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Early specification and development of rabbit neural crest cells

Erin Betters, **Rebekah M. Charney**, **Martín I. Garcia-Castro***

School of Medicine Division of Biomedical Sciences, University of California, Riverside, California, 92521 USA

Abstract

The phenomenal migratory and differentiation capacity of neural crest cells has been well established across model organisms. While the earliest stages of neural crest development have been investigated in non-mammalian model systems such as Xenopus and Aves, the early specification of this cell population has not been evaluated in mammalian embryos, of which the murine model is the most prevalent. Towards a more comprehensive understanding of mammalian neural crest formation and human comparative studies, we have used the rabbit as a mammalian system for the study of early neural crest specification and development. We examine the expression profile of well-characterized neural crest markers in rabbit embryos across developmental time from early gastrula to later neurula stages, and provide a comparison to markers of migratory neural crest in the chick. Importantly, we have applied explant specification assays to address the pivotal question of mammalian neural crest ontogeny, and provide the first evidence that a specified population of neural crest cells exists in the rabbit gastrula prior to the overt expression of neural crest markers. Finally, we demonstrate that FGF signaling is necessary for early rabbit neural crest formation, as SU5402 treatment strongly represses neural crest marker expression in explant assays. This study pioneers the rabbit as a model for neural crest development, and provides the first demonstration of mammalian neural crest specification and the requirement of FGF signaling in this process.

Keywords

Neural crest; rabbit; Pax7; Pax3; Sox9; Sox10; AP2α; FGF signaling; SU5402; mammalian embryogenesis; specification; neural plate border

Introduction

The multiple achievements and recognitions of Wilhelm His include his contributions to the neuron doctrine, a much-improved microtome, his unwavering support to the study of embryology in its own right – calling to understand the mechanisms behind the transformation from zygote to organism – and of course, the topic honored in this special

*Correspondence: martin.garcia-castro@ucr.edu.

issue, the first description of the neural crest 150 years ago. While his original work describing the neural crest (NC) was performed using chick embryos, he soon moved to work with other organisms. This included a strong focus on human biology, stressing the need to understand human development to ameliorate human health conditions. Towards this goal, the work we provide here on rabbit NC development represents an appealing approach to amend the current gap in our understanding of the very early events leading to the formation of NC in mammals.

NC cells are a migratory population unique to vertebrates which gives rise to a variety of derivatives throughout the body, including neurons and glia of the peripheral nervous system, melanocytes, and much of the craniofacial skeleton and connective tissue (Le Douarin and Kalcheim, 1999). A large number of human health conditions are caused by defects in NC development, collectively known as “neurocristopathies” (Bolande, 1997), and include craniofacial malformations such as cleft lip and cleft palate, rare diseases such as Waardenburg syndrome, and aggressive cancers including melanoma and neuroblastoma (Etchevers et al., 2006; Farlie et al., 2004; Watt and Trainor, 2014). Therefore, the development and subsequent terminal differentiation of NC has been the subject of intense focus.

Considerable progress has been gained in our understanding of NC development using a variety of animal models, including chick, zebrafish, Xenopus, and mouse (Prasad et al., 2012; Sauka-Spengler and Bronner-Fraser, 2008; Simoes-Costa and Bronner, 2015; Stuhlmiller and García-Castro, 2012a), and more recently, lamprey and hagfish (Green and Bronner, 2014; Nikitina et al., 2008; Ota et al., 2007). Broad integrative efforts from these models have resulted in a postulated NC gene regulatory network representing hierarchical interactions between signaling molecules and transcription factors modulating the gene expression that ultimately governs NC development and differentiation. Further, advances in stem cell technologies have allowed for the interrogation of human NC formation and differentiation in vitro (Chambers et al., 2016; Fukuta et al., 2014; Hackland et al., 2017; Leung et al., 2016; Menendez et al., 2011; Mica et al., 2013; Umeda et al., 2015), and a few studies have interrogated NC cell morphology and gene expression in human embryos in vivo (Betters et al., 2010; Bondurand et al., 1998; O’Rahilly and Müller, 2007). However, major questions surrounding the formation of mammalian NC – in particular early NC ontogeny and the role of major signaling pathways – remain unanswered.

NC development is generally considered as a progression of states from the neural plate border (NPB), to NC specifier, migratory NC, and finally, to terminal differentiation (Prasad et al., 2012; Sauka-Spengler and Bronner-Fraser, 2008; Simoes-Costa and Bronner, 2015; Stuhlmiller and García-Castro, 2012a). NC induction is thought to initiate by the actions of WNT, BMP, FGF, and Notch signaling molecules, amongst others, leading to the establishment of the NPB, characterized by the expression of TFs including AP2⟨, Msx½, Pax3/7, and Zic1. These factors, in concert with signaling events, launch a distinct NC-specifier state marked by the expression of a suite of TFs at the neural folds including Snail2, Sox9, ETS1, and FoxD3. Subsequently, NC cells undergo an epithelial to mesenchymal transition, acquire migratory capacity and express transcription factors such like Sox10 and other markers such as HNK-1 and P75NTR. Finally, NC cells differentiate

into terminal derivatives at their final destinations. Most commonly, studies on the formation of NC cells have focused on their formation at the NPB during neurula stages of development, and their subsequent terminal differentiation.

More recently, however, a NC-specified population of cells has been identified in the gastrula embryo, prior to the formation of the neural plate. In this context, “specified” cells are those that have initiated a particular program of differentiation - in this case, towards a NC state. These specified cells will continue a progressive development towards the NC fate if permissive conditions are provided. Specification does not speak about commitment, and it remains possible that these cells could be redirected towards other fates upon alternative, strong instructive signals. In the chick, it was demonstrated that specific epiblast regions of Hamburger and Hamilton (HH) stage 3–4 gastrulae are specified to give rise to NC cells in culture, independent of mesoderm or neural tissue. This NC specified region resides in the intermediate territory between the primitive streak and the edge of the embryonic disc, and following culture, explants give rise to prospective NC cells expressing markers of pre-migratory and migratory NC, including Pax7, Snai2, and HNK-1 (Basch et al., 2006; Patthey et al., 2008). Using similar assays, subsequent studies have revealed chick NC specification at even earlier gastrula stages (HH2) (Patthey et al., 2009). Importantly, this early NC specification does not appear restricted to Aves. In Xenopus, a specified region of NC has been shown to exist coincident with the completion of gastrulation (Mancilla and Mayor, 1996). While other studies have addressed the specification of mammalian NC terminal derivatives beginning from a pre-migratory or early migratory NC-state (Chai et al., 2000; Li et al., 2000; Matsuoka et al., 2005; Billon et al., 2007; reviewed in Debbache et al., 2018), the in vivo specification of earlier stages of mammalian NC development - specifically, prior to the formation of the neural plate and the expression of the earliest known NC markers - is a major unanswered question.

