (Brachmann et al. 2005). Of the different isotypes of the p110 subunit, p110**δ** is primarily found in leukocytes (Vanhaesebroeck et al. 1997), whereas p110**α**, **β**, and **γ** all have roles relating to development and/or insulin (Hooshmand-Rad et al. 2000, Tups et al. 2010), but not exclusively. Regardless, their involvement in NC formation is evident among various model organisms, including xenopus, zebrafish, mice, human cell lines, and chicken. Interestingly, studies in xenopus show clear evidence of early MAPK function and subsequent Pi3K activation, with Pi3K and Akt phosphorylation phenocopying dnFGFR4 injection (Geary and Labonne 2018). Contradictory to this report, FGF signaling in zebrafish appears to preferentially signal via MAPK, as is evidenced by an absence of Akt activation by FGF (Ciarlo et al. 2017). However, they found that their drug, CAPE, only blocks Akt activity in the presence of FGF, which is supported by evidence of increased Akt phosphorylation after administration of a Mek inhibitor. This confounding unidirectional regulation of Pi3K by MAPK is likely the result of CAPE exploiting a previously undocumented ability for FGF to prime Mek for Akt regulation independent of Insulin signaling, which has since been supported in other studies (Xu et al. 2018). Furthermore, this study points to a non-FGF growth factor as responsible for activating Akt in NC development.

Downstream of Pi3K lies PDK1, which is directly phosphorylates Akt. As with other Insulin signaling components, a double knockout results in embryonic lethality, in this case at embryonic day 9.5 (Lawlor et al. 2002). These unviable fetuses exhibit developmental delay, along with complete lack of any heart structure, but more relevantly, they have no pharyngeal arches or DRG. All three of these structures rely on NC, either entirely or partially, to develop completely.

Additionally, the viable mice were much smaller in size than their control counterparts, but this was not due to lack of cells, but rather cell size. Furthermore, administration of insulin to the PDK1 homozygous or heterozygous knockouts reestablished normal activation of other Pi3K effectors. This indicates that propagation of Pi3K-mediated activation of Akt from high doses of insulin can bypass PDK1 in order to support development of NC derivatives, but does not reconcile problems with organ volume, or the fact that there were clear NC defects from PDK1 mutations.

Although there is very little information available about the role of FoxO1 during neural crest development, this is perhaps unsurprising in light of the degree to which insulin signaling has been overlooked in this field. However, FoxO1 has been shown to be involved in proper formation of NC derivatives, where mice lacking FoxO1 cannot form branchial arches (Furuyama et al. 2004), but this study investigates FoxO1 in the context of migration of endothelial cells at E9.5 and suggests it is under regulation by VEGF. Also, FoxO4 morpholinos are capable of reducing FoxD3 and TFAP2**α** expression and impeding NC migration, as well as heart and eye development (Schuff et al. 2010), but once again this study focuses on later stages of development, even declaring FoxOs as dispensable during gastrulation. Ironically, they show that FoxO1 transcript levels are higher than FoxO4 in early stages, as well as in the eye, heart, and gut, where NC is known to migrate to. Additionally, FoxOs appear to participate in regulation of congenital neuroblastomas, which arise from defects in NC (Mei et al. 2012). However, FoxO4 and FoxO1 are both implicated here, and they are tied to the expression of PDGF and propagation of its signal.

Chapter 2: Insulin Signaling Provides Instructive Cues for Blastula-Stage Neural Crest Specification

Abstract

Early events during Neural Crest (NC) formation have been reported to rely on Wnt, FGF, and BMP signaling contributions amongst others. However, a role for insulin during early NC formation has not been established, despite insulin implications in various neurocristopathies, and its consistent use as a component in chick and rabbit embryo NC specification assays, as well as in human and mouse models of NC formation from pluripotent stem cells (PSCs). Here, through a robust model of human NC formation and chick embryo experiments, we report that insulin is required for NC development, providing instructive cues, independent from its known functions in growth and metabolism. Experiments including absence of insulin ligand, various insulin inhibitors, and knock down conditions, suggest that insulin signaling is required at early stages during NC formation, before overt expression of definitive NC markers. Small molecule inhibition and RNAi knockdown of insulin receptor (IR) and insulin-like growth factor (IGF) receptor (IGFR), both individually or simultaneously, causes reduced expression of definitive NC markers, and in most cases does so without a significant effect on cell survival, suggesting the contribution of both receptors or a hybrid IR/IGFR during NC formation. Furthermore, we show evidence that these signals are propagated via Pi3K and PDK1, which lead to Akt phosphorylation of FoxO1, an insulin effector, which our results identify for the first time as a relevant contributor during early NC specification.

Introduction

NC cells are a transient population of cells unique to vertebrates that arise early in development and contribute to a diverse range of tissues and organs throughout the body. NC undergo an EMT and emigrate from their dorsal NT location, and engage in stereotypic and extensive migration in a rostrocaudal wave that corresponds to their sequential and progressive formation and maturation, starting from cranial regions, and followed by vagal, trunk, and sacral sections. Their multipotent differentiation capacity allows for contribution to numerous tissues, organs, and structures. Importantly, these contributions appear axially restricted, with melanocytes, neurons and glia of the peripheral nervous system, being generated by NC along the entire axis, while ectomesenchymal derivatives which include odontoblasts, and craniofacial bone and cartilage, amongst others, are unique to anterior NC. Disruption in formation or function of NC can result in a wide range of pathologies known as neurocristopathies, which include debilitating or fatal congenital defects, rare syndromes and aggressive cancers. To understand, treat, and prevent these developmental disorders it is essential to fully understand the formation of NC cells and the complex signaling contributions that direct NC specification, differentiation, and function.

Traditionally, NC has been suggested to arise after gastrulation, from the ectoderm, at the border between neural and non-neural ectoderm and above the underlying mesoderm, responding to converging signals emanating from these juxtaposed germ layers. However, early anterior NC has been shown to arise independently from surrounding signals conferred during or after gastrulation in chick embryos (Basch et al. 2006), and similar results were reproduced with rabbit embryos in the first demonstration of early mammalian anterior NC specification during gastrulation (Betters et al. 2018). More recent work has revealed that chick anterior NC cells are specified as early as the blastula stage (Prasad et al. 2019b), and in xenopus it is apparent that the NC program is linked to blastula cells (Buitrago et al. 2015). As the idea of blastula stage NC

specification continues to be proven across more model organisms, it forces reexamination into the roles of the growth factor program responsible for early NC specification.

Exploration into early stages of NC development has been made more feasible with the development of human NC induction models from PSCs. The first such model appeared almost fifteen years ago (Pomp et al. 2005) and relied on stromal cell co-cultures and complex KSR media. Since then a considerable number of variations have been generated by several groups, giving way to defined culture conditions in which culture components are known (Lee et al. 2007/2010, Menendez et al. 2011, Fukuta et al. 2014, Zeltner et al. 2014, Leung et al. 2016, Hackland 2017/2019, Gomez et al. 2019a/b). Our group has described a human NC formation model based on PSCs induced through Wnt signaling that generates anterior NC in 5 days (Leung et al. 2016). This model has been used to interrogate NC ontogeny, define the early acquisition of NC traits and departure from stemness state (Prasad et al. 2020, submitted), examine transcriptional profiles during NC formation and identify novel effectors of NC formation (Leung et al. 2019, BioRxive), generate xeno-free models of NC formation amenable for clinical translational efforts (Hackland et al. 2017/2019), establish the role of ECM1 mutants associated with human craniofacial defects in early NC development (Marquez et al. 2020), and define the epigenome of differentiating cranial NC (Wilderman et al. 2018). Additional efforts focused on the temporal and magnitude parameters of Wnt signaling during NC formation. These studies revealed the efficacy of a transient 2-day pulse of Wnt to efficiently generate NC from PSC in 5 days and exposed the modulation of NC axial identity by the magnitude of the WNT signal (Gomez et al 2019a/b).

Using the aforementioned human NC models and a variety of *in vivo* models, including chick, xenopus, zebrafish, rabbit, and axolotl, inductive signals contributing to NC formation have been well investigated, namely Wnt (Dickinson et al. 1995, Ikeya et al. 1997, Garcia-Castro et al. 2002, Steventon et al. 2009, Patthey et al. 2009, Stuhlmiller and Garcia-Castro 2012b, Gouti et al.

2014, Turner et al. 2014, Barriga et al. 2015, Leung et al. 2016, Hackland et al. 2017/2019, Gomez et al. 2019a/b), FGF (Monsoro-Burq et al. 2003, Yardley and Garcia-Castro 2012, Turner et al. 2014, Stuhlmiller and Garcia-Castro 2012a/b, Ciarlo et al. 2017, Betters et al. 2018), and BMP (Dudas et al. 2004, Linker et al. 2004, Steventon et al. 2009, Patthey et al. 2009, Hackland et al. 2017) and other signaling pathways, as well, including NOTCH (Milet and Monsoro-Burq 2012, Mayor and Theveneau 2013, Prasad et al. 2019a). With mouse and human models to study NC formation based on PSCs (Pomp et al. 2005, Lee et al. 2007/2010, Aihara et al. 2010, Menendez et al. 2011, Fukuta et al. 2014, Gouti et al. 2014, Turner et al. 2014, Zeltner et al. 2014, Leung et al. 2016, Hackland et al. 2017/2019, Gomez et al. 2019a/b, Odashima et al. 2019), there has been widescale interrogation into the earliest stages of NC development, and it is worth noting that all these models involve insulin for *in vitro* and *ex vivo* NC studies, yet no report has assessed its role in NC formation.

Insulin is classically known for modulating glucose metabolism, but also has a major role in maintaining homeostasis, survival cues, and signaling for proliferation and differentiation (Siddle 2011, Guo 2014, Boucher 2014). It is maternally sourced (Heyner et al. 1989, Ruiz-Palacios et al. 2017), required early in development (Rao et al. 1990, Fawzy et al. 2016), and imbalances in insulin levels or mutations in its signaling pathway have been associated with congenital defects, including known NC-associated orofacial clefts (1/800 births in entire population), and malformations of heart valves (Rao et al. 1990, Siman et al. 2000, Correa et al. 2008, Hrubec et al. 2009, Stott-Miller et al. 2010, Rahimov et al. 2012, Garcia et al. 2015, Leslie et al. 2015, Beaty et al. 2016, Dinsmore and Soriano 2018). Considering the diabetes epidemic, as the World Health Organization reports 422 million people worldwide were diabetic in 2014, while the International Diabetes Foundation measured over 463 million diabetic individuals in 2019 (9.3% of the

population), and the fact that 9.2% of healthy women develop gestational diabetes (Desisto et al 2014, Alfadhli 2015), it is imperative that we delve into the role of insulin during early NC formation.

Given the associations of insulin dysregulation with neurocristopathies, and insulin's constant presence in NC formation studies in chick (Bottenstein and Sato 1976, Tessier-Lavigne et al. 1988, Basler et al. 1993, Dickinson et al. 1995, Garcia-Castro et al. 2002, Patthey et al. 2008/2009, Stuhlmiller and Garcia-Castro 2012a, Prasad et al. 2019b), rabbit (Betters et al. 2018), mouse (Aihara et al. 2010, Gouti et al. 2014, Turner et al. 2014), and human models (Pomp et al. 2005, Lee et al. 2007/2010, Menendez et al. 2011, Fukuta et al. 2014, Zeltner et al. 2014, Leung et al. 2016, Hackland 2017/2019, Gomez et al. 2019a/b), including other models, it is remarkable that few studies have addressed its role in NC development. Insulin has been explored as a molecule promoting and directing differentiation of NC derivatives, in late stage embryos (Cederberg et al. 2003) and adult tissues (Nekrep et al. 2008, Olerud et al. 2009). However, no reports have defined the function and mechanism by which insulin may direct early events during NC formation.

Expression profiles of proinsulin, insulin, IGF, and their respective receptors (IR and IGFR) have been established in chick embryos as early as gastrulation, which occurs at embryonic day E0.5 (Alarcon et al. 1998, Hernandez-Sanchez et al. 2006). However, maternally sourced insulin is required for early embryo survival prior to gastrulation (Rao et al. 1990, Fawzy et al. 2016, Ruiz-Palacios et al. 2017). Furthermore, experimental studies of insulin dysregulation during pregnancy have shown a range of maladies presented in the offpsring, some of which are fatal. Reports of underdeveloped NC derivatives, including various facial malformation such as cleft lip/palate, and deformed aortic outflow tracts, which rely either completely or partially on NC for proper development, were observed in offspring of rat models of gestational diabetes but absent in control litters (Garcia et al. 2015). Specifically, outflow tract defects in diabetic mouse models were

linked to maternal hyperglycemia, resulting in poor migration of Pax3+ CNC (Morgan et al. 2017). These congenital defects could be a result of improper formation, survival, proliferation, and/or function (ie. migration) of NC cells. Interestingly, litters from diabetic mothers tend to be smaller, and those that are morphologically normal require prolonged gestation period (~23 days) compared to their control counterparts (21 days) (Garcia et al. 2015). Altogether, this suggests developmental delays, as opposed to complete inability to form NC derivatives.

The abnormalities in NC derivative formation seen in these experiments are also prevalent in studies where MAPK and Pi3K, the intracellular branches of insulin signaling, were modified (Dinsmore and Soriano 2018). The activation of these branches can be triggered by many other growth factors (including FGF, EGF, VEGF, PDGF, etc.), making dissection of the mechanistic contributions of insulin more difficult. Nonetheless, in the context of NC, MAPK is widely reported to be majorly utilized by FGF for its signaling contributions (Stuhlmiller and Garcia-Castro 2012a, Ciarlo et al. 2017, Geary and Labonne 2018, Dinsmore and Soriano 2018). Specification studies in chick showed FGF-mediated ppERK1/2 activation in prospective NC regions, with inhibition of FGF leading to a medial shift of Pax7 (Stuhlmiller and Garcia-Castro 2012a), and further studies also support the link between FGF and ERK activity during NC development (Geary and Labonne 2018). While Pi3K is also downstream of FGF, it has not been implicated as the intracellular pathway for signal propagation. Insulin, however, has been linked, although sparsely, in favoring transduction of its signal via Pi3K and its downstream targets, such as PDK1, in NC. While embryonic lethal by day E11, homozygous PDK1 knockout mouse embryos exhibit absent brachial arches and dorsal root ganglia, both NC derivatives, and impaired formation of heart tube, which NC contributes to, while maintaining normal closure of the neural tube (Lawlor et al. 2002). Furthermore, Akt, which is directly downstream of PDK1, has been shown to be required for early NC development in zebrafish (Ciarlo et al. 2017), although this study suggests their small molecule

inhibitor, CAPE, specifically inhibits FGF-mediated Akt activation. They do, however, show that FGF activates Erk preferentially over Akt, whereas insulin appears to stimulate higher levels of phospho-Akt.

The novel research presented here establishes a distinct role for insulin signaling in early NC specification events in chick and initial stages of human NC induction from pluripotent stem cells. Using chick NC specification assays, which test bisected chick explants in hypoinsulinemic conditions and in presence of small molecule inhibitors, we demonstrate the requirement for insulin and unimpeded intracellular Pi3K signaling for expression of definitive neural plate border specifier Pax7, the earliest visible NC marker in chick. Furthermore, experiments using our robust human NC model (Gomez et al. 2019a) also suggest a key role for insulin/Pi3K signaling during early NC formation. Small-molecule and RNAi screens indicate distinct temporal effects, with early NC markers (Pax7, Msx1, Pax3, and Zic1) being less sensitive to insulin signaling inhibition compared to late markers (SNAI2, Sox9, Sox10, FoxD3). Additionally, insulin regulation of FoxO1 nuclearization appears to contribute significantly to NC development, as both inhibition of FoxO1 and increased insulin levels in hNC lead to a sharp drop-off in expression of all NC markers tested.

Results

Insulin is required for NC Specification in Chick explant assays

Specification assays aim to reveal the initiation of a developmental path, before markers of such path are expressed and before that path has been attained. Specified cells have started the differentiation program to acquire a fate, and may do so if undisturbed, however, they are not committed to said program and may respond to deviating instructions by adopting alternative paths. Chick epiblast and gastrula explants have been extensively used to assess the specification of neural, neural crest and craniofacial placodes (Wilson et al. 2000/2001, Basch et al. 2006, Patthey et al. 2008/2009, Linker et al. 2009, Stuhlmiller and Garcia Castro 2011, Prasad et al. 2019b). These studies, as well as a majority of other NC studies in other models, have consistently used the N2 supplement as "supportive" non-inductive or "naïve" media component. N2 however, consists of 5 components (human transferrin, progesterone, putrescine, selenite, and insulin). To address the possible instructive contribution of insulin under these culture conditions, we performed NC specification assays with blastula stage chick embryo explants cut from an equatorial strip containing prospective NC, as previously reported (Prasad et al. 2019b) (Figure 1A-B). We sought to address if insulin alone, in the absence of all other N2 components, is able to promote NC specification in epiblast explants, which resulted in maintained robust Pax7 expression (N=16/16) (Figure 1C). Astoundingly, there appears to be no noticeable change in Pax7 expression or cell health with exposure of explants to 1:2 (N=6/6), 1:10 (N=4/4), and 1:100 (N=6/6) the concentration of insulin in N2 (860nM) (Figure 1C).

As determination of the necessity of insulin relies on the ability of the program to function without it, explants were incubated in N2 without insulin, resulting in total loss of Pax7 (N=14/14) (not shown). However, to better assess the loss of Pax7 we took a full strip of equatorial explants

and diagonally bisected them (Figure 1D), thus allowing exposure of each half to N2-supplemented media either with or without insulin. Bisections incubated in N2 expressed Pax7 as shown previously, while their counterparts incubated in N2 without insulin were unable to display Pax7 (N=6/6) (Figure 1E). Importantly, insulin depleted explants displayed normal nuclear counts and appearance, and no major effect could be identified based on DAPI nuclear staining (Figure 1E). Thus, removing only insulin from N2 media is enough to impede NC specification in a non-survival related capacity.

To explore the temporal requirement for insulin during NC specification, we performed experiments in which insulin was provided either during the first or the second 12.5 hours of the full 25 hours of incubation (Figure 1F). Immunofluorescence revealed that exposure during the first half of the culture was sufficient to enable progression of specified cells to express Pax7 (N=8/8), while explants cultured for the second half were less capable (N=4/8) (Figure 1G-H). Overall these experiments expose for the first time a requirement for insulin during early events of NC specification in chick embryos.

Human Neural Crest Formation from PSC also Requires Insulin

To assess if and how human NC formation is affected by insulin, we took advantage of our robust model of NC formation in which human PSCs exposed for the first 2 days to Wnt signaling efficiently generate NC after 5 days of culture under defined DMEM-B27 culture conditions (Leung et al. 2016, Gomez et al 2019a). In this model, insulin is included as a component of the B27 neurobasal supplement. To establish a role for insulin in this protocol, normal B27 was replaced with "B27 minus insulin" for the duration of the induction (condition henceforth referred to as "No Insulin"). After the 5-day protocol, total cell counts observed under no insulin conditions dropped to 45% compared to control cultures (Figure 2A/B). Importantly, the remaining cells did

not appear unhealthy, and few pyknotic nuclei were detected. Immunofluorescence for Pax7, and the premigratory/migratory NC marker Sox10 revealed robust NC formation under control conditions, but significantly reduced expression of these markers was found under no insulin treatment (19% Pax7+, 9% Sox10+, and 6% Pax7+/Sox10+ compared to normal values) (Figure 2C). Further analysis via RT-qPCR for a wider range of NC markers confirmed the requirement for insulin and revealed a dysregulated expression profile under no insulin condition compared to control cultures, with early NC markers Pax7, Msx1, Pax3, Sox9 and Zic1 all displaying increased expression levels, whereas late markers Sox10 and FoxD3 showed decreased expression levels, and SNAI2 was unaffected (Figure 2D). These results clearly demonstrate that insulin plays an important role in this human NC formation model.