Concurrent with the demonstration of a NC-specified region in lower vertebrate and amniote embryos, studies have also evaluated the roles of multiple signaling factors (FGF, BMP, and WNT) during early NC specification (Basch et al., 2006; Garcia-Castro et al., 2002; Mayor et al., 1995; Monsoro-Burq et al., 2003; Patthey et al., 2009; 2008; Steventon et al., 2009) (for review, see Patthey and Gunhaga, 2011; Stuhlmiller and García-Castro, 2012a). From studies in frog and chick, the role of BMP and WNT signaling pathways during NC specification has been divided into two steps: an early, pre-NPB “WNT-only” phase where prospective NC cells are thought to require a period of exposure solely to WNTs, followed by later dual exposure of prospective NC to both WNT and BMP during neurulation (Patthey et al., 2009; 2008; Steventon et al., 2009). Although FGF signaling is classically associated with neural development, this signaling pathway has also been implicated in Avian and frog NC formation (Hong et al., 2008; Monsoro-Burq et al., 2003; Stuhlmiller and García-Castro, 2012b). Interestingly, recent work in the chick demonstrated a more direct role for FGF signaling, activated and required in the epiblast during the pre-NPB “WNT-only” stage of NC development (Stuhlmiller and García-Castro, 2012b).

Despite this research focusing on the early facets of NC formation in Aves and frogs, early mammalian NC specification remains unanswered. Currently, the mouse stands as the sole embryonic model for mammalian NC development. While murine systems are powerful

tools for developmental biology and genetics, their small size and unique gastrula morphology have restricted its use in specification assays. To-date, no reports exist about early specification or induction in mouse that could validate or contest chick and Xenopus studies. In addition, although chimeric fate-mapping has provided evidence that a specific region of the E7.5 ectoderm can yield cranial NC cells (Tam, 1989), similar studies performed at earlier mouse gastrula stages (E6.5) have not commented on the generation of the NC (Lawson et al., 1991; Quinlan et al., 1995). Thus, the actual status of mammalian NC specification during gastrulation, and the interplay of NC-associated signaling factors therein, remains to be investigated.

In this report, we have turned to the rabbit to interrogate early mammalian NC specification and development. Prior to this work, rabbit NC formation has only been described based on cell morphology, and as early as the 10–12 somite stage (Berke, 1965). Here, we present the most detailed characterization to-date of rabbit NC formation by interrogating NC marker expression across a time course of rabbit embryogenesis from unprecedented early gastrula stages (stage 2–4+) through the end of neurulation (20–21 somites). We determine that NC markers Msx½, Pax7, AP2α, and limited Pax3 are expressed in the NPB as early as rabbit stage 6, prior to the appearance of definitive somites, and that the expression of these and additional genes (Pax3, Sox9, and Sox10) denote the pre-migratory or migratory NC cells of older embryos. Importantly, we propose that the rabbit embryo is highly amenable for mammalian early specification studies. Using explant assays, we demonstrate that rabbit NC is specified by gastrula stage 3+, and furthermore, provide evidence that Pax7 and Sox10 expression in specified explants is dependent on FGF signaling. Taken together, this work provides a novel characterization of NC formation in the rabbit, and critically, provides the first evidence of mammalian NC specification during gastrulation, prior to the expression of known NC markers.

Methods

Embryo Collection

Timed-pregnant New Zealand White rabbits (obtained from Millbrook Breeding Labs, Amherst, MA) were euthanized, and uteri removed and stored in 1x PBS at 4°C. Embryos were dissected from the uterine tissue in sterile 1x PBS containing 5% Fetal Bovine Serum (FBS) and 100 U/mL Penicillin-Streptomycin (Thermo Fisher #15140122, used at 1X). For immunohistochemistry, rabbit embryos were fixed in 4% paraformaldehyde for 30 minutes to 1.5 hours (depending on the stage) at room temperature. For explants, embryos were moved to fresh 1x PBS with 5% FBS and 100 U/mL Penicillin-Streptomycin and processed as described below. Embryos were staged as previously reported, with pre-gastrula embryos according to Viebahn et al. (Viebahn et al., 1995; 2002), and gastrula and neurula embryos according to chick developmental stages (Hamburger and Hamilton, 1951; Viebahn et al., 2002). Rabbit embryos were obtained and experiments performed at Yale University, following the approval and guidelines of IACUC. Chick embryos were collected from fertile hen eggs (Hardy's Hatchery, MA) and staged according to Hamburger and Hamilton (1951). For immunofluorescence, 10 somite stage chick embryos were fixed in 4% paraformaldehyde for 2 hours at 4°C.

Immunohistochemistry

Following collection and fixation, embryos were embedded in gelatin and cryosectioned (Leica CM1900), or subjected to whole mount immunofluorescence. Sections were stained for markers of NC (AP2α, Msx½, P75NTR, Pax3, Pax7, Sox9, and Sox10), mesoderm (T/Brachyury, Tbx6) and neural (Sox2/3) tissue, as previously described (Betters et al., 2010). Detailed antibody information is presented in Supplemental Table S1. Embryo sections were subsequently stained with DAPI and coverslips mounted with Permafluor. Whole mount immunofluorescence was performed in a similar manner with both primary and secondary antibody incubations carried out at 4°C overnight. All embryos and sections were imaged using Spot and processed in Photoshop.

Explant Generation, Culture, and Immunohistochemistry

All explant dissections were carried out on Sylgard 184-coated plates in sterile 1x PBS containing 5% FBS and 100 U/mL Penicillin-Streptomycin. Explants were generated from freshly collected rabbit embryos using hand-drawn glass needles or ultrafine tungsten needles (Roboz, 0.125mm diameter), coated with FBS (Gibco). Extraembryonic tissue was trimmed from the embryonic disc, and strips of epiblast/hypoblast tissue roughly ¼ - ⅓ the height of the embryo proper were dissected. Explants were embedded in collagen drops placed in 4-well plates (Thermo Scientific, 144444), and overlain with 500μl of culture media (DMEM/F12 supplemented with 200mM L-Glutamine, N2, 1% BSA and 100 U/mL Penicillin-Streptomycin). For FGF inhibition, SU5402 (Calbiochem) resuspended in DMSO was added to the culture media at a final concentration of 10 μM. Explants were cultured for 21–24h or 41–48h at 37°C with 5% CO2. Additional stage 3+ explants were immediately fixed without culture and designated as time zero (T_0). Antibody staining for collagen drops and explant imaging was performed as for immunohistochemistry described above. To serve as a positive control for endogenous expression of NC markers, sections derived from in vitro cultured whole rabbit embryos were immunostained. In brief, whole rabbit embryos between 1 and 3 somite stage were cultured using the “ring method” as previously described (Bitzer, 2008; Püschel et al., 2010a) at 37°C with 5% CO2 for 19–22. Following culture, embryos were cryosectioned and processed for immunofluorescence as described above. Successful cultures were marked as those embryos demonstrating normal morphology along the entire rostral-caudal axis. Explants images were acquired and processed under identical conditions, and compared to rabbit sections representing endogenous signal.

Results

Analysis of NC markers during early rabbit development

To investigate early mammalian NC development, we have made use of the rabbit, which, at ~600μm, offers a smaller embryo than the chick, yet considerably larger than the rodent embryo (Supplemental Figure S1). Additionally, the early rabbit embryo develops as a flat blastodisc, similar to Aves and humans. This is in clear contrast to the cup-shaped rodent gastrula, which is unique amongst mammals. These features make the rabbit embryo a particularly attractive model to advance our understanding of early mammalian development (Bitzer, 2008; Feistel and Blum, 2008; Fischer et al., 2002; Knoetgen et al., 1999; Piliszek et al., 2017; Viebahn et al., 2002; Plöger and Viebahn, 2018).

To characterize the development of the rabbit NC at the molecular level, we focused on molecules that report early NPB, NC-specifier, and NC migration statuses. AP2⟨, Msx½, Pax3, and Pax7 TFs are associated with the NPB status in other model organisms. We also interrogate the expression of Sox9, associated with early NC specification, and Sox10, which appears later in the NC-specifier state, and is also associated with an early NC migratory state. Finally, to better explore NC migration in rabbit embryos, we analyzed the markers P75NTR and HNK-1.