Human Neural Crest Formation Tolerates Significant Changes of Insulin Concentrations

The above experiments tested the effect of insulin during NC formation through the inclusion or exclusion of insulin from the B27 supplement used in culture media. The amount of insulin tested was based on the composition of the supplement, which was not designed to address NC formation (Brewer et al. 1993).

Therefore, we decided to assess the effect of different insulin concentrations for NC formation under our culture conditions. To this end we added increasing concentrations of insulin to the B27 insulin free media and monitored cell proliferation/survival via DAPI nuclei counts and NC formation through PAX7 and SOX10 immunofluorescence. We also performed a different insulin titration by diluting the B27 with insulin with the B27 without insulin and found similar results (data not shown). Overall, we tested a range of concentrations around the 3.125µg/ml found in the B27 supplement (Brewer et al. 1993, recipe from the Hanna lab at the Weizmann Institute 2016) from 8µg/ml to No Insulin. As before, No Insulin cultures revealed a drop of roughly 50%

in nuclei counts, and a sharp reduction of NC markers. Instead, while no significant drop in overall nuclei count was found from .04µg/ml to 8µg/ml of insulin, immunofluorescence for NC markers revealed a dynamic response (Figure 2E-G). There was, however, a significant increase in total cells in the 8µg/ml condition of 9% (Figure 2F). Compared to control cultures, insulin concentration from 0.04µg/ml to 4µg/ml display high NC marker expression, while at higher concentrations of 6µg/ml and 8µg/ml, a significant reduction was observed (Figure 2E-G). Interestingly, we noted that an apparent reduction could be appreciated at ~0.4µg/ml of insulin (34% Pax7+, 42% Sox10+, 23% Pax7+/Sox10+), but not at lower concentrations. In further analysis, qPCR data confirmed decreased expression of early NC markers Pax7, Msx1, and Zic1, but no apparent effect on Sox9, Sox10, FoxD3, or SNAI2 (data not shown). *In vitro* hNC studies have shown distinct concentrations of Wnt (Leung et al. 2016, Hackland et al. 2017/19, Gomez et al. 2019a/b), FGF (Hackland et al. 2019), and BMP (Menendez et al. 2011, Lee et al. 2010, Hackland et al. 2017) can generate robust NC marker expression and maintain derivative potential, yet Wnt modulation generates a double-peak of NC marker expression, with lower concentrations (2.5-3µM) (Leung et al. 2016, Gomez et al. 2019a)generating cranial NC and higher concentrations (10µM) forming trunk NC (Gomez et al. 2019b), and FGF levels can dictate axial identity in trunk crest. These variations in insulin that result in decreases of NC marker may represent a similar axial control or bimodal response, but further investigation is necessary.

Human Neural Crest is most Sensitive to Insulin Depletion During First Day of Induction

To better characterize the effects of insulin during hNC formation, we explored its temporal parameters by testing different deployment and duration times (Figure 2H). As before, providing insulin for the whole five days of culture provides robust NC formation assessed by Pax7 and Sox10 IF, also counting for cells coexpressing both markers. Instead, when insulin is added for the last 4

or last 3 days of culture, a significant and progressive 50% and 90% reduction of efficiency in NC formation was observed, respectively (Figure 2J-L). Remarkably, adding insulin only for the first 2 days of culture provided normal NC expression markers with no significant changes. To further analyze insulin requirement during the first two days we assessed hNC induction when insulin is added the 1st or 2nd day only. Adding insulin only for the first day of culture had a significant 70% reduction, while adding it on the 2nd day only provided a not significant 50% reduction of NC markers. These results suggested that perhaps the second day was more critical than the 1st day of insulin treatment, however, when insulin was provided during the 1st day and from the 2nd to the 5th days of culture, making insulin unavailable from day 1 to day 2, the expression of NC markers was normal. Comparing this result with absence of insulin only during the first day (insulin added from day 1 to day 5) where NC markers are significantly reduced, this suggests that while insulin alters hNC formation throughout the 5 day culture, it is the 1st 24 hours of treatment that are most sensitive to its absence.

Considering the importance of early insulin exposure for NC induction, a wealth of gene transcripts were measure at the end of the first day of culture using qPCR (Figure S1). These genes represent early NC markers (Pax7, Pax3, Gbx, Zeb2, Msx1, Msx2), FGF targets (Dusp6, Spry1, Spry2), BMP targets (Id1, Id3, Klf2, Klf5), Wnt targets (Axin2, Sp5, Dkk1, Lef1, Myc), and TGF**β** targets (Serpine1, Smad7, CDKN1A; including TGF**β**1). The transcript profile of No Insulin showed general downregulation of all NC markers after only 24 hours of culture. Two of the three FGF targets were upregulated, while Spry1 was downregulated, and BMP targets were upregulated, with the exception of Klf5. TGF**β** targets were downregulated, however, TGF**β**1 itself was upregulated. Of great importance to our induction model, Wnt targets showed very mixed range of effects, overall indicating dysregulation of Wnt signaling.

PKCε Increases Cell Numbers in Absence of Insulin Without Promoting Neural Crest

With Insulin being highly involved in cell health, from homeostasis to pro-survival cues, separating a potential instructive role from its biggest role, glucose metabolism, would allow for true assessment of the consequences of absent insulin signaling. In a search to understand insulin independent Glut-4 membrane translocation, studies have been conducted to isolate PKC activity in the absence of insulin. PKC lies downstream of PDK1 in the Pi3K branch and has a variety of isoforms. Using diacylglycerol (DAG), it has been established that specifically PKCε is capable of increasing Glut-4 membrane translocation independently of insulin signaling (Tsuchiya et al. 2013). Thus, administration of FR236924 (50nM), a highly specific PKCε activator, to hNC induction in hypoinsulinemic conditions should be capable of increasing the metabolic activity, and subsequently proliferation, without increasing NC due to a lack of instructive signal from insulin. Indeed, administration of PKCε activator at 50nM increased the population of cells by 51% compared to No Insulin control, while NC marker expression changes were negligible (Figure 3).

Receptor Inhibition Reveals Potential Utilization of Hybrid IR/IGFR by Insulin in Chick

Insulin signaling pathway is initiated by binding of the insulin ligand to one of several receptors, including multiple IR isoforms, IGFRs, and even hybrid IR/IGFR receptors (Bailyes et al. 1997, Hernandez-Sanchez et al. 2006, Benyoucef et al. 2007, Zhang et al. 2007, Belfiore et al. 2009, Siddle 2011). However, the specific receptor(s) that may contribute to insulin signaling in early NC development have not been documented. To approach this question, chick NC specification assays were executed with small molecule inhibitors specific for IR (HNMPA-(AM)3 40μM) (Baltensperger et al. 1992), IGFR (PQ 401 6 and 12μM) (Gable et al. 2006), or both simultaneously (BMS 536924 300nM) (Zhou 2015). Chick epiblast equatorial explants were bisected to provide one sample as control (DMSO), while the counterpart tissue was exposed to the

small molecule treatment, and assay was carried out as before. While control samples display the expected Pax7 signal for specified NC, the inhibition of IR (N=8/8), IGFR (N=10/10), or both receptors simultaneously (N=7/8) all lead to consistent Pax7 inhibition without noteworthy effect on cell health (Figure 4A-C). This result suggests that the contribution of insulin during early chick NC development, does not rely exclusively on IR or IGFR, but instead appears to utilize hybrid receptors. Alternatively, NC specification in our tests my require and utilize other endogenous growth factors like IGF. Further studies are needed to clarify this possibility.

Insulin Appears to Signal Predominantly via IGFR to Promote Human Neural Crest

To asses which insulin receptors participate during hNC formation we induced hNC under the presence of IR (HNMPA-(AM)3 at 35μM), IGFR (PQ 401 at 12μM), and IR-IGFR (BMS 536924 at 300μM) small molecule inhibitors, and NC markers were monitored by IF after 5 days of culture. IGFR inhibition resulted in a strongly impaired expression of NC markers (43% Pax7+, 38% Sox10+, 34% Pax7+/Sox10+) (Figure 4D-F). Instead, IR inhibition did not result in reduced expression of NC markers (103% Pax7+, 95% Sox10+, 102% Pax7+/Sox10+) (Figure 4D-F). Importantly, at the doses tested neither inhibitor triggered a considerable reduction of nuclear counts (95% and 92%, respectively). Simultaneous inhibition of IR and IGFR with BMS 536924 triggered significantly reduced NC marker expression (23% Pax7+, 8% Sox10+, 7% Pax7+/Sox10+), and nuclei counts (47%), to numbers matching the No Insulin trend, (Figure 4D-F). These results suggest that in our human NC model, insulin signaling relies on the IGFR, while in the chick explant model of NC specification, insulin may use both IR and IGFR to convey its effects.

To further analyze the influence each small molecule has on NC markers, mRNA levels were assessed via qPCR. All inhibition treatments led to abnormal expression profiles, with IGFR

inhibition leading to reduction in all markers tested, while IR increased transcript levels of all markers, some very substantially. Dual receptor inhibition generated a transcript profile that phenocopies No Insulin, with increased early markers and decreased late markers. While each profile is unique, both dual receptor and IGFR inhibition hindered mature NC marker expression, whereas dual receptor and IR inhibition both increase early marker expression. This suggests further that IGFR is more heavily involved in human NC formation, and that IR may act antagonistically to the NC program.

Alternative to inhibition studies, we used the natural mimic demethylasteriquinnone B1 (DB1) (Webster et al. 2003), which increases IR signaling via Pi3K but not Erk. Adding this molecule to our hNC cultures produced a reduction in Sox10 (70%) while Pax7 (117%) was increased (data not shown), which matches trends seen in various conditions where Sox10 exhibits greater sensitivity to insulin dysregulation than Pax7, however, the overall nature of this phenotype is unique. Furthermore, FGFR inhibition by PD173074 (PD17) (Leung et al. 2016) also reduced NC marker expression but differently than IR and IGFR inhibitors. Exposure to PD17 for the duration of induction reduced cell population dramatically (35%), but interestingly reduced Pax7 (6.7%) levels greater than Sox10 (16%) (data not shown). Exposure starting at later time points were less toxic but resulted in a similar phenotype with Sox10 being more resistant than Pax7 to FGF inhibition. While results from the Leung paper indicate stronger effect of PD17 on Sox10 than Pax7, CHIR exposure for that protocol extends for the full induction, whereas the protocol used here follows Gomez et al. 2019, utilizing a two-day CHIR treatment, suggesting a necessary balance between Wnt and FGF signaling in this protocol.

As with No Insulin, relevant targets of NC and known instructive pathways was assessed at 24 hours via qPCR (Figure S1), with the exception of IR inhibition. Dual receptor inhibition reduced all NC markers, matching No Insulin, while IGFR inhibition upregulated four of the six

targets. Regarding FGF targets, IGFR phenocopied No Insulin, while dual receptor inhibition deviated, but only with respect to one marker. Somewhat unsurprisingly, Dual receptor inhibition also showed downregulation of all BMP, Wnt, and TGF**β** targets, including TGF**β**1. IGFR inhibition presented a mixed profile of up and down regulation for BMP, Wnt, and TGF**β** targets, which was also the case in No Insulin, however, only half of the targets were dysregulated in a fashion similar to No Insulin, while the other half were inversely regulated.

RNAi Highlights Need for Functional IR and IGF2R in Early Human Neural Crest

To provide additional evidence of the role of the specific receptors mediating insulin signaling during hNC formation we turned to knockdown (KD) strategies through transcript-specific siRNA oligonucleotides. To this end, in an effort to inhibit receptor signaling at the earliest stages of induction, siRNAs were transfected into hPSCs using a reverse transfection method, thus exposing cells to siRNA at the beginning of our hNC formation protocol (time zero). A **β**-catenin specific siRNA was used as proof of concept, a tagged Cy-5 siRNA was used for transfection efficiency, and a scrambled non-specific siRNA as an experimental control. Surprising based on previous inhibitions in human yet unsurprising based on studies in chick, KD of IR produced minimal negative effect on Pax7 (78%) and greater reduction in Sox10 (45%) and double positive cells (54%) (Figure 4H-K). Interestingly, knockdown of IGF1R and IGF2R provided clarity of receptor preference during NC induction, with IGF1R knockdown improving induction percentages (110% Pax7+, 172% Sox10+, 194% Pax7+/Sox10+) and IGF2R knockdown phenocopying IR knockdown (93% Pax7+, 56% Sox10+, 60% Pax7+/Sox10+). The massive increase in NC markers after knockdown of IGF1R is an unexpected positive response, suggesting its presence and activity may be working against NC induction. Furthermore, mRNA expression profiles of IR and IGF2R siRNA knockdown matched the phenotype trend seen with small

molecules inhibitors for dual receptor inhibition and No Insulin, which is increased early markers, especially Pax7, Msx1, and Zic1, paired with decreased late markers, most notably Sox10 and Sox9 (Figure 4L).

The ability for either IR or IGF2R to yield similar results to one another speaks to the potential use of a hybrid receptor, unfortunately this does not totally agree with the results of IR inhibition with HNMPA-(AM)3 in human. As siRNA target transcripts and do not inhibit already formed proteins, its effects are mildly delayed compared to small molecules. This delay may resolve the conundrum of IR inhibition by small molecules increasing NC, since IR siRNA was successful in blocking NC, while HNMPA-(AM)3 had an opposite effect. Administration of HNMPA-(AM)3 at higher concentrations than used previously (40μM instead of 35μM) at timepoint 0 was lethal; however, administration at this concentration starting at later time points post induction and maintained until the end of the 5th day resulted in decreased NC markers without notable influence on cell survival, specifically at 3 hours (43% Pax7+, 58% Sox10+, 48% Pax7+/Sox10+) and 24 hours (28% Pax7+, 56% Sox10+, 30% Pax7+/Sox10+) (data not shown). Altogether this data provides evidence for the requirement of functional IR and IGF2R during NC formation in hPSCs, implicating ligand promiscuity and/or the use of IR/IGFR HRs.

Pi3K and PDK1 Inhibition Prevent Acquisition of Early Neural Crest State in Chick

Insulin has been shown to signal through MAPK/Erk and/or Pi3K. While the role of MAPK/Erk downstream of FGF has been well documented in NC formation, it appears to predominantly utilize MAPK (Yardley and Garcia-Castro 2012, Stuhlmiller and Garcia-Castro 2012ba, Ciarlo et al. 2017, Geary and LaBonne 2018, Xu et al. 2018). Consequently, little attention has been placed to the Pi3K branch. Here we sought to test if Pi3K and downstream PDK1 participate in NC formation. To this end we used Ly294002 (Ly29 at 1μM) (Bain et al. 2007) to

inhibit Pi3K in chick NC specification assays where it successfully ablated Pax7 expression (N=8/11), as did GSK2334470 (GSK at 1.2μM) (N=10/14), a potent PDK1 inhibitor (Najafov et al. 2011) (Figure 5A/B). While these results do not define Pi3K and PDK1 activation as a result of insulin signaling, they represent involvement of this intracellular branch during blastula stage NC specification independent of cell survival and match the results from earlier experiments which blocked insulin signaling.

Human Neural Crest Induction Requires Signaling via Pi3K and PDK1

As mentioned above, determining a functional inhibitory concentration of drugs which target metabolic and homeostatic processes can be difficult. While a variety of small molecules tested had no reportable results until reaching cytotoxicity (including PI-103 targeting Pi3K, Akti1/2 targeting Akt, and Rapamycin targeting mTor), Ly29 (6.6μM), GSK (900nM), and PD184352 (PD18), a selective Mek inhibitor, were able to modify NC marker expression without major insult to cell population (Figure 5C). Inability to obtain a functional phenotype with Akt inhibition is unsurprising, considering how many pathways interact with Akt to some degree, and other reported NC studies in which Akt dysregulation was embryonic lethal (Dinsmore and Soriano 2018).

For the small molecules that were not toxic beyond the levels of No Insulin, a clear distinction was seen in the NC marker phenotypes, with Pi3K inhibition following the No Insulin and IR/IGFR inhibition trends (52% Pax7, 25% Sox10+, 28% Pax7+/Sox10+) (Figure 5C-E), whereas Mek inhibition generated the opposite phenotype (58% Pax7+, 95% Sox10+, 50% Pax7+/Sox10+) (data not shown). PDK1 had a less pronounced effect on Pax7/Sox10 and results were variable, producing non-significant reductions, but a reproducible overall decrease in NC markers (54% Pax7+, 58% Sox10+, 64% Pax7+/Sox10+) (Figure 5C-E). Further quantification and

expansion of assessment of NC marker expression profile was achieved by qPCR analysis (Figure 5F). Unsurprisingly, Pi3K phenocopied dual receptor inhibition and No Insulin condition, with upregulated early markers Pax7 and Msx1, while all late markers were strongly decreased, especially Sox10 and FoxD3. PDK1 demonstrated a similar expression profile to IGFR inhibition, which resulted in decrease of all measured NC markers, albeit much less pronounced (Figure 5F).

Investigation of day 1 transcript levels revealed some familiar phenotypes (Figure S1), with both Pi3K and PDK1 inhibition downregulated all NC markers tested, with PDK1 inhibition also phenocopying No Insulin FGF transcript profile. FGF target profile of Pi3K inhibition matched the trend of dual receptor inhibition, with increase in Dusp6, and decrease in the other two targets. Interestingly, BMP profiles for both of these conditions showed opposite effects with complete downregulation of all genes, with the exception of Klf2 in PDK1 inhibition. Wnt targets for both conditions were mixed, however the Pi3K profile is the most similar to No Insulin of all conditions tested. This is also true for TGF**β** target transcript levels of Pi3K and No Insulin, while PDK1 downregulated all genes besides Serpine1, which was also the only upregulated gene after IGFR inhibition. Finally, TGF**β**1 itself was downregulated in both conditions, in opposition of No Insulin.

RNAi Reveals Distinct Pi3K Subunit and Downstream Effector for Insulin Signaling in Human Neural Crest

RNAi was employed to further clarify the involvement of the Pi3K branch, as well as which Pi3K subunit was most relevant to hNC formation. Of the two Pi3K major subunits, p110 and p85, p110 acts as the catalytic subunit. Having four isoforms, each one needed to be assessed, with the exception of p110-**δ** (PI3K-CD), which has been shown to be chiefly expressed in leukocytes (Vanhaesebroeck et al. 1997). Of the three tested subunits, only p110**β** gave a phenotype consistent with insulin dysregulation (50% Pax7+, 38% Sox10+, 42% Pax7+/Sox10+), while p110**α** and

p110**γ** produced an increase compared to controls (Figure 5G-J). Additionally, p110**α** was the only insulin related target tested to show any signs of toxicity (21% reduction in total cells) (Figure 5H). Moreover, PDK1 knockdown did not match GSK inhibition phenotype (Figure 5G-J). However, this result may not be entirely surprising, considering the variable results of GSK administration, suggesting that PDK1 may be involved but Pi3K may have a means to bypass PDK1 to activate Akt in NC. Analysis of transcript levels in Pi3K knockdown resulted in downregulation of Pax7 and Sox10 and Sox9, with moderate downregulation of Msx1, Zic1, and FoxD3, while both Pax3 and Slug were mildly upregulated (Figure 5H). This trend does not seem to completely agree with any previous conditions, but the overall result is decreases in NC markers, and none more than Pax7 and Sox10.