Interrogation of early NC markers in gastrula stage rabbit embryos

The expression of NC markers in classic model organisms (zebrafish, Xenopus, chick, and mouse) has been identified from early neurula stages onwards, but recent studies suggest that NC cells are specified in the gastrula prior to the overt expression of NC markers. In chick, specified NC cells have been identified in a territory in the equator of the embryo between the primitive streak and the edge of the embryo (area pellucida/area opaca border), referred to as the prospective NC territory (Basch et al., 2006). We therefore interrogated the very early expression of NC markers in gastrula stage rabbit embryos (Figure 1A–F; Supplemental Figure S2A–F). Similar to chick profiles, no definitive expression of Pax7, Pax3, AP2⟨, or Msx½ was observed in the equator of the embryo proper of the rabbit gastrula through stage 4+. Therefore, we conclude that no signal is found in the regions associated with prospective NC. However, we note that AP2⟨ expression was observed in the extraembryonic tissue (Figure 1D and F; Supplemental Figure S2F), and Msx½ expression was found in the posterior epiblast as well as the extraembryonic tissue (Figure 1F; Supplemental Figure S2F). Similar to previous reports in the rabbit, we observed expression of the mesodermal transcription factor T/Brachyury in the posterior epiblast and ingressing mesoderm at stage 3 (Figure 1A–C; Supplemental Figure S2A–E), with further expression seen in the node at stage 4 (Supplemental Figure S2E) (Viebahn et al., 2002). Finally, consistent with germ layer specification in the chick, in gastrula stage embryos, we also observed posterior epiblast expression of the mesodermal T-box transcription factor Tbx6 (Figure 1D) (Knezevic et al., 1997), and anterior expression of the neural markers Sox2/3 (Figure 1E) (Rex et al., 1997).

NC development in early neurula rabbit embryos

We next examined early neurula stage rabbit embryos for expression of NPB markers. Anterior sections from stage 5–6 embryos reveal the earliest NPB/presumptive NC territory expression of Pax7 (Figure 1G–I), Msx½ (Figure 1L, M; Supplemental Figure S2G), and AP2α (Figure 1N, O). We note that, while definitive Pax7 expression is detected in st. 5+ embryos (Figure 1H, I), Pax3 signal is not detected at this stage (Figure 1J, K). However, in sections taken from a slightly older embryo, we observe a small number of Pax3-positive cells in the neural plate border territory, with more robust expression in the underlying mesoderm (Figure 1L, M; Supplemental Figure S2G). Further, at both gastrula and early neurula stages, Sox2 expression was observed in the neuroectoderm (Figure 1E, H, I) in a pattern similar to that described for Sox2 and Sox3 in similarly-staged chick embryos (Rex et al., 1997). We also observed some overlap between Pax7 and Sox2 at the NPB of early neurula embryos (Figure 2H, I). In addition to its expression at the NPB, AP2α expression is also found in the non-neural ectoderm (Figure 1N, O). Taken together, analysis of early NC

makers reveals the earliest detectable expression of Pax7, Msx½, AP2α, and limited Pax3, in the NPB of early neurula stage 5–6 rabbit embryos.

Molecular markers associated with pre-migratory and migratory NC in neurula stage rabbit embryos

NC cells at the neural folds undergo an epithelial-to-mesenchymal transition and migrate away from the closing neural tube. Molecular markers of both pre-migratory and migratory NC have been extensively studied in model systems including chick, frog, fish, and mouse (Betancur et al., 2010; Sauka-Spengler and Bronner-Fraser, 2008; Simoes-Costa and Bronner, 2015; Stuhlmiller and García-Castro, 2012a). To assess pre-migratory and migratory stages of NC development in the rabbit embryo, we characterized the expression of multiple NC markers in rabbit embryos between the 6–7 somite stage (ss) and 20–21ss. At the edge of the anterior open neural plate of 6–7ss embryos (Figure 2A), cells in the neural folds display expression of AP2α, Sox9, Pax7, Pax3, and Msx½ (Figure 2B–E; Supplemental Figure S3A, B). A small number of Sox10-positive cells are also observed in the neural folds (Figure 2E). Expression of Sox9, AP2α, Pax7, Pax3, and Sox10 can also be seen in freshly emigrating NC. A few Msx½-positive migratory NC cells were also observed, with a small number of cells co-expressing Sox10 and Msx½ (Figure 2E; Supplemental Figure S3B). The expression of AP2α at this stage also marks the lateral non-neural ectoderm (Figure 2B; Supplemental Figure S3A), while Sox9 can be seen in axial mesoderm (Figure 2B; Supplemental Figure S3A). In slightly older 8–9ss embryos, NC cells marked by Sox9, Pax3, and/or Pax7 can be seen emerging from the level of the midbrain (Figure 2F, G; Supplemental Figure S3C), with strong Pax7-positive cells additionally populating the rostral-most cranial mesenchyme (Figure 2F, G; Supplemental Figure S3C). In the 8–9ss embryos examined, Pax7, Msx½, and AP2α expression was also maintained in pre-migratory NC cells within the neural folds along the rostral-caudal axis (Figure 2F; Supplemental Figure S3C; data not shown).

At later developmental stages, Sox9-positive cranial NC cells can be seen migrating in streams toward the branchial arches and frontonasal process in 15ss embryos, while Pax7 expression was observed primarily in NC cells populating the rostral cranial mesenchyme (Figure 2H; Supplemental Figure S3D). By the 20–21ss (Figure 2I), AP2α, Sox9, and Sox10 expression was observed in the NC-populated maxillary process (Figure 2J, L). We also observed Pax3, p75NTR, and Sox10 staining in the NC-derived cranial ganglia (Figure 2K, L), and Pax3-positive cells are noted in anterior regions of the cranial mesenchyme (Figure 2K inset). AP2α expression was detected in the anterior non-neural ectoderm (Figure 2J). Interestingly, Sox9 was detected throughout the otic vesicle (with stronger dorsal expression), and AP2α signal appeared in a graded fashion with higher ventral expression (Figure 2J). Caudally, Pax3, AP2α, Sox9, and Sox10 is maintained in 20–21ss trunk migratory NC cells (Figure 2M–O; Supplemental Figure S4). Further, we observe p75NTR expression in migratory NC cells (Figure 2M; Supplemental Figure S4). Robust Pax3 and Sox9 expression, and to a lesser extent Sox10, was also observed in pre-migratory NC in the dorsal aspect of the neural tube underlying the non-neural ectoderm. Definitive Pax7 and Msx½ expression was not observed in pre-migratory nor migratory NC cells in trunk sections (Figure 2N; Supplemental Figure S4E). Outside of NC cells, Pax3 and Pax7 was

observed in the dermomyotome (Figure 2M; Supplemental Figure S4B, E), AP2α was observed in the epidermis (Figure 2O; Supplemental Figure S4D), and Sox9 was observed in the paraxial mesoderm (Figure 2O; Supplemental Figure S4D).

Throughout these experiments we tested the immunogenicity of the HNK-1 antibody in embryos from 6–7 ss where we first identified migratory NC to the later stages 20 to 21 ss. While migratory NC were detected with multiple other markers, no HNK-1 positive NC cells were detected in the rabbit, while HNK1 revealed chick migratory NC as expected (data not shown).