NC Induction is Highly Sensitive to Increased FoxO1 Nuclearization

A distinctive feature of insulin's Pi3K signaling is its regulation of the FoxO1 transcription factor. In the absence of insulin, FoxO1 actively modulates the transcription of multiple targets, but upon insulin Pi3K signaling, FoxO1 is retained in the cytoplasm, thus ceasing its transcriptional activity (Carter and Brunet 2007, Gross et al. 2009, Guo 2014, Nies et al. 2016). While FoxO1 intracellular translocation is not only regulated by insulin (Essaghir et al. 2009), it's connection with insulin and glucose is well documented (Matsuzaki et al. 2003, Vogt et al. 2005, Zhang et al. 2015, McClelland Descalzo and Satoorian et al. 2016). Our previous experiments suggest that insulin signaling is active and instructive during the earlier hours and days of hNC formation, and thus we would expect FoxO1 to be negatively modulated by insulin.

As a first step to assess the regulation of FoxO1 by insulin during hNC formation, we monitored its distribution by immunofluorescence under normal and No Insulin conditions. IF analysis at 24 and 48 hours into our hNC formation protocol reveal both nuclear and cytoplasmic

presence of FoxO1. However, under the No insulin condition a nuclear shift in FoxO1 was evident at day 2 (Figure 6A-C). These results suggested that under our normal hNC formation cultures, in the presence of insulin, FoxO1 is not fully excluded from the nucleus, and instead, some FoxO1 remains in the nucleus and might be active, and thus insulin might be critical in its precise modulation.

FoxO1 Inhibition is Detrimental to Human Neural Crest

To assess the possible role of FoxO1 during hNC formation we used a specific small molecule FoxO1 inhibitor, AS1842856 (AS18) (Nagashima et al. 2010, Zhou et al. 2014). Inhibition of FoxO1 with AS18 at 33nM for the entire five day duration of the hNC formation protocol yielded significant decreases in NC markers (7% Pax7+, 7% Sox10+, 2% Pax7+/Sox10+), and interestingly significant increase in cell population with 5-day exposure (13% increase) (Figure 6C-E), which is similar to the significant population increase in high insulin conditions (Figure 2F). Furthermore, two-day treatment of FoxO1 inhibition also resulted in significant decreases in NC markers (13% Pax7+, 14% Sox10+, 4% Pax7+/Sox10+) while increasing cell population (8% increase) (Figure 6C-E). Analysis of mRNA levels at day five of hNC induction with either 5-day or 2-day AS18 exposure resulted in reductions across the board, and almost complete ablation of all NC markers tested, with the 2-day reduction trends matching the 5-day trends but to a slightly less severe degree (Figure 6F).

Analysis of early NC markers after one day of induction in the presence of FoxO1 inhibitor surprisingly upregulated all but one gene (Figure S2). However, as FoxO1 inhibition is most similar to increased insulin signaling, this inverted profile compared to No Insulin should be expected. Additionally, all FGF targets were downregulated, which is opposite from No Insulin in 2 of the 3 targets, and all BMP targets are expressed inversely from No Insulin, showing downregulation in

all genes except Klf5. While No Insulin Wnt targets were variable, FoxO1 inhibition led to definitive upregulation of all genes, a result which, if indicative of increased Wnt activity is expected to reduce NC (Gomez et al. 2019b). Finally, TGF**β** targets were mostly inversely expressed compared to No Insulin, with 2 of 3 targets upregulated, while TGF**β**1 itself was decreased, which is opposite of No Insulin and expected given that TGF**β**1 is a known target of FoxO1 (Zhang et al. 2015).

Knockdown of FoxO1 with siRNA led to a mild effect on Sox10, but interestingly gave a pronounced effect on Pax7, reducing it by half (52% Pax7+, 84% Sox10+, 63% Pax7+/Sox10+) (Figure 6 G-J), whereas FoxO3 knockdown has essentially no effect. Further analysis of mRNA profile of FoxO1 siRNA knockdown revealed reduction of all NC markers tested, with the greatest reductions in Pax7, Msx1, Sox10, and FoxD3 (Figure 6K), a similar trend to IGFR small molecule inhibition, and matches what was seen with AS18 exposure. These results strongly implicate FoxO1 in hNC development and correlate with involvement/requirement of insulin, suggesting that insulin signaling provides/modulates a specific level of FoxO1 transcriptional activity rather than completely blocking it by nuclear exclusion.

Discussion

Here we present the first designation of insulin as a NC-inducing growth factor, and furthermore, establish its novel role at the earliest stages of specification in both chick and human NC models. These results suggest that the contribution of insulin goes beyond metabolic and proliferative responsibilities and that it is a key ingredient in the delicate and complex recipe of growth factors that induces NC. In agreement with Prasad et al. 2019b, blastula stage chick explants showed the ability to form NC in the absence of exogenous growth factors and signals from juxtaposed neural and non-neural ectoderm, thus providing the most ideal model to identify potential contributions of insulin in NC specification. One caveat to the work reported here is that it implies that the inclusion of insulin in specification assays may not allow for growth in a "neutral and naïve" environment. However, insulin is different in that early exposure *in* vivo is supplied by the mother (Heyner et al. 1989, Ruiz-Palacios et al.2014). Furthermore, as we know from the myriad of studies into NC formation, there is no single growth factor that can generate NC alone. Formation of NC, as with virtually any other cell type, requires a host of contributions, generally at very precise concentrations. For example, the delicate endogenous FGF levels in the Leung et al. 2016 and Gomez et al. 2019a hNC induction protocols would not promote NC in the absence of Wnt, and vice-versa, and modulation of FGF in Hackland et al. 2019 protocol is the key determinant in axial identity of the generated trunk NC.

While the ideal length of insulin exposure in both chick and human models is for the duration of all assays conducted, it is clear from the data here that early insulin signaling (first two days in human; first 12.5 hours in chick) is sufficient to induce comparable NC. With prolonged insulin producing the best results, it is possible that the role of Insulin changes during the course of NC development or simply that its role benefits NC as a sustained signal. Yet, considering specification is the first step in differentiation, early requirement suggests its contribution is tied to

initiating the beginning of the earliest phase of NC formation. Furthermore, the result of insulin removal in hNC for only the second day of induction is equivalent to controls compared to insulin absence on only the first day causing a significant drop in NC but non-significant change in cell population, further instills the importance of insulin at the earliest moments of specification. More impressively, the evidence that insulin as the sole exogenous addition to basal growth media can stimulate NC formation in chick supports the necessity of proper maternal insulin levels considering they are almost exclusively maternally sourced.

Perhaps the most telling indication of insulin's unique contribution to NC is the steep drop-off of NC marker expression with increased concentrations of insulin. High insulin in culture media emulates the initial stages of type II and gestational diabetes, which starts with the body's inability to process glucose (or process enough glucose), leading to overproduction of insulin to compensate, followed by eventual insulin resistance and finally the inability to produce functional levels of insulin (Weir and Bonner-Weir 2004, Westgate et al. 2006). Although not significant, the reproducible dip in NC marker expression in human at roughly 1:10 normal insulin levels draws a clear line for optimal high and low levels of insulin. Furthermore, it produces a unique mRNA profile of reduced early NC markers and unaffected late NC markers (data not shown), which is not seen in any other conditions tested here. This may be the profile of a population that is accelerating NC development faster than other insulin concentrations or may be forming a slightly different cell population altogether, which is more likely considering the drop in NC marker protein expression. In any case, this intermediate concentration is substantially less debilitating to NC formation than completely absent insulin, and it supports the idea of a potentially bimodally responsive phenotype of hNC induction, similar to that described in Gomez et al. 2019. Thus, more investigation into the differences between NC induced from high versus low insulin is required to determine if there are distinguishing differences between them.

Further confirmation of the involvement of insulin in NC formation was established using multiple small molecules that inhibit receptors utilized by insulin. Besides its designated receptor, insulin has been shown to signal via IGFRs, although with less affinity, and hybrid IR/IGFR receptors (Bailyes et al. 1997, Hernandez-Sanchez et al. 2006, Benyoucef et al. 2007, Zhang et al. 2007, Belfiore et al. 2009, Siddle 2011) Intracellularly, they appear to activate similar transduction pathways, however it is very unlikely that binding of insulin or IGF to the other's receptor or a hybrid will result in the same signal as the canonical pathway. The existence of hybrid IR/IGFR receptors for the insulin ligand, and IGF ligands as well, suggest a synergistic and complementary nature in their signaling contributions, and modifications in the expression, formation, and/or activity of IR, IGFR, or the hybrid receptor can affect the signaling of the IR, IGFR, or hybrid receptors. These hybrid receptors make analysis of their functions in insulin signaling pathway more complicated. Nonetheless, attempts to establish one specific receptor in chick failed to distinguish if IR or IGFR is preferred, as individual inhibition of each blocked expression of Pax7. With dual inhibition yielding similar results, we can surmise that chick NC relies on either a combination of signaling from both receptors or hybrid receptors, which would be rendered non-functional upon inhibition of either IR or IGFR. If insulin explicitly used IR in NC specification, then inhibition of IGFR would have no effect, and vice-versa.

Investigation of potential involvement of both receptors, either simultaneously or as a hybrid receptor, in human overall yielded results in accordance with the chick, although not in each experiment. Dual receptor inhibition and IGFR inhibition by small molecules both generated a phenotype that reduced Pax7, Sox10, and Pax7/Sox10 double positive cells, showing Sox10 to be more sensitive. Alternatively, IR inhibition with the same small molecule that blocked Pax7 expression in chick failed to inhibit NC and, in fact, mildly increased both markers and double positive cells. Analysis of mRNA expression revealed dual receptor small molecule inhibition

matches what is seen in No Insulin, with increases in early markers and decreases in late markers, while small molecule inhibition of IR or IGFR individually were different, both from dual receptor inhibition and from each other. IR inhibition showed decreases or no effect in most markers, although mild compared to all other conditions, but an increase in Msx1, whereas IGFR inhibition decreased all markers. As confounding as these results are, it could be as simple as lower binding affinity for HNMPA-(AM)3 in human, potentially from a partially masked target in the formation of hybrid receptors, therefore it is likely only reducing insulin signaling to levels that still allow for substantial expression of Pax7 and Sox10 (similar to Figure 2G). However, administration of HNMPA-(AM)3 at concentrations higher than what is used here lead to toxicity and eventual cell death (not shown). The other potential rationale for this discrepancy between models is the greater abundance of IGFR compared to IR in both hES and hNC, as assessed by immunofluorescence (data not shown).

Employment of RNAi in hNC inductions provided more clarity, and these results were more aligned with what was seen in the chick. Knockdown of IR did successfully reduce Pax7 and Sox10 protein levels, even matching the trend of Sox10 being more sensitive to insulin signaling inhibition. Targeted knockdown of IGF2R, but not IGF1R, phenocopied IR siRNA knockdown cell counts, and matched the trends seen in small molecule inhibition screens in human, further propagating the notion that both receptors are needed in some capacity to allow for proper NC development. Interestingly, and in agreement with our hypothesis, mRNA levels of both IR and IGF2R siRNA knockdown increased early NC markers while decreasing late NC markers. Unsurprisingly, the ability of RNAi to more specifically knockdown a given target resulted in trend-fitting data that further suggests the need for signaling via both IR and IGFR, either separately or as a hybrid receptor, and provided evidence of their involvement without affecting the overall health and proliferation of the cells.

Whether IR, IGFR, or a hybrid receptor is involved in the mechanism of insulin's instructive role in early NC specification, they all utilize the same intracellular signaling branches. Across both chick and human, the data here reveals a clear necessity for functional Pi3K during early NC formation events, which insulin preferentially uses to propagate its signal. The downregulation of Pax7 expression in chick and Pax7/Sox10 from human upon inhibition of PDK1 reported in this study supports involvement of the Pi3K branch in hNC specification. Unfortunately, PDK1 inhibition produced a less pronounced phenotype in human than Pi3K, and PDK1 inhibition mRNA profile does not match No Insulin. However, Pi3K inhibition does match No Insulin in cell counts and mRNA profile, further suggesting Pi3K's involvement downstream of insulin and association with arrested development or severely delayed progression of the NC program. Moreover, the greater sensitivity of late NC markers compared to early markers agrees with the phenotypes of other reports that implicate Pi3K in NC development (Ciarlo et al. 2017), which show a similar trend, and also may explain why inhibition of FGFR and MAPK resulted in an inverted phenotype compared to Pi3K inhibition.

The preference of insulin to signal via Pi3K is partially indicative of its major role in glucose metabolism, as this branch leads to Glut4 membrane translocation. However, siRNA knockdown of p110**β** showed an ability for Pi3K to intervene with normal NC development without any significant change in expected proliferation and survival, both of which require functional glucose metabolism. In comparison, knockdown of other Pi3K isotypes p110**α** and p110**γ** resulted in either cell death or no effect, respectively. p110**α** and p110**β** have been shown to have partially overlapping and partially distinct roles downstream of insulin signaling in various cell types, but both are required for proper insulin signaling (Tups et al. 2010). Previous research suggests that p110**β** participates in proliferation, metabolism, and cell trafficking in tumors (Jia et al. 2008, Ciraolo et al. 2009), but that it has no function in development. Furthermore, p110**β** has non-

catalytic functions that are not involved in oxidative stress responses, while p110**α** does (Matheny Jr. and Adamo 2010). However, p110**β** has been shown to have functions unique to insulin-mediated activation (Hooshmand-Rad et al. 2000), and taken together with the data shown here, it indicates a much more significant role in hNC downstream of insulin than what has previously been found.

Importantly, insulin signaling may participate with, and be modulated by, other pathways know to contribute to NC development. Most significantly, it has been suggested that there is crosstalk between insulin and Wnt signaling, which is especially pertinent considering the role of Wnt in the hNC induction model used here (Gomez et al. 2019), which utilizes a GSK-3**β** inhibitor. Thankfully, the GSK-3**β** motif targeted by Pi3K and the site involved in formation of the **β**-catenin destruction complex do not overlap (Beurel et al. 2015), and the amount of GSK-3**β** required for formation of the destruction complex is relatively small (Voskas et al. 2010). In other words, changes in GSK-3**β** by either Pi3K or Wnt do not affect its role in the other pathway. Focusing on **β**-catenin, conflicting reports exist on the influence of IR and IGFR on **β**-catenin (Rota and Wood 2015, Zhang Q.Y. et al. 2015). Additionally, exogenous insulin exposure is capable of increasing embryo length, whereas inhibition of glycolysis, a function of insulin signaling, reduces tail bud elongation (Patwardhan et al. 2004, Oginuma et al. 2017). Inhibition of glycolysis reduces nuclear **β**-catenin levels and separately mimics MAPK inhibition in this system, whereas increased insulin leads to spikes in mesodermal and neural markers, indicating potential major regulation of two early, and very important NC induction contributors in Wnt and FGF. Additionally, increased insulin lead to higher expression of Noggin, a BMP antagonist, in the neural plate, suggesting insulin impacts the three major NC inducing pathways, and makes a case for it having the potential to be the first signal required to initiate NC specification. Even if insulin doesn't affect all three of these pathways, crosstalk between insulin and any one of these pathways could be a key to

understanding the significance of insulin's instructive role in NC development. However, it seems like the most instructive influence is on Wnt/**β**-catenin, and the answer may be in the function of FoxO1.

Probing for further clarification of insulin mediated Pi3K activity in NC, FoxOs were investigated, as they lie downstream of Akt and remain cytoplasmic upon phosphorylation. In the absence of insulin signaling or in hyperglycemic environments, FoxOs enter the nucleus and carry out their functions as transcription factors (Accili and Arden 2004, Batista et al. 2019). Thus, insulin signaling via Pi3K/Akt acts as a negative regulator to FoxO nuclear activity. Between the two most abundant FoxOs, FoxO3a appears to be more responsive to high glucose induced oxidative stress (McClelland Descalzo and Satoorian et al. 2016), while FoxO1 function has been suggested to be more distinctly regulated by insulin signaling (Zhang et al. 2015). There is even evidence of FoxO1 interacting with co-factor TCF/LEF in the nucleus, and thus affecting what **β**-catenin targets are expressed (Zhu et al. 2016, Doumpas et al. 2018).

Another potential instructive contribution from insulin signaling could result from IR and IGFR functioning as a transcription factors (Sarfstein et al. 2012, Hancock et al. 2019). The genes targeted by these receptors turned transcription factor in the adult liver tissue and breast cancer cells correlated to known insulin related events, autoregulation of each receptor, respectively, and indirect metabolic regulation. This function of IR and IGFR would negate the need for Pi3K, PDK1, and FoxO activity, and while the data here does not refute this ability of IR, it does imply a requirement for proper Pi3K function. In cases where IR is potentially acting as a transcription factor, IR target genes do not appear to overlap with FoxO1 target genes (Hancock et al. 2019), which supports the different mRNA expression profile after siRNA knockdown of IR and FoxO1 (Figure 4/6). Meta-analysis of FoxO family ChIP-seq data indicates that FoxOs are highly active across multiple model organisms, and a wide range of conserved targets have been found between

Drosophila, C. elegans, mouse, and human tissue (Webb et al. 2016). It is clear that FoxOs function differently in various tissues and their targets and co-factor interactions change with age, however, conserved binding motifs upstream of NC marker Sox9 is found in mouse and human. Alternatively, no other NC genes were found to be conserved FoxO targets in both mammalian models. Considering the gene regulatory network described in Prasad et al. 2019a, NC genes Gbx2, Sp5, Msx2, and Ets1 were targeting in human but not conserved in mouse, whereas data from mouse indicated Sox5, Id3, and Sox8 were targets of FoxOs. Despite this data not being specific for FoxO1, it implicates a potential involvement of FoxOs in NC gene expression. Further exploration into these genes and FoxO1 co-factors will be necessary to fully clarify the mechanism of its contribution to early NC development.

Considering FoxO1 targets a multitude of genes, outside of the potential NC genes it may target it is likely that none are as important to NC formation as TGF**β**1. FoxO1 nuclearization is increased in response to diabetic conditions and resulting environmental consequences, which not only increases FoxO1 nuclear activity but also affects which genes it targets (Zhang et al. 2015). Increased nuclear FoxO1 results in a spike in TGF**β**1 expression, however, prolonged hyperglycemic conditions interfere with FoxO1 nuclear activity and recruit it to drive expression of anti-migratory genes. Two major points from this: 1. Lack of insulin leads to increased TGF**β**1, which has been shown to partially dictate NC induction (Conway and Kaartinen 2013, Lee et al. 2010, Menendez et al. 2011); 2. Under diabetic conditions, FoxO1 nuclear activity is modified leading to novel interactions with different genes and likely different cofactors (Zhu et al. 2016). These interactions between FoxOs and **β**-catenin in the nucleus may regulate canonical Wnt signaling to a degree, but increased interactions between these two transcription factors in the presence of hyperglycemia related environmental factors (Zhang et al. 2015) is potentially the switch that hinders NC upon dysregulation of insulin signaling. In mouse ES cells, hyperglycemic

conditions lead to increased reactive oxygen species (ROS), which lead to a rise in FoxO1 and FoxO3a expression, nuclearization, and interaction with **β**-catenin (McClelland Descalzo and Satoorian et al. 2016). These interactions influenced target gene expression levels; however, this was the result of a hyperglycemic environment independent from changes in Akt phosphorylation, which is in agreement with the changes in nuclear FoxO1 in diabetic wound healing (Zhang et al. 2015). Furthermore, FoxO3a increased interactions with **β**-catenin in this high glucose, high ROS environment to a greater degree than FoxO1 (McClelland Descalzo and Satoorian et al. 2016), suggesting the function of FoxO3a is more responsive to homeostatic threats than FoxO1. Considering this, it is unsurprising that inhibition or knockdown of FoxO1 and subsequent mRNA analysis provided further confirmation that dysregulation of insulin and downstream effectors is detrimental to NC specification whereas FoxO3 knockdown gave no measurable phenotype.