Comparative analysis of cranial NC markers in chick and rabbit embryos

The chick has been utilized extensively as a model for amniote NC specification and development. We therefore directly compared the expression of known NC markers in 10ss rabbit and chick (HH10) cranial NC cells. In both rabbit and chick, Sox9, Sox10, and AP2α robustly mark migratory NC cells populating the cranial mesenchyme, with Sox9 and AP2α further found in pre-migratory NC cells (Figure 3A, D, E, H; Supplemental Figure S3F). Outside of the NC cells, AP2α also marks the non-neural ectoderm (Figure 3D, H). We next compared Pax7 and Pax3 expression in rabbit and chick NC cells. In the chick, strong Pax7 expression is observed in both pre-migratory and migratory cranial NC (Figure 3F). Pax3 expression is detected in both, albeit with reduced signal (Figure 3G). In the rabbit, we find Pax7 strongly expressed in pre-migratory NC cells located in the dorsal aspect of the fused neural tube with, in these sections, a smaller number of Pax7-positive migratory NC cells (Figure 3B; Supplemental Figure S3E). However, we note that Pax7 expression is detected in migratory NC cells emerging from the midbrain in a slightly younger whole embryo (Figure 2F, G). Further, at the 10ss, Pax3 expression is observed in both pre-migratory and migratory NC (Figure 3C; Supplemental Figure S3E). At this stage in the rabbit, definitive Msx½ expression is not detected in a more rostral section (Supplemental Figure S3F), while in chick is confined to pre-migratory NC and the non-neural ectoderm, with very few co-labeled Sox10 and Msx½ cells (Supplemental Figure S3G). Taken together, we observe broad conservation between rabbit and chick in the expression of markers of cranial NC cells, with some differences in the expression of Pax3 and Pax7.

Rabbit neural crest cells are specified in the gastrula embryo

Studies in chick embryos indicate that NC cells are specified prior to the appearance of the NPB, and before the detectable expression of NC marker genes. Specifically, a prospective NC domain has been identified in gastrula epiblast tissue. This region does not display definitive neural crest, neural, or mesodermal markers, but following culture, clearly displays neural crest, but not mesodermal or neural, markers (Basch et al., 2006; Patthey et al., 2009). Here, we took advantage of the flat and large rabbit embryo to assess if this early, gastrula-stage NC specification occurs in mammals. To this end, we generated explants from an equatorial strip of tissue from rabbit gastrula embryos (approximately stage 3+), including lateral explants adjoining the area opaca; medial/primitive streak explants either directly above or including the anterior tip of the primitive streak; and intermediate explants between the primitive streak and lateral territories (Figure 4A). Explants were transferred into collagen gels and cultured in isolation under non-inductive conditions. Explants were

fixed uncultured (T_0) or after 24 or 45 hours of culture, and evaluated for NC (Pax7 and Sox10) and mesoderm (Brachyury/T) marker expression. For our expression analysis, we used sections of rabbit embryos displaying endogenous marker expression as comparative controls (Figure 4J–L).

Uncultured explants (T_0) corresponding to lateral and intermediate regions do not reveal Brachyury expression (Figure 4B). Further, Brachyury signal did not appear in intermediate or lateral explants after 24h or 45h culture, but instead, medial explants (primitive streak) cultured for 24h or 45h display stronger Brachyury expression (Figure 4C–F; Supplemental Figure S5A). Consistent with our whole mount data, stage 3+ T_0 uncultured explants do not express Pax7 or Sox10, regardless of the region of the embryo from which explants are derived (Figure 4B; Figure 1; Supplemental Figure S2).

We next analyzed NC marker expression in explants cultured for 24h (n=4 embryos) and 45h (n=5 embryos). After 24h culture, Pax7 and Sox10 signal was not observed in explants adjacent and/or lateral to the primitive streak (Supplemental Figure S5A). Instead, following 45h of culture, we observed robust Pax7 and Sox10 expression in the intermediate explants (Figure 4C–F). These intermediate explants exhibited a heterogeneous population of Pax7, Sox10 and Pax7/Sox10 positive cells. Further, re-staining of the intermediate explant presented in Figure 4F revealed robust Sox9 expression, which significantly co-localized with Pax7 and Sox10 (Figure 4I; Supplemental Figure S5B). Additionally, weak Sox10 signal was often detected in lateral explants, although at much lower levels than those seen in intermediate explants. Taken together, these results suggest the presence of a NC-specified region in the rabbit gastrula embryo. This region does not initially express NC markers (Figure 4B; Figure 1A–C; Supplemental Figure S1A–E), but robustly does so following culture in the absence of exogenous “inductive” signals. Furthermore, medial and lateral regions of the gastrula embryo fail to express these NC markers when cultured under identical conditions. Finally, given the restricted distribution of strong Brachyury signal to the medial/primitive streak derived explants, these results suggest that early NC specification in the rabbit is mesoderm independent.

FGF signaling is required for NC formation in rabbit gastrula explants

We have previously demonstrated a requirement for FGF signaling in early Avian NC formation (Stuhlmiller and García-Castro, 2012b), and therefore we asked whether this signaling pathway is also required during rabbit NC formation. To this end, we used the FGF inhibitor SU5402, which blocks FGF receptors (Mohammadi et al., 1997), and which we have previously used to disrupt NC development in the chick (Stuhlmiller and García-Castro, 2012b). As above, strips of tissue were removed from approximately stage 3+ rabbit embryos (n=5 embryos), and explants were generated covering the lateral, intermediate, or medial/primitive streak regions. Explants were cultured in isolation in collagen gels for 45h with or without SU5402, fixed, and analyzed for Pax7 and Sox10 expression. Intermediate explants, which normally display robust Pax7/Sox10 expression, displayed a dramatic reduction in signal for these markers in 4 out of 5 stripes tested (Figure 4G). These results suggest that FGF signaling is necessary for the expression of Pax7 and Sox10 in the rabbit

presumptive NC territory, and highlights the role this pathway plays in the induction of rabbit NC cells.

Discussion

In this study, we explored rabbit NC formation and characterized for the first time the expression of multiple NC markers from gastrula to neurula stages of development (stage 2 through 20–21ss) (Figure 5). Taking advantage of the large size and flat structure of the rabbit gastrula, we performed explant assays to provide evidence that a specified population of NC cells exists in the rabbit gastrula, and further reveal that the FGF signaling pathway is required for the activation of NC markers in these explants. This is, to our knowledge, the first demonstration of early NC specification in a mammalian gastrula embryo.

The rabbit as a model for mammalian NC development

Murine models have provided powerful tools for the study of mammalian development, and the mouse currently serves as the sole embryonic model for mammalian NC formation. However, a void of information exists regarding early events in embryonic mammalian NC development. We and others have tried to replicate classic embryonic experiments to advance our understanding of NC formation using early mouse embryos (E6.5), before the establishment of the neural ectoderm, neural folds, and possible expression of NC markers. However, the small size (150μm) of the mouse embryo has represented a major challenge not currently surpassed. Despite the widespread use of explant assays in the chick (Basch et al., 2006; Patthey et al., 2009; Patthey et al., 2008; Wilson et al., 2001; Wilson et al., 2000), to the best of our knowledge, no study has successfully utilized this technique to identify a specified population of NC within the mouse gastrula. Murine genetic tools have led to considerable progress in studies of later NC development; for example, genetic tracing of the NC lineage, driven by NC cell-specific promoters (e.g. Sox10, Wnt1), have provided invaluable information regarding the contribution of mammalian NC cells to adipocytes, melanocytes, the outflow tract of the heart, and craniofacial structures, among others (Chai et al., 2000; Li et al., 2000; Matsuoka et al., 2005; Billon et al., 2007; reviewed in Debbache et al., 2018). However, no study to-date has addressed or revealed specific contributions to earlier events underlying the initial acquisition of NC markers in murine models.