While it is clear that FoxOs are relevant for NC derivative formation (Furuyama et al. 2004, Schuff et al. 2010, Mei et al. 2012), we believe the implications mentioned above, as well as the data presented here, promote FoxO1 as an essential early NC transcription factor, whose regulation by insulin heavily dictates the success and outcome of the NC program. While exposure to FoxO1 inhibitors for the full duration of induction was more successful in blocking NC, exposure for the first two days (matching the requirement for insulin and Wnt) in this protocol produced significantly reduced NC markers, as well as severe downregulation of all NC genes tested. Additionally, insulin at 8μg/ml and 5-day exposure to FoxO1 inhibitor resulted in a similarly significant increase in total cells and decrease in NC (Figure 2F/6E), supplying more evidence for a connection between them in NC. This result further supports FoxO1 relevance as an insulin effector in the early stages of NC development and gives more validity to potential crosstalk between insulin regulated FoxO1 and Wnt effectors.

Altogether, the data here represents the first evidence of insulin as a key NC contributor, and furthermore distinguishes its role in blastula stage specification, utilizing the Pi3K pathway and regulating FoxO1 nuclearization. The notion that premigratory/migratory markers are more sensitive than early neural plate border specifiers to most perturbations of insulin signaling points to two potential roles for insulin: 1. Severe delays of developmental timing; or 2. inability to progress to migratory/further differentiated/less stem-like stages. Defining the complete function and mechanism of Insulin in NC formation will require further investigation beyond specification and more robust transcriptional regulation examination. Nonetheless, with continuously increasing numbers of diagnosed and undiagnosed diabetes, including acquisition of gestational diabetes, the significance of these findings will hopefully raise awareness to the implications of diabetes in expecting mothers and improve treatment and prevention of embryogenic consequences of maternal diabetes.

Methods

Chick Embryos and Neural Crest Specification Assays

Fertilized hen eggs were incubated for 30 minutes to 3 hours for stage XI-XIV Eyal-Giladi and Kochav (EK) embryos. Embryos were collected by cracking the egg in the upper third, draining the albumin, centering the embryo in the hole of a punched filter paper, cutting the vitaline membrane, and removing the filter paper with the embryo from the yolk to place it in Ringer's solution. Under a stereoscopic microscope (Nikon), hypoblast was removed using a sharpened tungsten needle, and with the area pellucida lined up with the edges of a 20x20 ocular grid an equatorial strip at row 10 was dissected out. Each strip was dissected further into 12 explants (≈90mm^2 each) (Figure 1A). For most experiments, explants were bisected diagonally (Figure 1C) and kept in PB1 (NaCl, KCl, Na_2HPO_4. K_2HPO_4, Glucose, Sodium piruvate, Phenol red, $MgCl_2$, $CaCl_2$) in 96 well terisaki plates. This served as a holding place until explants and bisections were suspended in a 10µl collagen gel mixture consisting of 90µl of rat type I collagen (Collaborative Research, Waltham, Massachusetts), 10µl of 10X D-MEM and 5 µl of 7.5% sodium bicarbonate, vortexed for 20 seconds and briefly spun down. These collagen droplets were made in 4 well IVF plates (Falcon), and when the gels were polymerized, cultured media (DMEM-F12 (Invitrogen, nose, USA) supplemented with N2, N2-I, N2 with DMSO, or N2 small molecules was added. Cultures were kept in an incubator at 37°C for 25 or 30 hours, depending on the experiment.

Human Neural Crest Induction & Drug Administration

Confluent H1 hES cells were dissociated to single cells using Accutase and resuspended in a defined NC induction media compromised of 1x Glutamax (Gibco), 2% B27 (Gibco), 0.5% Bovine Serum Albumin (BSA) (Sigma Aldrich), 10µM ROCK inhibitor (Y-27632), and 2.75µM

CHIR 99021 (Tocris Bioscience), all diluted in Dulbecco's DMEM/F12. The cells will then be plated on Matrigel (Fisher Scientific, 08-774-552) coated plates at a low density of 20,000 cells/cm^2. The media will remain the same for the first two days, but on the third day, CHIR and ROCK inhibitor will not be added. See Gomez et al. 2018. B27 without insulin (Thermo Fisher Scientific, A1895601) will be used in place of normal B27 to create a controlled system lacking insulin. In small molecule inhibitor screens, the small molecules were added to the well prior to plating the cells, thus cells were plated on top of a small volume of induction media containing proper concentration of the appropriate drug. This mimics the reverse transfection method used for RNAi (see below).

Immunofluorescence

Chick explants were immunostained in accordance with previously published methods (Basch et al. 2006, Stuhlmiller and Garcia-Castro 2012a, Prasad et al. 2019b). Explants in collagen gels were fixed with 4% paraformaldehyde at RT for 15 minutes and then rinsed three times with PBS. Permeabilization step was done with PBT (PBS with 1% BSA and 0.1% tween). Block was done using PBTS (PBT with 10% fetal bovine serum added). Primary Pax7 antibody (mouse IgG1 anti-Pax7, Developmental Studies Hybridoma Bank) diluted in PBTS at 1:10 was added and incubated overnight at 4° C. Three 1-hour washes were done with PBT at RT, and goat anti-mouse IgG1 568 secondary antibody diluted in PBTS at 1:2000 was added and incubated overnight at 4° C. Two rinses and three 1-hour washes were done with PBT. Explants were then placed on slides and sealed with DAPI Fluoromount.

Human cells were immunostained in accordance with previously published methods (Leung et al. 2016, Gomez et al. 2018, Prasad et al. 2019b). Cells in 24-well plates, 96-well plates, or Nunc Labtek Permanox Chamber Slides (Thermo Scientific, 177410) were fixed for 10 minutes

at RT in 4% paraformaldehyde and then rinsed 3 times in PBS. Permeabilization was done using PBS with 0.04% Triton. Triton was rinsed with PT (PBS with 0.05% tween). Block was done with PTS (PT with 10% fetal bovine serum). Double staining was done with Pax7 primary antibody (as described above) at 1:10 and Sox10 primary antibody (mouse IgM anti-Sox10, Santa Cruz Biotech, SC271163) at 1:200 in PTS, incubated overnight at 4° C. Primary was washed with PT 3 times for 10 minutes at RT. Secondary antibody mixture in PTS included goat anti-mouseIgG1 568 and goat anti-mouseIgM 488 at 1:1000. This was incubated for 1 hour at RT and then washed with PT 3 times for 10 minutes at RT. DAPI was added to MilliQ water at 5µg/mL and then added to the cells.

Imaging

Chick explants were imaged on Nikon Eclipse 80i microscope and processed in Adobe Photoshop CC 2019. Images of each explant were taken with fluorescent filters, using the same exposure for each sample imaged for each channel. Images were composed into a grid in Photoshop, merged, and levels adjusted evenly, with threshold being set using a positive control for nuclear staining.

Human cells were imaged on Nikon Eclipse Ti inverted microscope at the UCR Stem Cell Core. All images were taken with same exposure parameters for each antibody. Images were converted to be processed in Photoshop as a collection in a grid and merged to adjust levels evenly. Cell counting was performed as previously published (Gomez et al. 2019a/b, Hackland et al. 2019, Prasad et al. 2019b). Bright spot detection script in Nikon elements counted positive nuclear stain based on threshold determined by a negative control incubated without primary antibody.

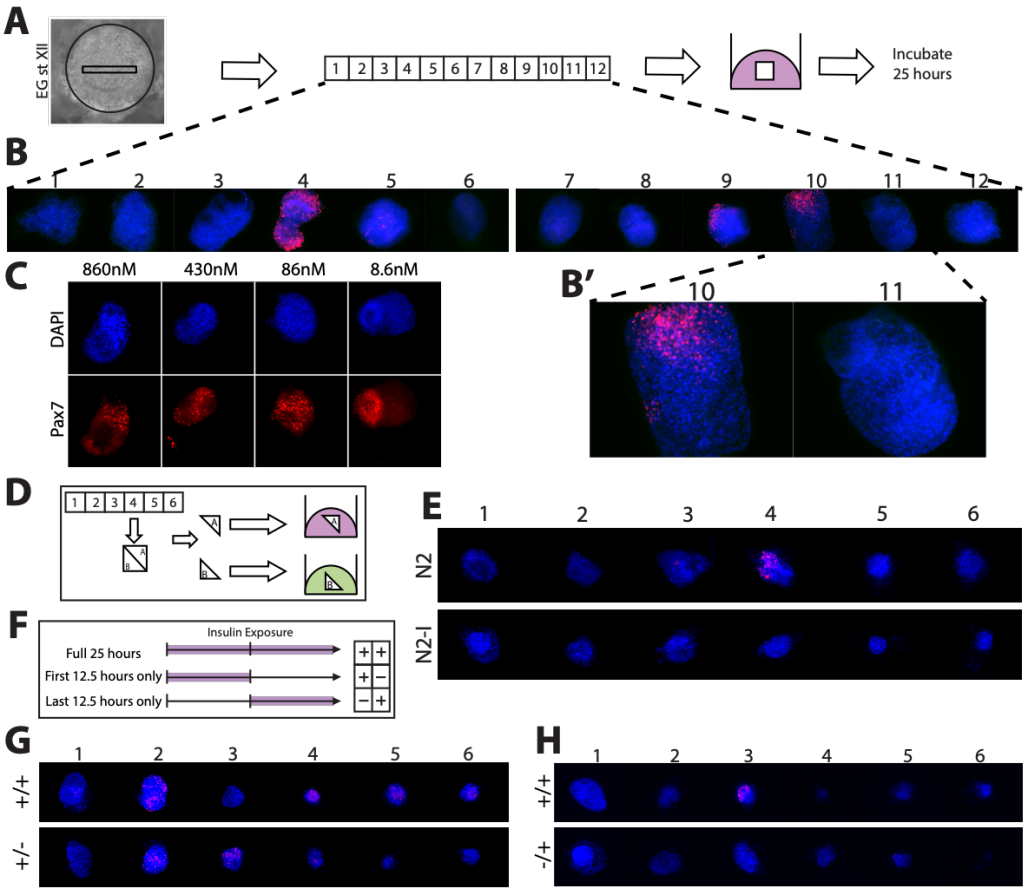
RNAi

siRNAs purchased from Dharmacon and Lipofectamine RNAiMax reagent was purchased from Invitrogen. Reverse transfection technique was used, which requires short incubation of siRNA and Lipofectamine in the well prior to addition of cells. Final concentrations in 96-well plate were 1.2 pMole per well of siRNA and 0.05µL per well of Lipofectamine. Before start of induction, siRNA was diluted at 6 pMole per 100µL OptiMEM, and separately Lipofectamine was diluted at 0.5µL per 100µL of OptiMEM. H1 cells were then accutased and hNC induction protocol was followed as described previously. During induction, Matrigel was aspirated from wells followed by addition of 10µL of Lipofectamine mixture and 20µL of siRNA mixture (30µL total to ensure coverage of the well). These mixtures incubated in each well at RT for 10-20 minutes, after which cells were plated. To accommodate for extra 30µL volume, 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). Media was changed after 24hours, in accordance with normal hNC induction protocol. Control siRNAs included a scrambled siRNA, as well as Cy3-tagged siRNA to check transfection efficiency.

RT-qPCR

RNA was collected in TRIzol (Thermo Fisher Scientific), isolated using a Zymo Direct-zol RNA kit, and cDNA was prepared with Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Fisher Scientific). RT-qPCR was performed on an Applied Biosystems Step One Plus system using SYBR Green Premix Ex Taq II (Clontech). Analysis was done using ΔΔ-Ct method.

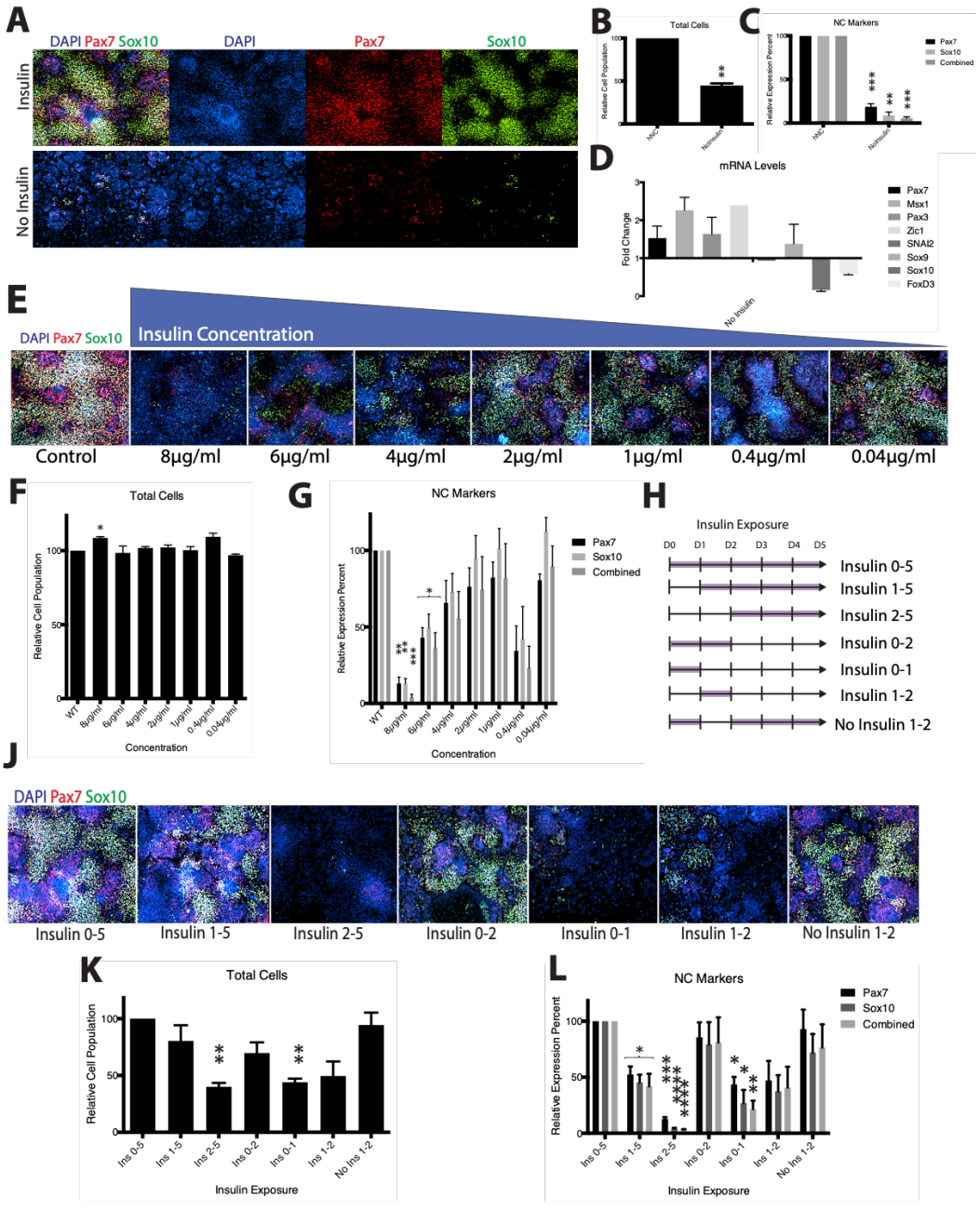
Chapter 2: Figure 1



Chapter 2: Figure 1 – Insulin is required in chick NC specification at blastula stage

Chick NC specification assays were executed with Eyal-Giladi stage XII chick embryos. Embryos were removed from the eggs, and an equatorial strip was cut within the area pellucida. This strip was cut into twelve explants, each of which were incubated individually in a collagen suspension for 25 hours (A). IF of fixed chick explants after 25 hours incubation showing robust Pax7 expression (Red) in intermediate explants (B) (Highlighted in B' with a Pax7+ and Pax7- explant). Incubation of explants in the presence of insulin only, starting at the concentration found in N2 (860nM), and lower concentrations shows expression as low as 1:100 normal insulin (C). IF for Pax7 (Red) shown only for pNC explants. Alternatively, testing in the presence of all N2 components minus insulin, explants were bisected (D) and each half exposed to either N2 or N2 minus insulin (N2-I). Bisected controls expressed Pax7 in the expected intermediate range, while Pax7 was absent from N2-I bisections (E), without obvious detriment to health of explants. Dividing up the incubation period into early and late stages, bisections were exposed to normal N2 for only the first half or second half of incubation (F). Insulin exposure during the 12.5 hours resulted in comparable Pax7 (G), while insulin for only the second 12.5 hours resulted in loss of Pax7 expression in bisected counterparts (H).

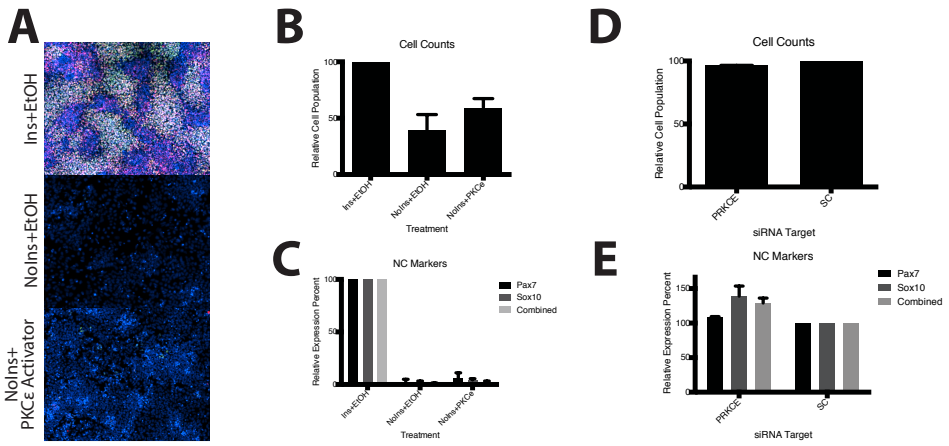
Chapter 2: Figure 2



Chapter 2: Figure 2 – Insulin is required in Human Neural Crest and displays optimal temporal and concentration ranges

Using our hNC induction protocol, cells were cultured in normal B27 and B27 minus insulin (referred to as "No Insulin"). IF of Pax7 (Red) and Sox10 (Green), with nuclear stain DAPI (Blue) shows tremendous reduction in both markers (A). Cell counting shows a significant drop in total cells at 45% compared to normal conditions (B). NC marker cell counts, which include a count for coexpression of both markers, were more significantly reduced (19% Pax7+, 9% Sox10+, and 6% Pax7+/Sox10+) (C). Using Pax7, Msx1, Pax3, and Zic1 to represent early NC markers and SNAI2, Sox9, Sox10, and FoxD3 to represent late NC markers, mRNA levels of NC markers by qPCR displayed a unique phenotype of upregulated early markers and generally downregulated late markers (D). Determination of a concentration window for insulin compared normal B27 conditions (insulin estimated ~3µg/ml) seen in the control (E). Insulin was tested above (8, 6, and 4µg/ml) and below (2, 1, 0.4, and 0.04µg/ml) this concentration, showing no drops in total cells (F), but revealing a statistically significant increase at 8µg/ml (9%). NC marker cells counts at 6 and 8µg/ml were significantly reduced ((42% Pax7+, 49% Sox10+, 36% Pax7+/Sox10+) and (13% Pax7+, 13% Sox10+, 4% Pax7+/Sox10+) respectively), while no other concentration yielded statistically notable result (G). 0.4µg/ml did produce a not significant drop (34% Pax7+, 42% Sox10+, 23% Pax7+/Sox10+), which was rescued at 0.04µg/ml. Clarification of temporal requirements for insulin by exposing NC for various durations (H) revealed an early need for successful induction (J). With total cell counts (K) revealing significant drops with the shortest durations of insulin (Ins 2-5 at 40% and Ins 0-1 at 44%), NC marker counts (L) of insulin for only the last 4 (52% Pax7+, 45%Sox10+, 41% Pax7+/Sox10+) or last 3 days (12% Pax7+, 4%Sox10+, 4% Pax7+/Sox10+) of induction produce significant loss of NC marker expression. On the other hand, insulin for only the first 2 days was sufficient to support NC program (86% Pax7+, 79%Sox10+, 81% Pax7+/Sox10+). Insulin for only the first day (44%) or only the second day (49%) produced similar NC marker counts, while removal of insulin for only the second day resulted in ample cell counts (94%) and NC marker expression (92% Pax7+, 72%Sox10+, 77% Pax7+/Sox10+).