Here we suggest that the rabbit is an excellent alternative for embryonic mammalian early NC studies. Used in classic embryology studies (Waddington and Waterman, 1933), the large size, flat blastodisc, and standard mammalian germ layer arrangement of the rabbit embryo provides powerful traits as an experimental model organism for early development. While the rabbit blastula and gastrula (~600μm) are approximately one-third the size of the chick (~2.5 mm), they are considerably larger than the mouse (150μm, and 300μm when flattened, at E6.0–6.5) (Figure S1). This large embryonic size provides enough spatial resolution to address important questions in early mammalian development (Behringer et al., 2013; Idkowiak et al., 2004; Tam and Gad, 2004; Viebahn, 2004; Viebahn et al., 2002). Other studies have already capitalized on the large size of the rabbit embryo to perform classic “cut-and-paste” experiments. Grafts from the rabbit node, primitive streak and visceral endoderm have all been reported, and these tissues have been successfully

introduced into chick gastrulae (Knoetgen et al., 1999; Knoetgen et al., 2000). In this report, we have made use of these traits to demonstrate, for the first time, gastrula-stage specification of mammalian NC cells.

Furthermore, an area of preeminent discrepancy in our current perception of NC development emanates from differences regarding the proposed requirement for key NC genes and signaling molecules which were established by studies in non-mammalian model systems, but that have been contested by studies in the mouse (Barriga et al., 2015; Stuhlmiller and García-Castro, 2012a). For example, the critical role of BMP and Snail genes in chick and Xenopus embryos appear dispensable for early NC induction in the mouse (Correia et al., 2007; Murray and Gridley, 2006; Stottmann and Klingensmith, 2011). Current appreciation of variation has been further boosted by recent findings not related to NC, in which prominent variation in early cell-lineage decisions between mammalian species where identified (Berg et al., 2011; Rossant, 2011). Importantly, the growth in the use of human embryonic stem cell-based models of human NC to interrogate their formation and differentiation potential (Chambers et al., 2016; Fukuta et al., 2014; Hackland et al., 2017; Leung et al., 2016; Menendez et al., 2011; Mica et al., 2013; Umeda et al., 2015) has empowered hopes for translational efforts (Betters et al., 2014). However, the pursuit of a comprehensive understanding of in vivo mammalian NC formation using alternative models is critical to inform a broader perspective of mammalian NC conservation and divergence – which has deep health implications for human embryo development.

The rabbit has been widely used in biomedical research as a model for human disease (Shiomi, 2009), and a number of qualities place the rabbit close to human biology. These include the kinetics of embryonic gene activation (e Silva et al., 2012; Shi et al., 2004), early embryo topography (Viebahn et al., 1995; Waddington and Waterman, 1933), placental development (Carter, 2007; Enders and Carter, 2004; Samuel et al., 1975), and physiological properties including lipid metabolism, autoimmunity, heart development, and pathology (Bosze and Houdebine, 2006; Püschel et al., 2010b; Rogel-Gaillard et al., 2009). The case of thalidomide is an excellent example where rodents failed to reveal the dramatic teratogenic effect seen in humans and rabbits (McBride, 1977; Pearn and Vickers, 1966; Schumacher et al., 1968). Taken together, these characteristics support the use of the rabbit as a powerful complementary model of mammalian development towards human comparative studies, and the work presented here represents a crucial step in addressing early embryonic mammalian NC development.

Molecular markers of early rabbit NC development

We assessed NC marker expression in early gastrula rabbit embryos, which reveal no specific signal for NC markers (Pax3, Pax7, Sox9, Sox10, Msx½ and AP2α) associated with future NC territories up through stage 4+ (Figure 1A–F; Supplemental Figure S2A–E; and data not shown). Previously, a histological analysis of rabbit NC development, devoid of molecular markers, had failed to identify NC in rabbit embryos younger than 10–12ss (Berke, 1965). Here we provide a thorough characterization of NC marker expression at neurula stage 5–6, and report the expression of Pax7, AP2α, Msx½, and limited Pax3 in the NPB/presumptive NC territory (Figure 1G–O). These expression patterns are similar to

those we observed in the chick (data not shown), and to what has been previously reported in the literature for Avian HH5–6 and mouse E7.5 embryos (Basch et al., 2006; Khudyakov and Bronner-Fraser, 2009; Shen et al., 1997). Interestingly, in stage 5+ embryos, we observed Pax7 expression at the NPB, but no overt Pax3 expression (Figure 1G–K). In slightly older stage 6 embryos we observed only a small number of Pax3-positive cells in the rabbit NPB (n=3 embryos) (Figure 1L, M; Supplemental Figure S2G), in contrast to reports of robust Pax3 expression in Aves and mouse (Bang et al., 1997; Basch et al., 2006). These findings suggest that Pax7 expression in the rabbit proceeds that of Pax3. However, Pax3 is robustly expressed in the pre-migratory and migratory NC cells of older rabbit embryos, and is additionally found in NC derivatives such as the cranial ganglia (Figure 2; Figure 3; Supplemental Figures S3 and S4). Future expression analyses, paired with functional assays, will help to clarify the precise roles of Pax3 and Pax7 in the formation of the rabbit NC.

As rabbit embryogenesis proceeds, it has been reported that the neural folds begin to fuse at the 8–9ss, with the completion of primary neurulation occurring at the 22ss (Peeters et al., 1998). Consistent with this, at early somite stages, we typically observe well-established but unfused neural folds. Pax7, Pax3, AP2α, Msx½, and Sox9 are all expressed in cranial pre-migratory NC cells at the tips of the unfused neural folds (Figure 2B–E). A small number of Sox10-positive pre-migratory NC were also observed, but our observations indicate that Sox10 more robustly marks rabbit migratory NC. In agreement with reports that NC migration begins prior to the fusion of the neural folds in both the mouse and human (Nichols, 1981; O'Rahilly and Müller, 2007), in the rabbit we observe migratory NC cells adjacent to the unfused neural folds at the 6–7ss (Figure 2B–E). These freshly migrating NC cells are marked by Pax7, Pax3, AP2α, Sox9, and Sox10, with a few cells weakly positive for Msx½. Importantly, the 6–7ss also marks the first point during our studies where we observe definitive Pax3, Sox9, and Sox10 expression in presumptive rabbit NC cells. This is consistent with reports that Sox9 and Sox10 first appear in Avian NC at HH9–10 (McKeown et al., 2005; Suzuki et al., 2006); however, Pax3 is involved in earlier stages of NC development in both the mouse and chick (Bang et al., 1997; Basch et al., 2006). As the neural folds begin to close at the 8–9ss, Pax3, Pax7, and/or Sox9 positive cranial migratory NC cells are visible adjacent to the midbrain neuroepithelium (Figure 2F–G). We also observe migratory NC cells emerging from the posterior region of the forebrain (Figure 2F, G; Supplemental Figure S3F), as has been reported in other model organisms and human embryos (Couly and Le Douarin, 1987; O'Rahilly and Müller, 2007; Serbedzija et al., 1992). More caudally at these early somite stages, the expression of Pax3, Pax7, Msx½, and AP-2α is maintained in the pre-migratory NC located in the dorsal tips of the unfused neural folds (data not shown).