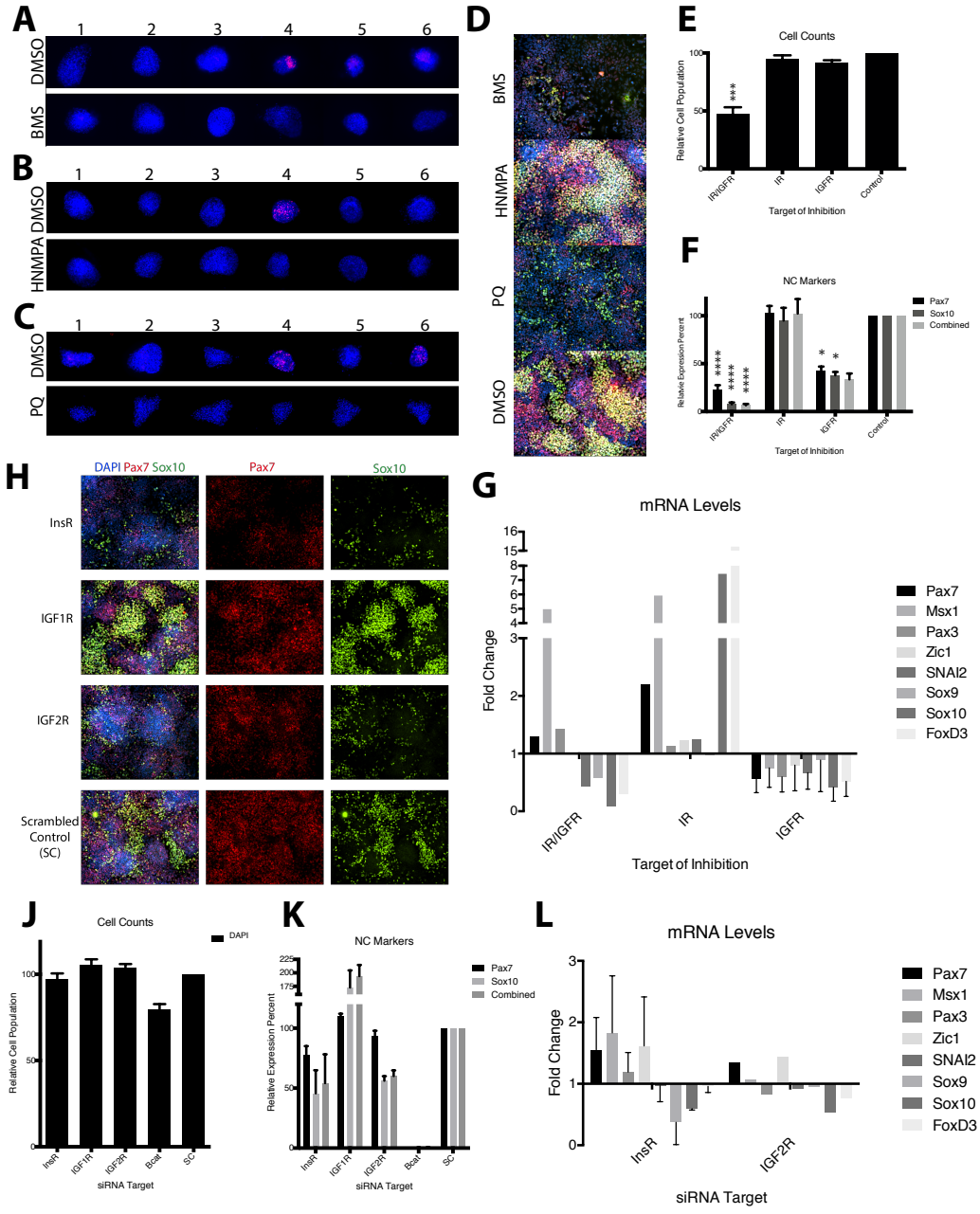
Chapter 2: Figure 3



Chapter 2: Figure 3 – Separation of insulin and glucose metabolism

A major caveat of this research endeavor is the difficulty of modifying insulin signaling without influencing the cell's ability to satisfy its metabolic requirements. Use of a PKCε activator (50nM) diluted in ethanol was added to a No Insulin NC induction (A). Cell counts were increased by 51% compared to No Insulin control (with ethanol), but still not quite at normal levels (B). This was matched with maintained low NC markers (C). Additionally, inhibition of PKCε with siRNA did not negatively infuence total cells (D) or NC markers (E).

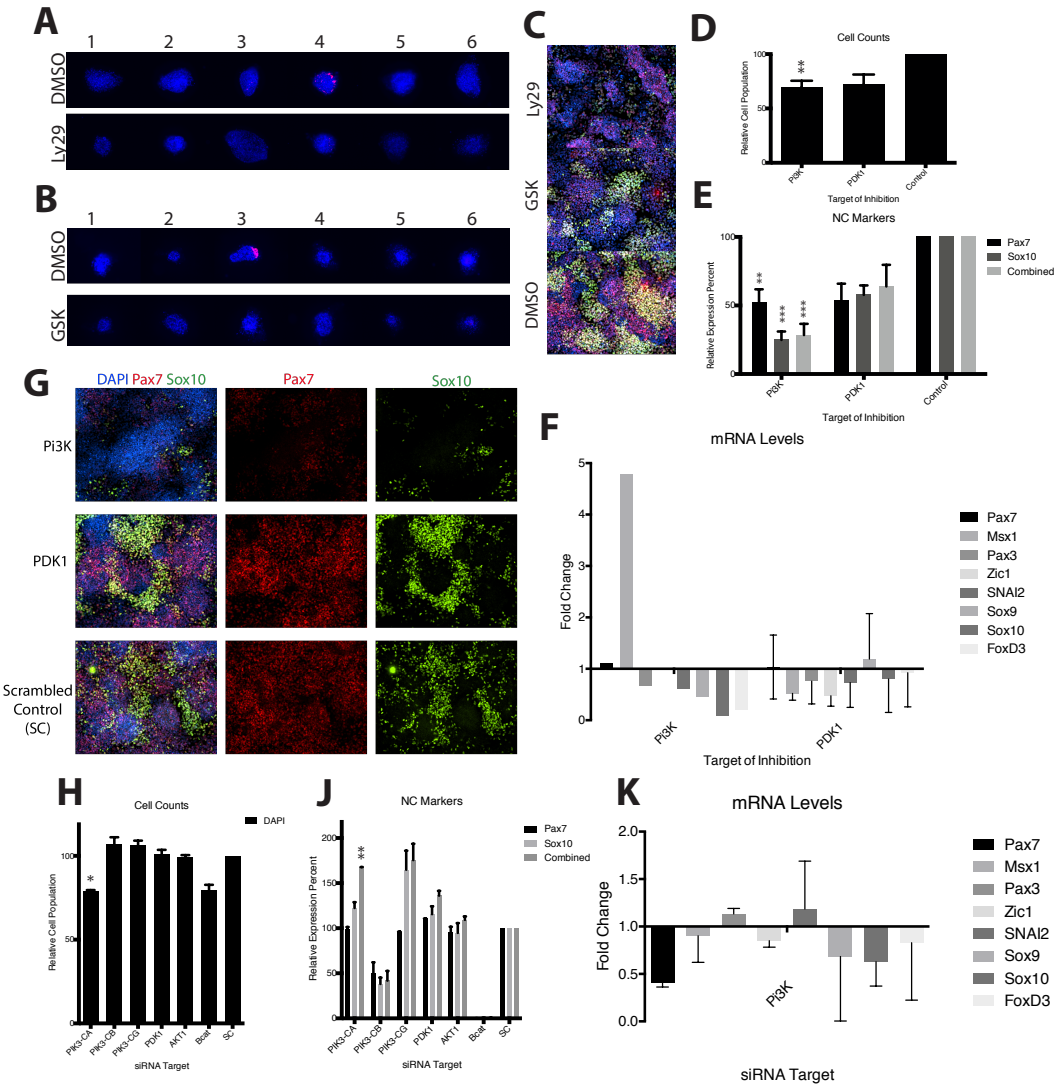
Chapter 2: Figure 4



Chapter 2: Figure 4 – IR and IGFR are both required for NC induction

Small molecule inhibitors were used to probe the requirement of insulin related receptors in bisected chick explants from blastula stage (A-C). Dual IR/IGFR inhibition with BMS 536924 (referred to as BMS) at 300nM showed reduction of Pax7 in exposed bisections (N=7/8) (A). Use of specific IR inhibitor HNMPA-(AM)3 (referred to as HNMPA) at 40μM produced a similar result (N=8/8) (B), as did IGFR inhibition with PQ 401 (referred to as PQ) at 6 and 12μM (N=10/10) (C). Exposure of the same small molecules in hNC produced different results with each small molecule (D). BMS at 300nM in hNC induction produced a similar drop in total cells (47%) as No Insulin (E), while HNMPA at 35μM and PQ at 12μM had no effect. However, both BMS expression (23% Pax7+, 8% Sox10+, 7% Pax7+/Sox10+)and PQ (43% Pax7+, 38% Sox10+, 34% Pax7+/Sox10+) reduced NC markers significantly (F), while HNMPA actually increased NC markers (103% Pax7+, 95% Sox10+, 102% Pax7+/Sox10+). Transcript analysis via qPCR revealed that dual receptor inhibition upregulated early NC genes and downregulated late genes (G). Matching cell counts, IR inhibition upregulated all markers, while IGFR downregulated all markers. RNAi targeting IR, IGF1R, and IGF2R, compared to a scrambled control (H) showed a requirement for IR and IGF2R in NC induction. No effects were seen in total cell counts (J), however, NC marker reductions were seen in IR (78% Pax7+, 45% Sox10+, 54% Pax7+/Sox10+) and IGF2R (93% Pax7+, 56% Sox10+, 60% Pax7+/Sox10+) while IGF1R increased NC markers (110% Pax7+, 172% Sox10+, 194% Pax7+/Sox10+) (K). Transcript analysis revealed upregulated early and downregulated late NC markers with IR inhibition (L), while IGFR produced mixed results, but generally matched upregulated early and downregulated late markers.

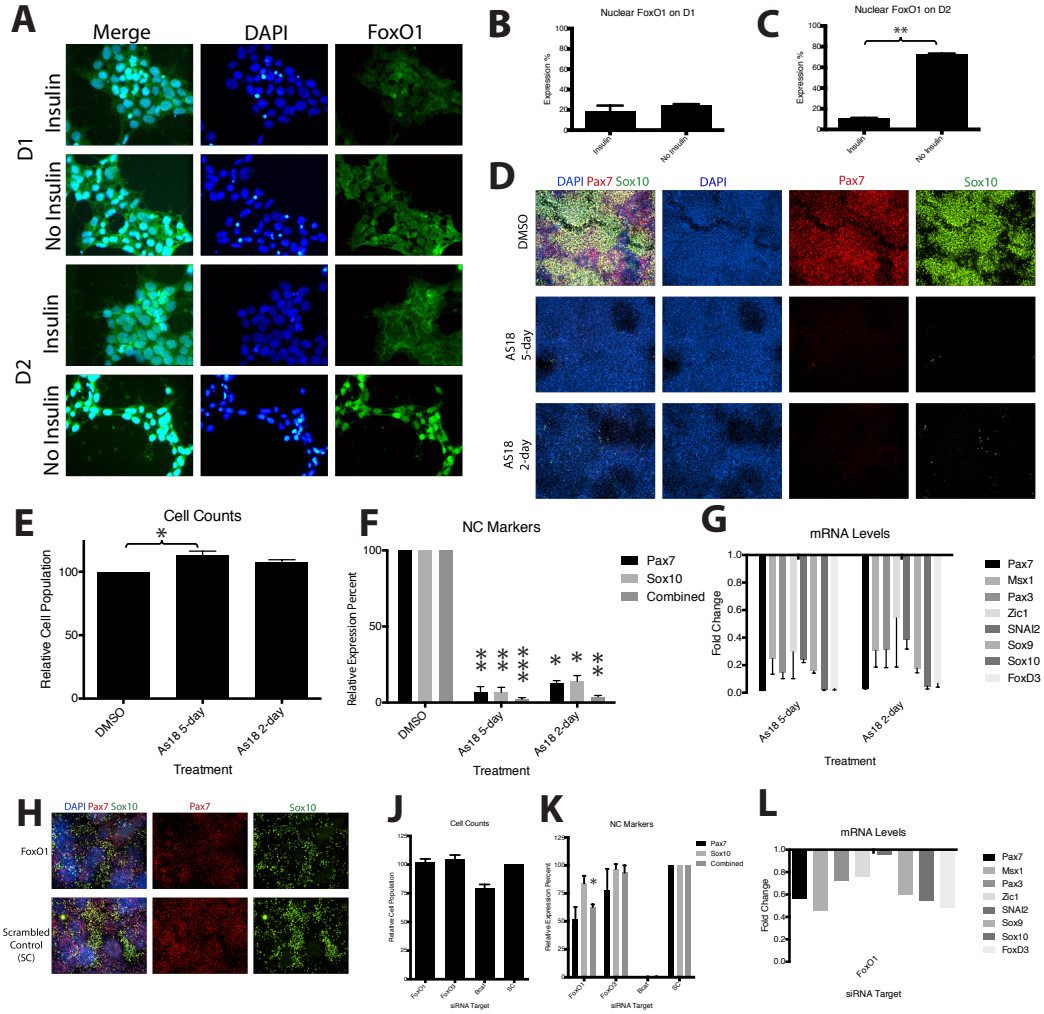
Chapter 2: Figure 5



Chapter 2: Figure 5 – Pi3K functionality is required for successful NC induction

Blastula chick NC specification assay employed to test Pi3K inhibition by Ly294002 (Ly29) at 1μM and PDK1 inhibition by GSK2334470 (GSK) at 1.2μM. Ly29 ablated Pax7 in bisected counterparts (N=8/11) (A), as did GSK (N=10/14) (B). Exposure of these drugs to hNC (Ly29 at 6.6μM; GSK at 900nM) also reduced NC markers (C). Pi3K inhibition produced a similar drop in total cells to PDK1 (D), however the drop in Ly29 was deemed significant while PDK1 was not. NC marker counts show reductions after PDK1 inhibition (54% Pax7+, 58% Sox10+, 64% Pax7+/Sox10+), and significant reductions with Pi3K inhibition (52% Pax7, 25% Sox10+, 28% Pax7+/Sox10+) (E). Transcript analysis displayed upregulated Pax7 and Msx1 in Pi3K inhibition, while all other targets were downregulated (F). On the other hand, PDK1 downregulated all markers except Pax7 and Sox9. RNAi targeting Pi3K and PDK1 showed a need for Pi3K but PDK1 inhibition had no effect (G). Multiple subunits of Pi3K were tested along with PDK1, with PiK3-CA producing the only significant drop in total cells (79%) (H). However, PiK3-CB revealed itself as the only subunit capable of reducing NC marker expression (50% Pax7+, 38% Sox10+, 42% Pax7+/Sox10+) (J), while PiK3-CA and PiK3-CG showed increased counts and PDK1 had no effect. Transcript levels were downregulated for all genes except Pax3 and SNAI2 (K).

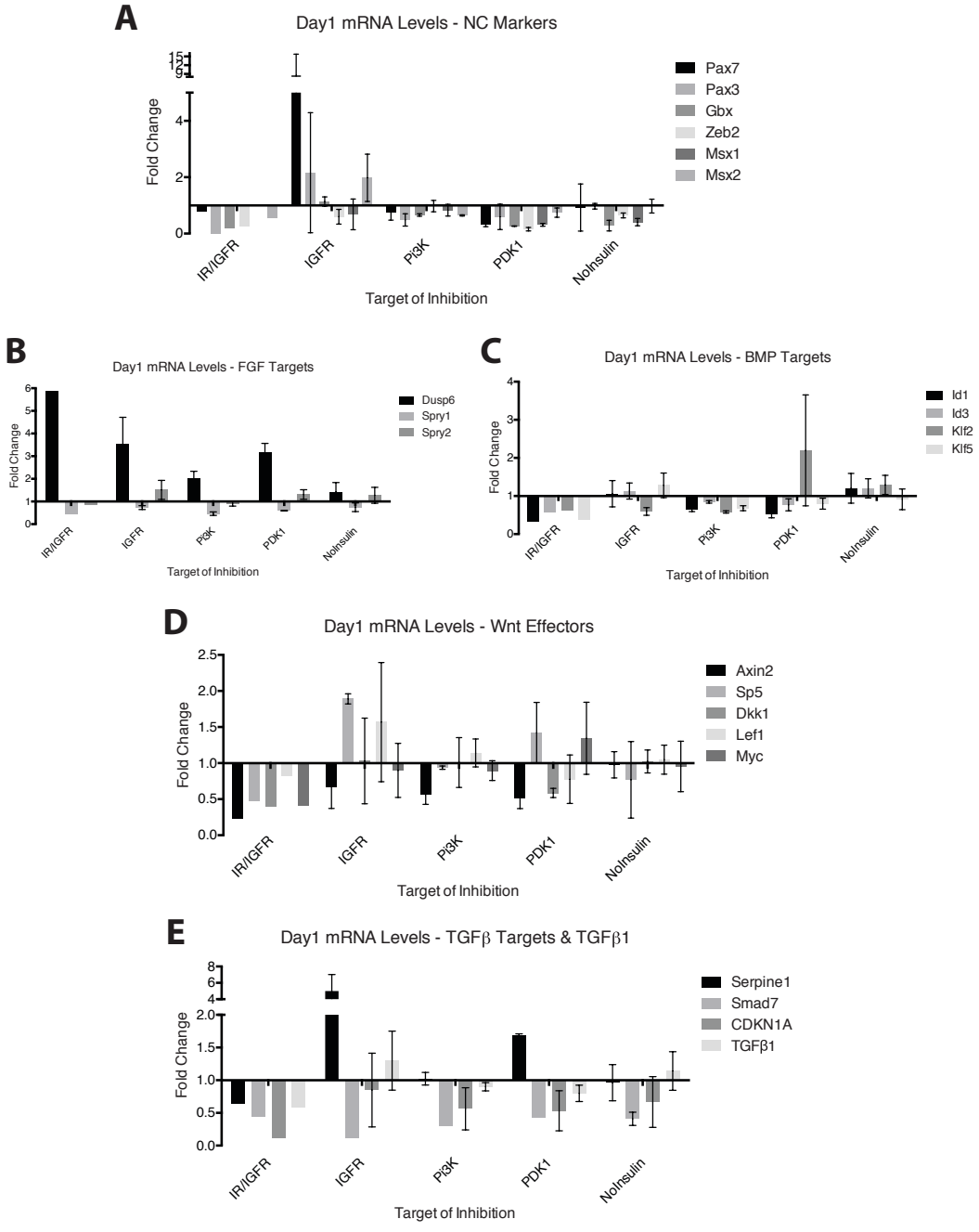
Chapter 2: Figure 6



Chapter 2: Figure 6 – hNC is sensitive to FoxO1 and its localization is regulated by insulin

Determination of insulin regulation on known effector FoxO1 revealed that Foxo1 is mostly cytoplasmic under normal conditions but by day 2 of No Insulin, FoxO1 is mainly found in the nucleus (A). Cell counts at day 1 reveal almost no difference in nuclear FoxO1 (19% nuclear with insulin, 24% nuclear with No Insulin) (B). However, at day 2, there is a stark contrast between the conditions, with a significant difference in nuclear FoxO1 between insulin (11%) and No Insulin (73%) (C). IF of hNC induction exposed to FoxO1 inhibitor AS1842856 (AS18) at 33nM (D) did not reduce cell counts, but actually significantly increased them with 5-day exposure (13%) (E). NC markers were significantly reduced in both cases (5-day (7% Pax7+, 7% Sox10+, 2% Pax7+/Sox10+); 2-day (13% Pax7+, 14% Sox10+, 4% Pax7+/Sox10+)) (F). qPCR showed massive downregulation of all genes tested after 5- and 2-day exposure to AS18 (G). IF of FoxO1 siRNA in hNC induction showed decreased NC markers (H) without affecting total cells (J). Loss of NC markers was not seen in FoxO3 knockdown (K) but was evident in FoxO1 knockdown (52% Pax7+, 84% Sox10+, 63% Pax7+/Sox10+). Additionally, the mRNA profile of FoxO1 knockdown downregulated all markers tested (L).