In the 10ss rabbit embryo, as the anterior neural folds fuse, migratory mesenchymal NC as well as premigratory NC display expression of Pax3, Pax7, Sox9, and AP2α, with Sox10 marking migratory NC in more rostral posterior forebrain sections (Figure 3; Supplemental Figure S3F). Interestingly, the 10ss is the first stage when p75NTR – a marker that has been used to identify NC cells in chick, mouse, and human embryos (Anderson et al., 2006; Lee et al., 2007; Morrison et al., 1999; Wilson et al., 2004) – appears in the rabbit NC, although broad expression is seen throughout the ectoderm and ingressing mesoderm of caudal sections at this and earlier stages (stage 6 and 6–7ss, data not shown). At the 10ss, p75NTR

predominantly labels cranial migratory NC cells, rather than the pre-migratory NC (data not shown). We also note that in our analysis, the HNK-1 antibody failed to recognize rabbit NC cells (data not shown), in contrast to the chick (Bronner-Fraser, 1986; Tucker et al., 1984). These results are consistent with reports that HNK-1 is largely non-specific for both murine and human NC cells in vivo (Betters et al., 2010; Tucker et al., 1984; Tucker et al., 1988).

Following the closure of the rostral neuropore, by the 15ss cranial NC cells have migrated into the frontonasal process, with Pax7 expressed more strongly in the rostral-most cranial mesenchyme compared to Sox9 (Figure 2H). This is consistent with our observation of Pax7-positive cells in a similar territory at the 8–9ss (Figure 2F, G). More caudally at the 15ss, Sox9 labels migratory NC cells traveling in streams towards the branchial arches (Figure 2H). This pattern of migration has been well-characterized in both the chick and the mouse, and has been described in human embryos (Lumsden et al., 1991; O'Rahilly and Müller, 2007; Serbedzija et al., 1992). Taken together, we provide a molecular characterization of rabbit NC development from gastrula to early neural stages, examining a battery of markers frequently used in other model organisms (Figure 5). Our results suggest that AP2α and Msx½ appear early in the prospective border, but that their expression is extended laterally into the non-neural ectoderm. Instead, the expression of Pax7 appears to be the first marker with expression restricted to the prospective neural crest region of the ectoderm, between the neural and non-neural ectoderm. As the border matures, restricted expression of Pax3, Sox9, and Sox10 are displayed in anterior territories. Concurrently, the early marker Msx½ becomes more restricted to the NPB, while AP2α expression remains in the lateral non-neural ectoderm. Soon after, emigration of NC cells is apparent - well before the neural folds meet at the midline to close the neural tube. Finally, we also note that P75NTR, but not HNK-1, label rabbit migratory NC at these stages.

Rabbit NC cells are specified as early as gastrula stage 3+ and requires FGF signaling

Cell fate decisions involve a progressive series of steps, with the initial event commonly known as specification. This state represents a point in which a cell has started on the path towards a particular lineage. Specified cells may not yet express markers associated with later stages of differentiation, but would acquire these markers if development is allowed to continue in the absence of disruptive signals. Specified cells may express specific markers for that early stage. At this moment, we do not know of early specification markers of neural crest cells prior to the early neurula stage when Pax7 becomes restricted to prospective NC. It is therefore likely that specification relies on epigenetic marks that modulate expression profiles, differentiating the specified cells from their precursors and alternative fates, and promoting the future expression of genes associated with the specified fate. These suggested epigenetic marks of the early specification of NC also remain to be elucidated, and are the focus of ongoing efforts. At later stages, following activation of the gene network, the specified cell may become committed towards that fate (neural crest) and be unable to adopt alternative programs. In the case of the multipotent neural crest, pre- and migratory NC retain plasticity towards multiple NC-terminal derivatives on one hand, and published evidence suggest that they can also adopt apparent CNS character including ventral neural tube (Ruffins et al., 1998). However, the earlier specification state does not attest to cell fate commitment, but instead reflects the initiation of the progression towards that fate.

A specified population of NC has been demonstrated in both Avian and Xenopus embryos, either during gastrula stages in the chick, or coincident with the completion of gastrulation in frogs (Basch et al., 2006; Mancilla and Mayor, 1996; Patthey et al., 2009; 2008). In the flat disc of the chick embryo, this NC-specified region is located between the center and lateral edge of the embryonic disc (Basch et al., 2006; Patthey et al., 2009; Patthey et al., 2008). More lateral territories are specified as presumptive epidermis, whereas medial explants yield neural tissue (Patthey et al., 2009; Wilson et al., 2001; Wilson et al., 2000). Despite this progress in Xenopus and Avian embryos, to our knowledge no study has successfully evaluated early NC specification in an embryonic mammalian system. Importantly, our human model of NC development based on embryonic stem cells suggests that human NC formation is independent of neural and mesodermal tissues (Leung et al., 2016), lending support to the model of the early specification of mammalian NC.

In this report, we have successfully applied embryonic microdissections and explant specification assays to the flat and relatively large rabbit embryo. Our results demonstrate that tissue directly adjacent to the primitive streak – designated as the “intermediate” territory – does not display NC-marker expression at stage 3+ in the rabbit (Figure 1; Figure 4B). However, after 45 hours of culture in isolation under non-inducing conditions, intermediate explants robustly express NC markers Pax7, Sox9, and Sox10 (Figure 4C–F). These results strongly suggest that, in the mammalian rabbit embryo, the intermediate gastrula is specified to form NC. This presumptive rabbit NC territory is similar, but slightly proximal, to the NC-specified region observed in the chick (Basch et al., 2006; Patthey et al., 2008). Importantly, the lack of Brachyury expression in these intermediate explants, compared to medial/primitive streak explants, suggest that rabbit NC specification occurs independent of mesoderm.

Here, we refer to the early specification events underlying NC development, and thus, “specified NC” cells are those that have initiated the NC gene program and will continue a progressive development towards the NC fate, via the acquisition of NC marker expression, if permissive conditions persist. Nevertheless, it is important to reiterate that these assays do not attest to cellular commitment to the NC fate, and it remains possible that these cells could be redirected towards other fates upon alternative instructive signals. Further, we note that these experiments clearly identify a regionally segregated cell population specified towards the NC fate, and expose the heterogeneity at the explant level. Simultaneously, the expression of NC markers is not homogeneous within the specified explants, and thus heterogeneity within the explant could have led to inductive interactions during culture. Our current experiments cannot establish if the NC cells were induced during or before culture. It will be critical for future experiments to further address the temporal specification and possible alternatives to the classic model of induction.

Finally, we observed the loss of definitive Pax7 and Sox10 expression in intermediate explants following the inhibition of FGF signaling (Figure 4G). FGF signaling has been associated with NC development in Xenopus and Avian embryos (Hong et al., 2008; Monsoro-Burq et al., 2003; Stuhlmiller and García-Castro, 2012b), and mice carrying mutations in FGF8 demonstrate NC defects at later developmental time points (Abu-Issa et al., 2002; Frank et al., 2002). However, the role of FGF signaling in early mammalian NC

specification or induction is poorly understood. In Xenopus it had been proposed that FGFs activate WNTs in the mesoderm, and thus participate indirectly in NC formation; instead in chick, FGF was shown to be required in the ectoderm during early NC formation (Stuhlmiller and García-Castro, 2012b). Our findings suggest that rabbit NC formation, similar to chick, is dependent upon FGF signaling in prospective NC territories.