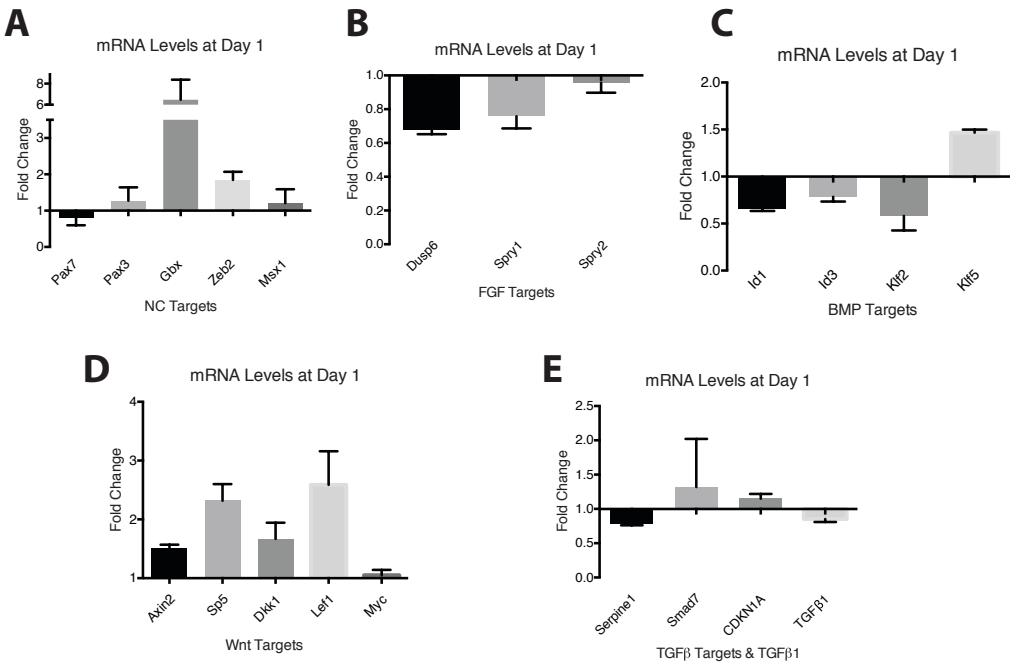
Chapter 2: Supplemental Figure 1



Chapter 2: Figure S1 – Transcript profiles at Day 1 of hNC induction with small molecule inhibitors

RNA from dual IR/IGFR inhibition, IGFR inhibition, Pi3K inhibition, PDK1 inhibition, and No Insulin condition were analyzed after 24 hours of culture for changes in mRNA profiles of targets of known NC contributing growth factors. Pax7, Pax3, Gbx, Zeb2, Msx1, and Msx2 were analyzed for early NC genes (A). Dusp6, Spry1, and Spry2 were analyzed for FGF targets (B). Id1, Id3, Klf2, and Klf5 represent BMP targets (C). Axin2, Sp5, Dkk1, Lef1, and Myc were assessed as Wnt targets (D). And finally, TGF**β** targets Serpine1, Smad7, and CDKN1A were measured, as was TGF**β**1 (E).

Chapter 2: Supplemental Figure 2



Chapter 2: Figure S2 – Transcript profiles at Day 1 of hNC induction with FoxO1 inhibitor

RNA from FoxO1 inhibition in hNC was analyzed after 24 hours of culture for changes in mRNA profiles of targets of known NC contributing growth factors. Similar to Figure S1, Pax7, Pax3, Gbx, Zeb2, Msx1, and Msx2 represent early NC genes (A). Dusp6, Spry1, and Spry2 signify FGF targets (B). Id1, Id3, Klf2, and Klf5 were measured as BMP targets (C). Axin2, Sp5, Dkk1, Lef1, and Myc levels represent Wnt targets (D). And Serpine1, Smad7, and CDKN1A denote TGF**β** targets (E). TGF**β**1 was also measured. It is worth noting that almost all genes display opposite regulation from No Insulin.

Chapter 3: Exploring the Potency of Avian Neural Crest

Abstract

Neural crest (NC) cells are a developmental phenomenon due to their wide range of derivatives and extreme migratory capabilities, allowing them to contribute to a variety of tissues and organs in vertebrates, specifically the predatory adaptations in the craniofacial region. As a multipotent population that exists during gastrulation, they exhibit supreme capabilities in their derivative potential. It has been long accepted that NC plasticity excludes the endodermal lineage, yet, being so closely related to pluripotent cells, it is feasible that NC cells retain the capacity to form endoderm under proper conditions. Recently, pre-gastrula NC in xenopus has shown the ability to express endodermal markers, and later stage NC can also do so with activation of specific early NC markers (Buitrago-Delgado et al. 2015). However, if this ability requires abnormally high levels of endoderm inducing signal then it may not be a retention of endodermal potential but rather a microcosm of the multipotent potential of NC and a close relationship of these specified cells to pluripotent cells. Unpublished work from our lab contradicts this notion, showing that hNC requires high levels of Activin to express endodermal markers at a level similar to hES, while low concentrations fail to guide hNC towards endoderm. The research presented here represents *in vivo* work in blastula stage avian explants which supports the limited potential of NC to form endoderm unless exposed to high levels of Activin, solidifying blastula stage NC as a specified cell state with wide ranging plasticity but limited capacity in endogenous environments.

Introduction

A pluripotent cell has unlimited capacity to differentiate into cells of any germ layer and any derivatives of each lineage. As differentiation progresses, pluripotent cells become multipotent and their potency continually becomes more and more restricted until the cell reaches its terminal fate. However, with the proper signaling at specific levels, some restricted cell types can be coerced into changing course or reprogramming their function. This is different in some respects between multipotent cells (ie. neural crest (NC)) and reprogramming of terminal differentiated cells back into induced pluripotent stem cells. Limited potency of a cell type suggests its innate ability to respond to endogenous cues, rather the potential to pivot in the presence of overwhelming force.

As a multipotent cell population, the NC is very capable of forming certain derivatives that are within the mesodermal and ectodermal lineages (Gans and Northcutt 1983, Baker and Bronner-Fraser 1997, Kalcheim 2006, Schlosser 2006,), which have helped form the unique predatory devices developed in vertebrates. They do not, however, show an ability to form derivatives of endodermal lineage (Le Douarin and Kalcheim 1999). The multi-germ layer potential of NC cells, as well as various reports of specification at or before gastrulation in chick, rabbit, xenopus (Monsoro-Burq et al. 2005, Basch et al. 2006, Buitrago-Delgado et al. 2015, Geary and Labonne 2018, Betters et al. 2018, Prasad et al. 2019b) and hNC induction protocols that define the stages of NC development from pluripotent hESCs via intermediates of ectomesenchymal nature (Leung et al. 2016, Prasad et al. 2019a, Prasad et al. 2020, submitted), are indicative of specification of NC before the presence of other germ layers. This evidence does not discredit or invalidate studies that show specification or induction occurring in various ways, and it is highly possible that the heterogeneity of NC cells relies on continued signaling from various sources, but blastula stage specification does suggest an early segregation and initiation of a lineage program of the NC population distinct from pluripotent cells.

Lineage segregation is the initial branching off and differentiation of various cell types from a common progenitor cell or population, and even between cranial and trunk crest. Cross-species grafts of cranial quail crest into the trunk crest region of chick showed an ability of committed cranial crest to form melanocytes, neurons, glia, and cartilage, which the trunk NC is not capable of forming (Rothstein et al. 2018). Alternatively, trunk quail crest grafted into the chick cranial crest region formed all derivatives except cartilage. This is a clear indication that even with endogenous levels able to promote NC derived cartilage, trunk crest is restricted at that stage to a point that it is not capable of forming this cranial NC derivative. This type of restriction has also been seen in homotopic trunk to cranial grafts (Couly et al. 2002, Le Douarin et al. 2004), where membrane bones arise only from non-Hox gene expressing NC, which is essentially the cranial NC. Additionally, these findings indicate a relationship between developing NC and surrounding endoderm, suggesting specific plasticity of trunk and cranial NC regarding their ability to respond to the endogenous signaling from surrounding tissue. Yet, a dependency does not indicate a shared potential.

At the differentiation stages where a cell is committed, prior to lineage segregation, the state of specification is much more pliable and responsive to inductive signals. As the search for early NC specification has expanded, multiple reports have shown coexpression of NC markers with pluripotency factors, including a similar transcriptome profile between pluripotent cells and pNC at the pre-border state (Trevors et al. 2018). Additionally, our lab has published multiple papers based on our original robust hPSC to hNC induction (Leung et al. 2016), where the status of early NC markers and transcripts are investigated (Gomez et al. 2019a/b, Hackland et al. 2019, Prasad et al. 2020 submitted). Additionally, these studies investigated loss of pluripotency during the transition to NC. While human and chick models cannot be directly compared, we do know from our hNC induction model that on day three of the 5-day protocol, Oct4 expression is still

moderate while Pax7 expression has become visible (Leung et al. 2016). Additionally, other Yamanaka factors (Takahashi and Yamanaka 2006) are not completely absent as NC genes begin to show up (Gomez et al. 2019a, Prasad et al. 2020 submitted). The retention of these pluripotency markers, along with recently published data suggesting xenopus NC retain pluripotency (Buitrago-Delgado et al. 2015), lead to exploration of potency in our hNC model (Prasad et al. 2020 submitted). Exposure of high and low levels of ActivinA and FGF to test for endodermal and mesodermal potential, respectively, it became evident that in our hNC model, high levels of either growth factor was able to promote alternative differentiation, as evidenced by IF and qPCR at various timepoints. However, at low concentrations more similar to endogenous levels, these growth factors failed to induce endoderm or mesoderm, yet they did interrupt the normal NC program.

Use of chick explants has been used previously to test potency of specific regions in response to exogenous signaling (Wilson et al. 2000/2001, Patthey et al. 2008/2009), resulting in conversion of pNC into neural after BMP or Wnt inhibition. Alternatively, BMP too early convert pNC into epidermal cells, as did early Wnt, altogether showing that modifications of major NC inducing signals converts these cells into different cell types related to their normal program, but endodermal potential is not apparent in these studies (Patthey et al. 2008). Other modifications of FGF and BMP allow for variations in NC marker expression, but do not push NC towards mesodermal or endodermal (Wilson et al. 2000). Therefore, in light of this new and contradictory evidence, as well as the notion that early NC states may in fact represent a pluripotent state, we sought to investigate if the status of the chick cells we target for NC specification assays were in fact pluripotent. To this end, we investigated high mesodermal and endodermal inducing levels of FGF and Activin, respectively, as well as lower, more endogenous levels of each.

Results

High Activin induces FoxA2 in multiple regions

Determination of the responsiveness of prospective NC to ectopic signaling is enhanced by isolating the tissue to ensure there is no interaction from endogenous antagonistic signals. To assess the plasticity of specified NC cells, chick NC specification assays were employed at blastula stage when NC is reported to have expression of pluripotency markers and be more responsive to reprogramming cues. In accordance with previously described methods, we targeted an equatorial strip from blastula stage chick embryos for explant culture. However, beyond culture of prospective NC (labeled as L1 and L2 for both lateral regions), an anterior (A) and koller's sickle (KS) explant were also collected and cultured, as well as a medial (M) explant (Figure 1A). A KS explant was chosen for its known ability to form cells from each germ layer, as well as to serve as a control for responsiveness to endoderm inducing signals, considering evidence that KS expresses definitive endodermal markers (Chapman et al. 2007). Anterior explants also show potential for endodermal markers, although less potent in comparison to KS (Wilson et al. 2000, Patthey et al. 2009). Each of the five regions cultured were slightly larger than normal explants to ensure the intended region was not missed. For example, the KS explant contains regions both above and below KS, and L1 and L2 cover a region that contains pNC but also potentially contains regions more medial and lateral.

The establishment of responsive levels of inductive signaling requires exploration of high and low levels for numerous durations, and results were assessed by immunostaining with FoxA2. FoxA2 is expressed primarily in KS (Chapman et al. 2002), but as development continues, it shows expression in areas lateral to the pNC, as well as more anterior regions. All five regions were collected and cultured as previously described, in F12 media with ActivinA. 41 hours of incubation resulted in robust expression of definitive endoderm marker FoxA2 in KS or A explants and one

or both of the lateral explants at high (100ng), medium (50ng) and low (10ng) levels of ActivinA (Figure 1B). A similar result was seen at 36 hours for concentrations of 10ng and 1ng, indicating that even 1ng of ActivinA for a prolonged period may be high; however, 24-hour exposure at 50ng and 25ng gave no sign of FoxA2 expression, suggesting these high concentrations may lead to more widespread expression but require more time to develop, or that FoxA2 expression is transient and not seen at 24-hours (Hatada and Stern 1994) (data not shown). Knowing FoxA2 shows up on *in situ hybridization* screens at the blastula stage suggests that an earlier assessment of expression may be necessary, so we cultured the explants for 18-hours at relatively low levels of ActivinA (Figure 1B). Considering that 1ng seemed to still to induce widespread FoxA2, we used that as our high concentration, and went as low as 1pg. Out of 9 replicates in either 25pg, 10pg, or 1pg of Activin, only 1 replicate showed expression of FoxA2 in more than 1 explant (10pg had one replicate with expression in L1 and A). On the contrary, of the 6 embryos exposed to the high concentration of 1ng for 18 hours, only one failed to show expression in multiple explants, while two replicates induced expression of FoxA2 in three explants, including both lateral explants. A control condition was cultured with F12 only and without any growth factor, resulting in expression of FoxA2 in only one (L1 only) or two (L1 and KS) explants (data not shown). Using this as the baseline, the only concentration capable of inducing expression in more than two explants of the same embryo was the 1ng condition.

High FGF induces Brachyury in all regions

The same approach was applied to test the mesodermal potential of NC cells with respect to determining ideal high and endogenous levels of concentrations and the appropriate duration to measure its effect, and BraT was used to assess mesodermal conversion. BraT is expressed in the region of KS and continues around the lateral edge of the area pellucida (Mikawa et al., 2004),

suggesting its proteins will be found lateral to the pNC. Using FGF in media to culture explants, a high concentration of 5µg was exposed to explants for 41 hours, which lead to expression of definitive mesodermal marker Brachyury (BraT) in all five explants (Figure 1C). Lower concentrations of 1µg and 500ng for 41 hours showed no expression whatsoever. Reducing the duration, 5µg for 36 hours resulted in expression of BraT only in explant A, while 2.5µg for the same amount of time resulted in expression in both lateral explants and KS. Twenty-four-hour exposure at 1µg and 500ng led to BraT expression in two explants, either both lateral explants or KS and L1. As with FoxA2, it appears that the expression of BraT may be transient. Thus, in our search to find the earliest inducible signal we tested various concentrations of high and low FGF at 18 hours of incubation, resulting in a clear difference between the response of each region. At 2µg of FGF for 18 hours, BraT was seen in all explants, with the exception of the medial explants which once again died more often than they survived. Out of six embryos, this high concentration resulted in 4 BraT positive explants in 1 embryo, 3 positive explants in 2 embryos, and 2 positive explants in 2 embryos. Again, almost all medial explants at all concentrations did not survive, therefore, 4 positive explants in one embryo represents BraT expression in all surviving explants. In two embryos cultured with the with 1µg FGF resulted in expression of BraT in only one explant at a time. In three embryos at 500ng FGF, 2 embryos had expression in 2 explants while one embryo showed no expression. Of the two replicates with positive results, KS was positive in both, while the other positive explant was L1 or A. However, at 250ng (3 embryos) or 100ng (4 embryos), expression of BraT was only seen in KS, with the exception of one embryo at 250ng that had expression in KS and L1. Lower concentration of 10ng or no growth factor resulted in no noticeable BraT staining. Overall this indicates that there does exist a high level of FGF that can induce expression of BraT in any of the non-medial regions of blastula stage embryos, but lower concentrations only induce expression in expected regions.

Low levels of Activin block NC program but cannot induce Endoderm

To address potential shifts in NC marker expression (Stuhlmiller et al. 2012), as well as assess the influence of Activin in a more geographically accurate model, full equatorial strips cut into 12 explants were cultured and stained with endoderm or mesoderm markers and Pax7 (Figure 2A). Additionally, a vertical strip cut into six explants was included to check expression in A and KS and for orientation insurance (data not shown). Due to the isotype of our Pax7 antibody, we switched our endoderm marker from FoxA2 to Sox17. Similar to BraT, Sox17 is expressed in the region of KS and continues around the lateral edge of the area pellucida (Chapman et al., 2007). Since the left and right side of an embryo are biologically identical at this stage, each individual side was counted as its own replicate, and thus each side of the embryo was scored with 1 as the lateral most explant and 6 as the medial most explant.

Establishment of natural locations of our germ layer markers with respect to Pax7 in a normal NC inducing environment was necessary to set a baseline and see potential co-expression of these markers in the same region. Thus, we cultured explants in F12 with N2 for 25 hours, which has been established as a reliable early timepoint for Pax7 expression after blastula stage explant culture (Prasad et al. 2019b). In six embryos representing 12 replicates, Sox17 was consistently expressed in the two most lateral explants (explant 1 and 2) of each replicate (Figure 2B-C). The exception is that one replicate expressed Sox17 in explants 1 and 3 instead of 1 and 2, and another replicate expressed Sox17 in the three most lateral explants (1, 2, and 3). Pax7 expression was consistently found in the intermediate explants (explant 4 and 5) with one embryo showing additional expression in explant 3, and two other embryos showing additional expression in the medial explant (explant 6). Despite the occasional medial expression, Pax7 positive explants left a gap of at least one explant in expression in their bilateral counterpart. Yet, the more noteworthy gap is that left between Pax7 expressing explants and Sox17 expressing explants in 10/12 replicates.