Conclusions

In this report, we have established the rabbit as a model for mammalian NC development. We analyzed the expression of 8 markers of NC cells frequently reported in other model organisms, detailing for the first time their association with rabbit NC development from early gastrula to mid-neurula stages, and provide comparative insights toward other model organisms. Furthermore, we provide evidence that rabbit intermediate explants (adjacent to the primitive streak) from gastrula embryos are indeed specified to launch NC development. These explants do not express NC markers after dissection; however, after 45h of culture under non-inducing conditions, they display robust expression of NC markers Pax7, Sox9, and Sox10. Using this specification assay, we also demonstrate a role for FGF signaling in early rabbit NC formation. To our knowledge, this is the first demonstration of early NC specification in a mammalian system, and the role of FGF signaling in mammalian early NC formation. Collectively, this work provides an exciting basis for future research evaluating rabbit NC formation, which together with the completion of the rabbit genome (Carneiro et al., 2014) and the ability to perform functional analyses via bead implantation (Fischer et al., 2002) or electroporation, will enable the characterization of the cellular and molecular events governing the early formation of mammalian NC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Abu-Issa R, Smyth G, Smoak I, Yamamura K-I, Meyers EN, 2002 Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. Development 129, 4613–4625. [PubMed: 12223417]

Anderson RB, Stewart AL, Young HM, 2006 Phenotypes of neural-crest-derived cells in vagal and sacral pathways. Cell Tissue Res. 323, 11–25. doi:10.1007/s00441-005-0047-6 [PubMed: 16133146]

Bang AG, Papalopulu N, Kintner C, Goulding MD, 1997 Expression of Pax-3 is initiated in the early neural plate by posteriorizing signals produced by the organizer and by posterior non-axial mesoderm. Development 124, 2075–2085. [PubMed: 9169853]

Barriga EH, Trainor PA, Bronner M, Mayor R, 2015 Animal models for studying neural crest development: is the mouse different? Development 142, 1555–1560. doi:10.1242/dev.121590 [PubMed: 25922521]

Basch ML, Bronner-Fraser M, García-Castro MI, 2006 Specification of the neural crest occurs during gastrulation and requires Pax7. Nature 441, 218–222. doi:10.1038/nature04684 [PubMed: 16688176]

Behringer R, Gertsenstein M, Nagy KV, Nagy A, 2013 Manipulating the Mouse Embryo.

Berg DK, Smith CS, Pearton DJ, Wells DN, Broadhurst R, Donnison M, Pfeffer PL, 2011 Trophectoderm Lineage Determination in Cattle. Dev. Cell 20, 244–255. doi:10.1016/j.devcel.2011.01.003 [PubMed: 21316591]

Berke JD, 1965 The development of the rostral neuraxis and neural crest in the rabbit embryo up to 16 somites. Bol Inst Estud Med Biol Univ Nac Auton Mex 23, 185–212. [PubMed: 5297348]

Betancur P, Bronner-Fraser M, Sauka-Spengler T, 2010 Assembling Neural Crest Regulatory Circuits into a Gene Regulatory Network. Annu. Rev. Cell Dev. Biol. 26, 581–603. doi:10.1146/annurev.cellbio.042308.113245 [PubMed: 19575671]

Betters E, Liu Y, Kjaeldgaard A, Sundström E, García-Castro MI, 2010 Analysis of early human neural crest development. Developmental Biology 344, 578–592. doi:10.1016/j.ydbio.2010.05.012 [PubMed: 20478300]

Betters E, Murdoch B, Leung AW, García-Castro MI, 2014 Human Neural Crest Cells and Stem Cell-Based Models, in: Neural Crest Cells. Elsevier, pp. 395–412. doi:10.1016/B978-0-12-401730-6.00019-3

Billon N, Iannarelli P, Monteiro MC, Glavieux-Pardanaud C, Richardson WD, Kessaris N, Dani C, Dupin E, 2007 The generation of adipocytes by the neural crest. Development 134, 2283–2292. doi:10.1242/dev.002642 [PubMed: 17507398]

Bitzer ES, 2008 Establishment of a new in vitro culture system and functional analysis of Sonic Hedgehog and FGF8 in the determination of laterality in the rabbit embryo.

Bolande RP, 1997 Neurocristopathy: its growth and development in 20 years. Pediatr Pathol Lab Med 17, 1–25. [PubMed: 9050057]

Bondurand N, Kobetz A, Pingault V, Lemort N, Encha-Razavi F, Couly G, Goerich DE, Wegner M, Abitbol M, Goossens M, 1998 Expression of the SOX10 gene during human development. FEBS Lett 432, 168–172. doi:10.1016/S0014-5793(98)00843-6 [PubMed: 9720918]

Bosze Z, Houdebine LM, 2006 Application of rabbits in biomedical research: A review. World Rabbit Science 14, 1–14.

Bronner-Fraser M, 1986 Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. Developmental Biology 115, 44–55. [PubMed: 3516760]

Carneiro M, Rubin C-J, Di Palma F, Albert FW, Alföldi J, Martinez Barrio A, Pielberg G, Rafati N, Sayyab S, Turner-Maier J, Younis S, Afonso S, Aken B, Alves JM, Barrell D, Bolet G, Boucher S, Burbano HA, Campos R, Chang JL, Duranthon V, Fontanesi L, Garreau H, Heiman D, Johnson J, Mage RG, Peng Z, Queney G, Rogel-Gaillard C, Ruffier M, Searle S, Villafuerte R, Xiong A, Young S, Forsberg-Nilsson K, Good JM, Lander ES, Ferrand N, Lindblad-Toh K, Andersson L, 2014 Rabbit genome analysis reveals a polygenic basis for phenotypic change during domestication. 345, 1074–1079. doi:10.1126/science.1253714

Carter AM, 2007 Animal Models of Human Placentation - A Review. Placenta 28, S41–7. doi:10.1016/j.placenta.2006.11.002 [PubMed: 17196252]

Chai Y, Jiang X, Ito Y, Bringas P, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM, 2000 Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 127, 1671–1679. [PubMed: 10725243]

Chambers SM, Mica Y, Lee G, Studer L, Tomishima MJ, 2016 Dual-SMAD Inhibition/WNT Activation-Based Methods to Induce Neural Crest and Derivatives from Human Pluripotent Stem Cells. Methods Mol. Biol. 1307, 329–343. doi:10.1007/7651_2013_59 [PubMed: 24301074]

Correia AC, Costa M, Moraes F, Bom J, Nóvoa A, Mallo M, 2007 Bmp2 is required for migration but not for induction of neural crest cells in the mouse. Dev. Dyn. 236, 2493–2501. doi:10.1002/dvdy.21256 [PubMed: 17676634]

Couly GF, Le Douarin NM, 1987 Mapping of the early neural primordium in quail-chick chimeras. II. The prosencephalic neural plate and neural folds: implications for the genesis of cephalic human congenital abnormalities. Developmental Biology 120, 198–214. [PubMed: 3817289]