This reinforces the major takeaway here, which is that no explant exhibited coexpression of Pax7 and Sox17, signifying a clear segregation of endodermal and NC specification at the blastula stage. Additionally, vertical explants did not show any coexpression of Sox17 with Pax7 (data not shown).

The purpose of these experiments is to test the plasticity of NC to respond to ectopic inductive signals, thus we added ActivinA at the low concentration of 25pg to the F12 media, with and without N2. To check for early Sox17 signal, cultures were stopped at 18 hours and stained for Pax7 and Sox17 (Figure 2D), however, as mentioned previously, Pax7 expression is reliably found at 25 hours, so more embryos were tested in the same media but taken to 25 hours of incubation (Figure 2E). With 3 embryos at 18 hours, representing 6 replicates (N=4 in F12+N2+Activin; N=2 in F12+Activin), Sox17 expression was once again found in the lateral most explants, with 2 replicates showing expression in explant 3. This indicates no change from the regions of Sox17 expression in the absence of exogenous ActivinA. At 25-hours, four embryos representing 8 replicates (N=4 in F12+N2+Activin; N=4 in F12+Activin), Sox17 expression was once again maintained in the lateral most explants, with two replicates showing expression in explant 3. Neither 25-hour nor 18-hour incubation exhibited Pax7 expression. Thus, in the presence of ActivinA at low levels, Pax7 expression was completely lost and these pNC regions were unable to express definitive endoderm marker Sox17.

Low levels of FGF can re-program NC into Mesoderm

Once again, the same approach was utilized to assess the ability of pNC to express mesodermal markers in the presence of FGF. In normal NC inducing conditions, with F12+N2 media and 25-hour incubation, BraT expression was generally found more lateral to Pax7 expression. In the five embryos representing 10 replicates, 4 replicates had Pax7 with no BraT, 3 replicates had Pax7 medial to BraT, and two replicates had no Pax7 expression. Interestingly, two

of the 10 replicates show coexpression of Pax7 and BraT in the same explant (Figure 2F-G), which is not uncommon (Yardley and Garcia-Castro 2012). This indicates shared potential between regions of NC and mesoderm. However, no vertical explants showed any coexpression of BraT with Pax7 (data not shown).

In the presence of low levels of FGF (175ng) in F12 media with or without N2, four embryos representing 8 replicates (N=4 in F12+N2+FGF; N=4 in F12+FGF) were used for 18-hour incubation (Figure 2H)and the same replicate representation was used for 25-hour incubation (Figure 2J). It is worth noting that the 18-hour time-point had more widespread BraT expression, further supporting the notion of transient expression. Regardless, both time-points exhibited BraT expression potential in all explants, with no expression of Pax7 in any of the 16 half-strips. Most replicates only expressed BraT in one or two of the explants, however, at 18-hours one replicate expressed BraT in four explants, another in all 6 explants, and another in three explants. After 25-hour incubation, only one replicate expressed BraT in more than two explants, but the location of expression was not restricted to the original lateral location BraT was identified. Altogether this data highlights the capacity for pNC to express definitive mesodermal markers in the presence of low level FGF, doing so at the expense of NC markers.

Discussion

The question of NC plasticity is a delicate issue in that NC cells inherently have more potential derivatives than many other cell types and can differentiate into tissue that is normally formed by either ectodermal or mesodermal cells, (Baker and Bronner-Fraser 1997, Le Douarin et al. 2004, Kalcheim 2006, Schlosser 2006). Thus, the real answer of NC plasticity lies in the capability of specified NC to express definitive endodermal markers, showing a retention of full pluripotent potential. In other words, testing NC cell plasticity higher on Waddington's epigenetic landscape will give us a greater chance of defining the full potential of this cell type once it is specified. Thus, the ability of pNC to express markers associated with mesoderm is to be expected, especially with endogenous levels of inductive signaling, considering the NC potential to form derivatives of each of this. With expression of endodermal markers being inducible in a wide variety of pre-gastrula locations (Hatada and Stern 1994), the inability of chick NC to form endoderm in our assay and other studies (Wilson et al. 2000, Patthey et al. 2008) is very telling of its potency at the stage of specification. This further validates the classification of NC as some combination of ectoderm and mesoderm, dubbed "ectomesechyme" or "mesectoderm", distinguishing it from the three classic germ layers (Hall 2000), as opposed to an alternative or intermediate pluripotent state (Buitrago-Delgado et al. 2015). However, the revelation of NC potential to express endodermal markers under endoderm inducing conditions, high or low, is novel and requires further investigation to establish whether this plasticity is innate or artifactual.

Focusing on the earliest and thus most plastic stages of development, assessment of the spatial expression patterns of Pax7, Sox17, and BraT exposed the exclusion of endodermal markers from pNC regions, compared to the potential of pNC to express mesodermal markers. Considering the sensitive and complex concentrations, temporal exposure, and combination of various growth factors required to induce NC (Wilson et al. 2000/2001, Garcia-Castro et al. 2002, Monsoro-Burq

et al. 2003, Pera et al. 2003, Linker et al. 2004, Steventon et al. 2009, Patthey et al. 2009, Stuhlmiller and Garcia-Castro 2012b), it is predictable that exposure of pNC to increased levels of FGF or Activin lead to complete loss of all Pax7 expression. However, the restriction of Sox17 to regions lateral to pNC regardless of Activin exposure is suggestive of the degree of plasticity within specified NC cells. Alternatively, increased FGF leads to expansion of the region that is capable of expressing BraT, showing capability of pNC to form mesoderm, but not endoderm. While hNC studies indicate a gradual decrease of pluripotency during early NC development (Leung et al. 2016, Gomez et al. 2019), this would need to be further investigated in chick before it can be deemed relevant to the rigid segregation of NC lineage specification. Nonetheless, despite similar expression of certain transcription factors in ES and preborder cells, specified cells of various regions eventually diverge towards distinct differentiation directions (Trevors et al. 2018). Thus, these regions have already been exposed to a range of patterning signals that have predestined their potential even before their transcriptome can convey this. Furthermore, as alluded to above, almost every medial explant died in these experiments. These medial explants are specified towards neural development (Patthey et al. 2008, Prasad et al. 2019b), but it is worth noting that there was no N2 in the media, and thus no exposure to insulin, which has been suggested as a requirement for neural development (Pera et al. 2001, Rhee et al. 2013).

Based on *in situ hybridization* of FoxA2, BraT, and Sox17 in blastula stage chick embryos (Chapman et al. 2002/2007, Mikawa et al., 2004), the responsiveness of the first set of explants which included two lateral regions (L1 and L2), a medial region (M), an anterior region (A), and koller's sickle (KS) is in agreement with expected spatial expression profiles. Yet, not all regions were consistently induced to express endodermal or mesodermal markers at all concentrations of ActivinA or FGF. With high concentrations resulting in expression capabilities in all surviving explants, it suggests that at early stages of development there is enough plasticity to coerce almost

any cell type to change course if provided with enough persuasive signal. It was also apparent that expression of these signals in each region may not be maintained temporally, which is unsurprising considering the transient nature of endodermal markers (Hatada and Stern 1994). Yet, the consistent expression of endoderm markers in lateral explants of the full strip but absence in intermediate explants (ie. pNC) supports the accuracy of explants cultured in figures 1-3, since L1 and L2 were unable to express an endodermal marker at low levels of Activin, similar to the pNC in the full strip.

The revelation that 18 hours of culture was enough time for high levels of FGF or Activin to induce expression in multiple regions, whereas low levels for 41 hours were less successful in reprogramming differentiation is a clear indication that with enough force any blastula stage cell is incredibly plastic, but that more endogenous levels are likely not sufficient to convert already specified cells. However, the loss of Pax7 expression in all regions is novel. In Buitrago-Delgado et al. 2015, in opposition of previous data (Basch et al. 2006), they suggest that Pax7 is a pluripotency retention indicator, as opposed to a NC marker. However, our data shows the explants expressing Pax7 were unable to convert to endoderm, while showing an ability to respond to mesodermal cues, contradicting the notion that Pax7 expression is correlated with pluripotency.

Loss of Pax7 in pNC response to FGF modification has been seen before in chick, but with gastrula stage explants and inhibition of FGF. In this instance, Pax7 expression shifted medially into neural regions (Stuhlmiller and Garcia-Castro 2012b). Additionally, beads coated with FGF and placed in the area opaca have shown the ability induce ectopic Pax7 (Yardley and Garcia-Castro 2012). Taken together, the results of these published experiments and the data here stresses the importance of FGF for NC formation, but more importantly indicates the existence of an ideal concentration. In any case, changes in FGF affect more than just the FGF effectors, but also influence the signaling of other growth factors that are regulated by FGF, specifically Wnt and

BMP, and even insulin (Wilson et al. 2000/2001, Pera et al. 2003, Ciarlo et al. 2017). Interestingly, this effect of FGF inhibition on pNC and Pax7 expression was only seen at early stages (Stuhlmiller and Garcia-Castro 2012ba), further supporting the early specified state of NC paired with the gradually decreasing levels of pluripotency markers. Therefore, finding that pNC retains potential to respond to inductive signals forceful enough to push them from one valley in Waddington's landscape, over the hill, and into another valley is within reason, especially considering the reported relationship between pluripotency markers and regulation of vital NC growth factor signaling (Ying et al. 2015). However, the chances of pNC encountering these forceful levels endogenously is unlikely.

The interchangeable expression in either A or KS, or even embryos where expression was only seen in one lateral explant and nowhere else, could be indicative of improper orientation of the embryo prior to dissection, as identification of the collection of cells that forms KS is not always clear (Hamburger and Hamilton 1951). However, the inability for lower concentrations to induce expression in as many explants as high concentrations, regardless of orientation confirms the persuasive power of high signal versus low signal. Yet, one intriguing outcome is the common result of expression of FoxA2 and BraT in both KS and one of two lateral explants in the same replicate. This is likely the result of isolating explants, leading to reductions of surrounding enhancing and inhibiting signals. An embryo dissection study (Torlopp et al. 2014) shows after removal of the area of KS (bottom half of the embryo), that a new primitive streak (PS) forms from the general area that we would expect to see NC. Importantly, only one new PS forms in the remaining top half of the embryo, but either side is capable of formation of this new PS. This means that this region (lateral to where the NC arises from) has the capacity to alter its differentiation and convert to a KS region, which is capable of expressing endodermal markers. In explants, the isolation reduces signals that may normally antagonize endodermal growth, thus leading to a more

receptive environment to endodermal potential. Additionally, the lack of surrounding inductive cues allows the usually transient Sox17 signals to remain in this region. This was also shown by hypoblast rotation experiments that rotated the hypoblast 180 degrees, leading to anterior forming PS, indicating that the cells at this early stage are non-committed and responsive to various cues. Therefore, explant experiments which isolate at the stages of specification will show Sox17 in lateral explants, and potentially slightly more medial regions as the expression has potential to spread with a lack of possible antagonists. Nonetheless, this potential to spread medially stopped before the pNC region.

Overall, the results here indicate that the chick blastula stage specification of NC represents precisely that: a specified NC state. While the result from Buitrago-Delgado et al. 2015 is intriguing and novel, it may be a unique attribute of xenopus NC that is not applicable to chick, as evidenced by the work presented here and previous studies (Wilson et al. 2000, Patthey et al. 2008). These cells retain the potential to express markers which they naturally wound not under normal conditions but are only able to under sufficiently forceful levels of influential signal. The most significant takeaway is the resistance of pNC to form endoderm in the presence of endogenous levels of Activin compared to the willingness to form mesoderm. This clear lineage segregation indicates the branching of NC from the other germ layers at the blastula stage, specifically endoderm, supporting the notion of NC as the fourth germ layer. Additionally, this provides supplementary evidence for NC as a specified cell state at the blastula stage, being able to change course in response to inductive cues but otherwise carrying on its normal program.

Methods

Chick Embryos and NC specification assays

Fertilized hen eggs were incubated for 30 minutes to 3 hours for stage XI-XIV Eyal-Giladi and Kochav (EK) embryos. Embryos were collected by cracking the egg in the upper third, draining the albumin, centering the embryo in the hole of a punched filter paper, cutting the vitaline membrane, and removing the filter paper with the embryo from the yolk to place it in Ringer's solution. Under a stereoscopic microscope (Nikon), hypoblast was removed using a sharpened tungsten needle and the area pellucida lined up with the edges of a 20x20 ocular grid. For figure 1, 5 explants were removed from the embryo as represented in figure 1A (≈180mm^2 each), with M, L1, and L2 lining up between rows 9-11. For figure 2, an equatorial strip at row 10 of the 20x20 grid was dissected out. Each strip was dissected further into 12 explants (≈90mm^2 each) (Figure 2A). After cutting, explants were placed in PB1 (NaCl, KCl, Na_2HPO_4. K_2HPO_4, Glucose, Sodium piruvate, Phenol red, $MgCl_2$, $CaCl_2$) in 96 well terisaki plates. This served as a holding place until explants were suspended in a 10µl collagen gel mixture consisting of 90µl of rat type I collagen (Collaborative Research, Waltham, Massachusetts), 10µl of 10X D-MEM and 5 µl of 7.5% sodium bicarbonate, vortexed for 20 seconds and briefly spun down. These collagen droplets were made in 4 well IVF plates (Falcon), and when the gels were polymerized, cultured media (DMEM-F12 (Invitrogen, nose, USA) supplemented with FGF, ActivinA, N2, FGF+N2, or ActivinA+N2 was added. Cultures were kept in an incubator at 37°C for various durations ranging from 18 to 41 hours, depending on the experiment.

Immunofluorescence

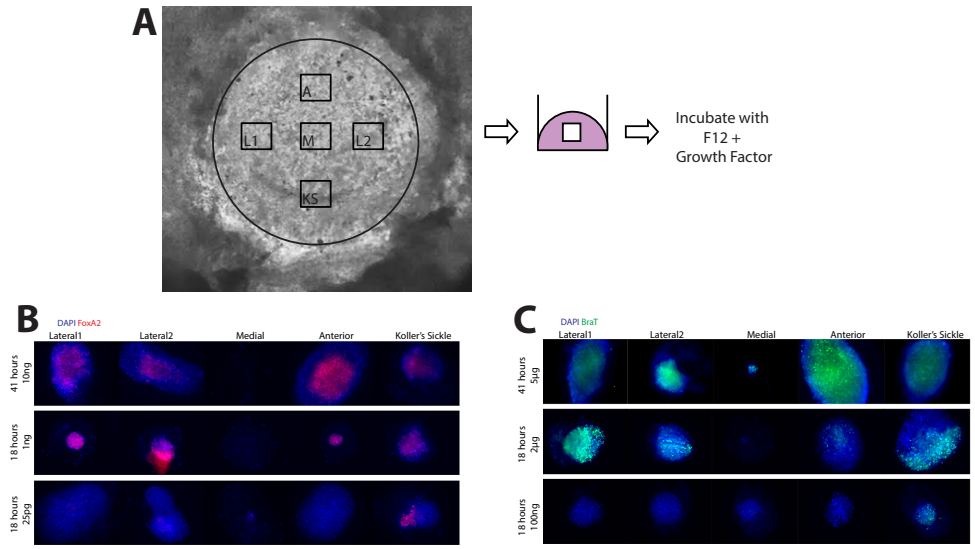
Chick explants were immunostained in accordance with previously published methods (Basch et al. 2006, Stuhlmiller and Garcia-Castro 2012ba, Prasad et al. 2019b). Explants in collagen

gels were fixed with 4% paraformaldehyde at RT for 15 minutes and then rinsed three times with PBS. Permeabilization step was done with PBT (PBS with 1% BSA and 0.1% tween). Block was done using PBTS (PBT with 10% fetal bovine serum added). Primary antibody (mouse IgG1 anti-Pax7 at 1:10, Developmental Studies Hybridoma Bank; mouse IgG1 anti-FoxA2 at 1:10, Developmental Studies Hybridoma Bank; rabbit IgG anti-Brachyury at 1:10, gift from Dr. Susan Mackem; goat IgG anti-Sox17 at 1:100, R&D AF1924) diluted in PBTS, added to the culture, and incubated overnight at 4° C. Three 1-hour washes were done with PBT at RT, and secondary antibody (goat anti-mouse IgG1 568; goat anti-rabbit IgG 488; rabbit anti-mouse IgG1 568; rabbit anti-goat IgG 488) was diluted in PBTS at 1:2000 was added and incubated overnight at 4° C. Two rinses and three 1-hour washes were done with PBT. Explants were then placed on slides and sealed with DAPI Fluoromount.

Imaging

Chick explants were imaged on Nikon Eclipse 80i microscope and processed in Adobe Photoshop CC 2019. Images of each explant were taken with fluorescent filters, using the same exposure for each sample imaged for each channel. Images were composed into a grid in Photoshop, merged, and levels adjusted evenly, with threshold being set using a positive control for nuclear staining.

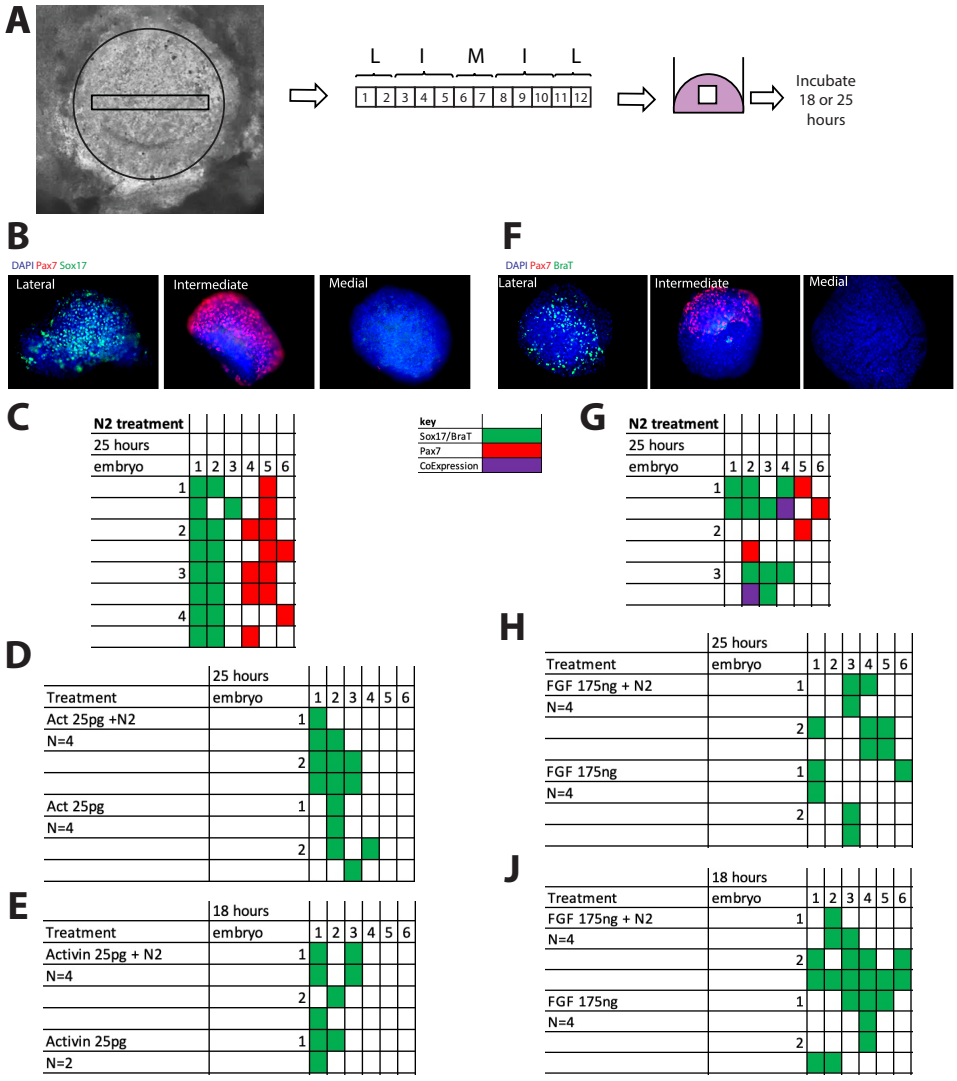
Chapter 3: Figure 1



Chapter 3: Figure 1 – High growth factor and long exposure promotes lineage changes

Five regions were taken from blastula stage chick embryos representing lateral pNC regions (L1 and L2), a medial region (M), an anterior region (A), and a section containing Koller's sickle (KS) (A). These explants were then incubated as previously explained. Exposure to high levels of Activin for 41 hours lead to expression of FoxA2 (Red) in all surviving explants, with DAPI used as a nuclear stain (Blue) (B), as did exposure to relatively high levels for 18 hours. However, exposure to low levels of Activin for 18 hours only supported FoxA2 expression in KS. Addition of high FGF to explants in a 41-hour culture supported Brachyury (BraT) (Green) in all explants (C), a trend which was also seen after 18-hour culture in the presence of moderately high FGF. Yet, lower levels of FGF in 18-hour culture revealed expression of BraT was only promoted in KS.

Chapter 3: Figure 2



Chapter 3: Figure 2 – Sox17 is restricted to regions lateral of pNC under low Activin conditions

Full equatorial strips were removed from blastula chick explants and cultured as before (A). In normal conditions with F12+N2, Pax7 (Red) is seen in intermediate explants while endoderm marker Sox17 (Green) remains lateral (B). Explant marker counts show this distinction between Sox17 and Pax7, with at least one explant of separation between these regions (C). Addition of Activin at 25pg, either with or without N2, for 25 hours (D) or 18 hours (E) of culture prevented Pax7 expression, however, pNC regions did not show the ability to express Sox17, which remained in explants lateral to where Pax7 was expected. The same technique was applied when staining for BraT (Green) (F), showing more variable locations, as well as coexpression of Pax7 and BraT. This is portrayed in explant counts with F12+N2 for 25-hour cultures (G) where BraT was generally found lateral to Pax7. Addition of FGF to 25- (H) or 18-hour (J) cultures, either with or without N2 led to complete loss of Pax7. However, unlike Sox17, BraT expression was seen in more regions, especially those where Pax7 expression is expected.

Chapter 4: Conclusions and Outlook

The data presented in chapter two addresses the gap in knowledge discussed in chapter 1 regarding the role of insulin in NC development and its mechanism. No study had defined the role of insulin beyond glucose metabolism and pro-survival cues, much less elucidated a mechanism for its instructive contribution to NC development at any stage. The data shown describes the involvement of insulin in both avian and hNC development during the earliest stages of induction, as well as the requirement for functional Pi3K to propagate insulin signaling and the regulation of FoxO1. Blastula stage NC specification has been alluded to in many publications before, but the work published from our lab in both chick and human NC (Leung et al. 2016, Gomez et al. 2019, Prasad et al. 2019b) further opened the door for investigation into this early NC state and the growth factors that contribute to specification. Of the potential contributors, insulin is the least discussed in terms of its instructive involvement at any stage of NC development, as it was generally assumed to simply be a facilitator of NC survival and proliferation.

Neural Crest is Sensitive to Insulin Dysregulation

In blastula stage avian NC specification assays, insulin showed itself to be required and sufficient to induce expression of Pax7 (Chapter 2, Figure 1E). This phenotype is expected but the nuances appear to be novel, yet supportive of the idea that improper insulin signaling acts to delay NC development (Garcia et al. 2015). To fully establish the characteristics of potential developmental arrest, delay, or reprogramming of these cells, an RNA-seq of this protocol carried out in the absence of insulin would be very revealing. The hNC model used here is reliant on Wnt signaling and has shown an inability to develop in NC without the Wnt mimc CHIR (Leung et al. 2016, Gomez et al. 2019a), thus we posit that insulin alone will not be enough to convert hES to

hNC. However, between chick blastula pNC and hNC induction there appears to be a common state of specified NC that requires additional insulin signaling to promote the NC program. If response to insulin is the determinant (Chapter 2, Figure 1H-J/2K-M) it would arguably be within the first 24 hours of hNC induction, as removal of insulin after this point significantly reduces NC markers, whereas 48-hour exposure is sufficient to induce NC.

Beyond being required early and sufficient, NC development appears unimpeded in chick in the presence of insulin as low as 1:100 the concentration found in N2 media supplement (Chapter 2, Figure 1G). Interestingly, as insulin levels approach zero in hNC, there does not appear to be a dose dependent decrease, in agreement with results from avian insulin titrations. However, a reproducible dip is seen between 0.3-0.4μg of insulin (1:10 normal levels), which was rescued at lower concentrations (Chapter 2, Figure 2E). While this result was not statistically significant, it did downregulate transcript levels of early hNC genes, but it will require further investigation involving additional concentrations close to this range and lower than the tested levels. Further dilutions had been tested, which include insulin at 0.04μg (~1:100), as well as an equivalent to insulin at 1:250 in a B27 insulin titration (data not shown). Additionally, high insulin at 4, 6, and 8μg in hNC resulted in a sharp decline of NC markers without affecting the cell population, which is not the first instance of reported insulin toxicity (Rhee et al. 2013), but most importantly shows the delicate relationship between optimal insulin signaling and successful NC induction. This high insulin effect must be explored in chick, as well as expanded in human and investigated with other methods such as RT-qPCR. Moreover, chick explants must be investigated beyond Pax7 expression. Other NC markers such as TFAP2**α**, Msx1, Sox9, and Sox10 must be examined, and in order to analyze later NC markers longer cultures will be required (Chapter 2, Figure 1F). Additionally, IF for for prosurvival and anti-apoptotic markers will help identify the health and proliferation of explants and hNC in all conditions.

It is possible that the dip at the 1:10 range of normal insulin in hNC represents a valley in a bimodal response to insulin, akin to Gomez et al. 2019, although the steep drop off at levels above normal is more likely the middle range, thus exploring much higher concentrations is necessary until insulin becomes toxic to cell health. Whether or not a bimodal response exists, the population resulting from insulin above normal B27 levels must represent some type of developmental population. If FGF inhibition leads to medial expression of Pax7 in chick (Stuhlmiller and Garcia-Castro 2012ba) and FGF does in fact regulate insulin (Ciarlo et al. 2017), then it is possible that this resulting population is similar to what would be lateral to NC (ie. non-neural ectoderm). Additionally, at the lower concentrations of insulin tolerable by NC, it is not beyond reason to question if these different concentrations influence axial patterning akin to the changes in trunk crest in response to FGF (Hackland et al. 2019) and Wnt (Gomez et al. 2019b), prompting more experimentation into defining what region of NC is being generated, and what populations result from insulin modulation.

In opposition of insulin signaling is glucose levels, as insulin is the main regulator of glucose metabolism (Guo et al. 2014). Modifications to glucose levels analogous to diabetic conditions have shown the ability to drastically influence cellular behavior during development (Boucher et al. 2014, Zhang et al. 2015, Zhu et al. 2016, Descalzo and Satoorian et al. 2016), prompting studies to parallel insulin modulation regarding the role of high and low glucose in NC development.

IR is Not the Primary Receptor, or May Not be the Only Receptor, for Insulin in Neural Crest

While efforts to define the preferred receptor utilized by insulin in early NC development were successful in showing an ability to reduce NC marker expression by targeting receptors known to interact with insulin, they did not completely clarify the mechanism. Previous studies have

investigated insulin in NC derivatives (Cederberg et al. 2003, Nekrep et al. 2008, Olerud et al. 2009) but the data presented here represents the first investigation into the functional receptor responsible for insulin's contribution to NC. Inhibition screens in chick revealed requirements for both IR and IGFR (Chapter 2, figure 4A-C). This does not definitively implicate one receptor or the other, but rather points to the use of an HR (Bailyes et al. 1997, Hernandez-Sanchez et al. 2006, Benyoucef et al. 2007, Zhang et al. 2007, Belfiore et al. 2009, Siddle 2011) While inhibition screens in hNC displayed a preference for IGFR, with IR inhibition consistently increasing NC markers (Chapter 2, Figure 4D-G), RNAi targeting IR and IGF2R both reduced NC marker expression (Chapter 2, Figure 4H-L), thus reinforcing the notion of an HR. IF of hPSC and hNC displayed a vast difference in the expression of IR and IGFR (data not shown), with IGFR greatly outnumbering IR, however, siRNA targeting IR was still able to hinder NC development similarly to IGFR knockdown, so this difference in expression may not be relevant for NC.

Continued investigation into the preferred receptor for insulin's instructive contribution for NC will be difficult considering insulin's ability to signal via different combinations of homodimerized IR and IGFR as well as heterodimer IR/IGFR HRs. To determine receptor involvement in chick with a secondary method, dominant-negative (dn) IR and IGFR plasmids can be electroporated into whole chick embryos at the blastula stage, unilaterally targeting pNC regions. This technique will allow for development *in vivo* as opposed to the *ex vivo* explants and will also provide more specific effects than small molecule inhibitors, which can have offsite affects and potential toxicity. Preliminary experiments using a dn-IR plasmid in HH3 chick embryos resulted in no visible change in morphology of the NF, and showed coexpression of the plasmid reporter (GFP) with Pax7, however, use of the WT-IR plasmid showed unilateral knockdown of Pax7 in regions expressing the plasmid reporter (data not shown). This initial outcome is surprising, yet also validates the effect of increased insulin in hNC being toxic to NC, and further emphasizes the

need to test high levels of insulin in chick NC specification assays. Additionally, with dn's or small molecules, relying on FoxO1 translocation as a reporter, we must determine which receptor phenocopies No Insulin conditions. However, these methods alone will not be sufficient to determine the dominant receptor, especially if both lead to a similar phenotype, indicating use of HR. Co-Immunoprecipitation first targeting the insulin ligand and then either receptor with subsequent Western Blot will help determine the levels of insulin binding, but once again will not rule out HRs. To assess HRs, a Co-IP of IR and IGFR can be done, followed by Western blot targeting insulin. This can be done in with chick explants or whole chick embryos but will be more relevant in hNC as a larger population of early NC will be more attainable and will lend itself to testing changes in binding levels with various concentrations of insulin, including No Insulin. Also, with increasing and decreasing levels of insulin, IPs and CoIPs can be done for IR, IGFR, and HRs, using an antibody for phosphorylation of the intracellular tyrosine domains, measuring changes in activation based on level of insulin.

Another topic that must be addressed is the role of IR and IGFR as transcription factors (Sarfstein et al. 2012, Hancock et al. 2019). While the data presented here suggests the requirement of Pi3K and other intracellular components, it does not nullify this potential function of these receptors. IF in the presence and absence of insulin would be a simple way to test for changes in nuclear translocation. However, a nuclear fractionation followed by Western blot may be more affective. Additionally, a ChIP-seq may be run to determine widescale targets during early NC development, but to justify running a ChI-seq, it would be worthwhile to know the preferred receptor for insulin in NC and have evidence of its nuclear translocation.

Neural Crest requires Functional Pi3K

The ability of insulin to drive NC formation, even with limited early exposure in chick and human, (Chapter 2, Figure 1H-J and Figure 2H-L), displays the early requirement for insulin, suggesting that insulin signaling may coincide with early FGF (Wilson et al. 2000, Monsoro-Burq et al. 2003, Ciarlo et al. 2017) or precede it. Finding common temporal profiles of insulin and FGF signaling would further fuel the debate of their shared intracellular signaling branches and which growth factor prefers which branch. *In situ hybridization* targeting activated FGF and insulin receptors may help clear this up. FGF utilization of MAPK is well documented, especially as it pertains to NC development (Stuhlmiller and Garcia-Castro 2012a, Ciarlo et al. 2017), however, while Pi3K has been deemed vital to NC formation, it has not been directly associated with a specific growth factor (Dinsmore and Soriano 2018).

Via small molecule inhibition in chick and human, RNAi knockdown in human, and transcript analysis of human experiments, Pi3K was validated as vital and phenocopied the results of conditions lacking insulin or with inhibited IR or IGFR (Chapter 2, Figure 5). Looking downstream of Pi3K, inhibition of Akt via small molecule administration proved lethal, whereas siRNA knockdown of Akt appeared to have no effect on hNC induction compared to scrambled control (Figure 5H-J). It could be that the level of Akt present is unaffected by siRNA targeting, and small molecule inhibition became toxic because of the plethora of responsibilities attributed to Akt from various signaling pathways. To definitively associate the Pi3K branch with insulin signaling, Western blots looking at phospho-Akt levels in the presence and absence of insulin, IR and IGFR inhibitors, and Pi3K inhibitors must be executed. Additionally, the idea that FGF regulates insulin via Pi3K (Ciarlo et al. 2017) was not explored but monitoring Pi3K/Akt activation, FoxO1 nuclearization, and insulin target transcript levels in the presence of FGF inhibitors and activators would help to elucidate this. Seeing no effect would of course implicate insulin as the

primary employer of the Pi3K branch, however, an increase in its activity in the absence of FGF, and inverse reaction in presence of higher signaling would denote some sort of regulation between the two.

FoxO1 Regulation is Vital to Neural Crest Development Under Regulation by Insulin

The connection between FoxO1, insulin, and NC development is not without its caveats, but the data presented here showing proper regulation of FoxO1 is clearly vital for NC development is novel and supports FoxO1 as an effector of insulin signaling. As expected, lack of insulin signaling leads to significantly higher levels of nuclear FoxO1 (Chapter 2, Figure 6A-C) (Gross et al. 2009, Guo 2014, Nies et al. 2016), but surprisingly this change is not visible by IF until day 2 of culture. Nuclear fractionation and Western blot for FoxO1 will give true levels of nuclear FoxO1, as well as more accurately differentiate between nuclear levels at day 1, but the fact that there exists some level of nuclear FoxO1 in normal insulin conditions suggests that insulin's role in NC development is to regulate levels of nuclear FoxO1, as opposed to wholesale inhibition.

Use of a FoxO1 small molecule inhibitor gave further indications of the delicate relationship FoxO1 and NC, however it is important to distinguish that FoxO1 inhibition relates to high insulin levels, as increased insulin signaling will act to further remove FoxO1 from the nucleus (Gross et al. 2009). Similar to increased insulin in hNC induction, FoxO1 inhibition almost entirely ablated NC marker expression, and even significantly reduced it after only two days of exposure (Chapter 2, Figure 6D-G). Even more convincing is the extreme downregulation of all eight NC markers transcripts tested for both 5- and 2-day exposure. While siRNA knockdown did not approach the same levels of inhibition as the inhibitor molecule, it did emulate the results (Chapter 2, Figure 6H-L). The overall effect on NC is undeniable but will require further investigation into exactly what FoxO1 is targeting as a transcription factor. Of its known transcriptional targets,

TGFβ1 stands out (Zhang et al. 2015) considering the role of TGFs in NC, including the detriment that early BMP (a member of the TGF superfamily) has on NC development (Patthey et al. 2008/2009, Yardley and Garcia-Castro 2012, Hackland et al. 2017). A ChIP-seq of FoxO1 in NC would likely reveal relevant targets to further investigate and determine the role of FoxO1 in NC development, as well as potential connections to insulin signaling. However, FoxO1 is capable of binding to Wnt cofactors and its nuclear activity can change depending on the circumstances (Zhang et al. 2015, Zhu et al. 2016, Doumpas et al. 2018), thus FoxO1 ChIP-seq in No Insulin conditions compared to regular conditions would likely unveil the nature of FoxO1's altered activity in diabetic conditions related to neurocristopathies.

Attempts to address FoxO1 inhibition in chick NC specification assays have thus far proven futile. The inhibitor was used at 66μM, double the recommended concentration but still under 100μM where FoxO3 and FoxO4 are said to be affected (Nagashima et al. 2010, Zhou et al. 2014), and preliminary results indicate an increase in expression of Pax7 and Sox9 (data not shown). It is common that the optimal concentration of inhibition is the not the same between chick and human cells, so further exploration with higher concentrations will be necessary. Furthermore, considering the FoxO1 inhibitor mimics high insulin in both increase in total cells and decrease in NC markers (Figure 2E-G/6D-F), this result must be confirmed with high insulin in chick NC specification assays. The similarities between FoxO1 inhibition and high insulin are striking in that both severely reduce NC markers while increasing cell population by around 10%. These statistically significant results bring to light the question of what cell type is being produced in place of NC when insulin levels spike and FoxO1 is heavily excluded from the nucleus.

Alternatively, a FoxO1 activator must be established and investigated in these models to more aptly compare to all other inhibition screens, since the effect on FoxO1 of lowered insulin signaling is more nuclear, and thus, active FoxO1 (Chapter 2, Figure 6A-C). While an activator that targets

only FoxO1 and no other FoxO family members does not exist, it would be feasible to use LOM612, which has been shown to increase FoxO1 and FoxO3a nuclearization (Cautain et al. 2016). While targeting FoxO3a may cloud the validity of the results, siRNA knockdown of FoxO3 did not have any significant influence on NC transcript levels (Chapter 2, Figure5-H-K), thus it may not have a substantial role in NC development. A more specific and accurate method would be electroporation of a plasmid expressing constitutively active FoxO1 or development of a cell line with inducible expression of a constitutively active FoxO1.

Neural Crest is Highly Potent but Requires Proper Force to Generate Endoderm

The ability of blastula stage chick explants to respond to high levels of Activin and FGF to form endoderm and mesoderm, respectively, indicates plasticity that is not unexpected for cells this early in development (Chapter 3, Figure 1). However, their response to low level signaling for 18 hours points to the innate capacity of pNC (Chapter 3, Figure 2). While maintenance of pluripotency factors makes pNC more likely to convert to other cell types (Leung et al. 2016, Buitrago-Delgado et al. 2016, Gomez et al. 2019a), the results presented here indicate that their native retained potential is limited, excluding endoderm. This supports unpublished data from our lab (Prasad et al. 2020, submitted), investigating the same potential in our hNC model. Further experiments to test ectodermal potential will help define the full germ layer capacity of chick pNC, and expansion of IF targets to include more endodermal, mesodermal, and NC markers will definitively outline the potential of this specified state.

This data highlights the sensitivity of early NC to changes in the growth factor repertoire, which has shown its requirement for the involvement of insulin. While insulin was not investigated here, the presence of absence of N2 in full equatorial strips (Chapter 3, Figure 2D-E/H-J) did not appear to change the plasticity of pNC regions. It did, however, moderately expand the number of explants

expressing BraT in the presence of FGF (Chapter 3, Figure 2H-J), compared to FGF only. We can speculate that the presence of insulin influenced FGF's ability to convert more cells into mesoderm, which is not beyond reason considering their reported potential interactions (Ciarlo et al. 2017). However, it is more likely that insulin may have acted here in a more metabolic, pro-survival manner, simply improving the efficacy of FGF exposure and propagating development. More investigation will be required to establish this, which would mirror many of the chick experiments done in chapter 2, such as small molecule inhibition targeting relevant insulin receptors and intracellular pathways while exposing explants to high and low levels of FGF, as well as staining for a wider range of mesodermal markers.

In conclusion, the results presented here show for the first time a role for insulin in early NC specification beyond metabolism and survival and indicate optimal temporal and concentration ranges that influence formation of NC. Additionally, this data supports insulin's use of the Pi3K branch as a mechanism for signal propagation, and the discovery of hNC sensitivity to changes in FoxO1 translocation and function imply a delicate relationship between the two, which is likely mediated by insulin.

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