Debbache J, Parfejevs V, Sommer L, 2018 Cre-driver lines used for genetic fate mapping of neural crest cells in the mouse: An overview. Genesis e23105. doi:10.1002/dvg.23105 [PubMed: 29673028]

e Silva ARR, Bruno C, Fleurot R, Daniel N, Archilla C, Peynot N, Lucci CM, Beaujean N, Duranthon V, 2012 Alteration of DNA demethylation dynamics by in vitro culture conditions in rabbit pre-implantation embryos. Epigenetics 7, 440–446. doi:10.4161/epi.19563 [PubMed: 22419129]

Enders AC, Carter AM, 2004 What can comparative studies of placental structure tell us? - A review. Placenta 25, S3–9. doi:10.1016/j.placenta.2004.01.011 [PubMed: 15033300]

Etchevers HC, Amiel J, Lyonnet S, 2006 Molecular bases of human neurocristopathies. Adv. Exp. Med. Biol. 589, 213–234. doi:10.1007/978-0-387-46954-6_14 [PubMed: 17076285]

Farlie PG, McKeown SJ, Newgreen DF, 2004 The neural crest: basic biology and clinical relationships in the craniofacial and enteric nervous systems. Birth Defects Res. C Embryo Today 72, 173–189. doi:10.1002/bdrc.20013 [PubMed: 15269891]

Feistel K, Blum M, 2008 Gap junctions relay FGF8-mediated right-sided repression of Nodal in rabbit. Dev. Dyn. 237, 3516–3527. doi:10.1002/dvdy.21535 [PubMed: 18553642]

Fischer A, Viebahn C, Blum M, 2002 FGF8 acts as a right determinant during establishment of the left-right axis in the rabbit. Current Biology 12, 1807–1816. doi:10.1016/S0960-9822(02)01222-8 [PubMed: 12419180]

Frank DU, Fotheringham LK, Brewer JA, Muglia LJ, Tristani-Firouzi M, Capecchi MR, Moon AM, 2002 An Fgf8 mouse mutant phenocopies human 22q11 deletion syndrome. Development 129, 4591–4603. [PubMed: 12223415]

Fukuta M, Nakai Y, Kirino K, Nakagawa M, Sekiguchi K, Nagata S, Matsumoto Y, Yamamoto T, Umeda K, Heike T, Okumura N, Koizumi N, Sato T, Nakahata T, Saito M, Otsuka T, Kinoshita S, Ueno M, Ikeya M, Toguchida J, 2014 Derivation of mesenchymal stromal cells from pluripotent stem cells through a neural crest lineage using small molecule compounds with defined media. PLoS ONE 9, e112291. doi:10.1371/journal.pone.0112291 [PubMed: 25464501]

Garcia-Castro MI, Marcelle C, Bronner-Fraser M, 2002 Ectodermal Wnt function as a neural crest inducer 297, 848–851. doi:10.1126/science.1070824

Green SA, Bronner ME, 2014 The lamprey: a jawless vertebrate model system for examining origin of the neural crest and other vertebrate traits. Differentiation 87, 44–51. doi:10.1016/j.diff.2014.02.001 [PubMed: 24560767]

Hackland JOS, Frith TJR, Thompson O, Marin Navarro A, García-Castro MI, Unger C, Andrews PW, 2017 Top-Down Inhibition of BMP Signaling Enables Robust Induction of hPSCs Into Neural Crest in Fully Defined, Xeno-free Conditions. Stem Cell Reports 9, 1043–1052. doi:10.1016/j.stemcr.2017.08.008 [PubMed: 28919261]

Hamburger V, Hamilton HL, 1951 A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49–92. [PubMed: 24539719]

Hong CS, Park BY, Saint-Jeannet J-P, 2008 Fgf8a induces neural crest indirectly through the activation of Wnt8 in the paraxial mesoderm. Development 135, 3903–3910. doi:10.1242/dev.026229 [PubMed: 18997112]

Idkowiak J, Weisheit G, Plitzner J, Viebahn C, 2004 Hypoblast controls mesoderm generation and axial patterning in the gastrulating rabbit embryo. Dev. Genes Evol. 214, 591–605. doi:10.1007/s00427-004-0436-y [PubMed: 15480760]

Khudyakov J, Bronner-Fraser M, 2009 Comprehensive spatiotemporal analysis of early chick neural crest network genes. Dev. Dyn. 238, 716–723. doi:10.1002/dvdy.21881 [PubMed: 19235729]

Knezevic V, De Santo R, Mackem S, 1997 Two novel chick T-box genes related to mouse Brachyury are expressed in different, non-overlapping mesodermal domains during gastrulation. Development 124, 411–419. [PubMed: 9053317]

Knoetgen H, Teichmann U, Wittler L, Viebahn C, Kessel M, 2000 Anterior Neural Induction by Nodes from Rabbits and Mice. Developmental Biology 225, 370–380. doi:10.1006/dbio.2000.9834 [PubMed: 10985856]

Knoetgen H, Viebahn C, Kessel M, 1999 Head induction in the chick by primitive endoderm of mammalian, but not avian origin. Development 126, 815–825. [PubMed: 9895328]

Lawson KA, Meneses JJ, Pedersen RA, 1991 Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. Development 113, 891–911. [PubMed: 1821858]

Le Douarin N, Kalcheim C, 1999 The Neural Crest, 2nd ed. Cambridge University Press.

Lee G, Kim H, Elkabetz Y, Shamy Al, Panagiotakos G, Barberi G, Tabar T, V, Studer L., 2007 Isolation and directed differentiation of neural crest stem cells derived from human embryonic stem cells. Nature Biotechnology 25, 1468–1475. doi:10.1038/nbt1365

Leung AW, Murdoch B, Salem AF, Prasad MS, Gomez GA, García-Castro MI, 2016 WNT/β-catenin signaling mediates human neural crest induction via a pre-neural border intermediate. Development 143, 398–410. doi:10.1242/dev.130849 [PubMed: 26839343]

Li J, Chen F, Epstein JA, 2000 Neural crest expression of Cre recombinase directed by the proximal Pax3 promoter in transgenic mice. Genesis 26, 162–164. [PubMed: 10686619]

Lumsden A, Sprawson N, Graham A, 1991 Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. Development 113, 1281–1291. [PubMed: 1811942]

Mancilla A, Mayor R, 1996 Neural crest formation in Xenopus laevis: mechanisms of Xslug induction. Developmental Biology 177, 580–589. doi:10.1006/dbio.1996.0187 [PubMed: 8806833]

Matsuoka T, Ahlberg PE, Kessaris N, Iannarelli P, Dennehy U, Richardson WD, McMahon AP, Koentges G, 2005 Neural crest origins of the neck and shoulder. Nature 436, 347–355. doi:10.1038/nature03837 [PubMed: 16034409]

Mayor R, Morgan R, Sargent MG, 1995 Induction of the Prospective Neural Crest of Xenopus. Development 121, 767–777. [PubMed: 7720581]

McBride WG, 1977 Thalidomide embryopathy. Teratology 16, 79–82. doi:10.1002/tera.1420160113 [PubMed: 331548]

McKeown SJ, Lee VM, Bronner-Fraser M, Newgreen DF, Farlie PG, 2005 Sox10 overexpression induces neural crest-like cells from all dorsoventral levels of the neural tube but inhibits differentiation. Dev. Dyn. 233, 430–444. doi:10.1002/dvdy.20341 [PubMed: 15768395]

Menendez L, Yatskievych TA, Antin PB, Dalton S, 2011 Wnt signaling and a Smad pathway blockade direct the differentiation of human pluripotent stem cells to multipotent neural crest cells. Proc. Natl. Acad. Sci. U.S.A. 108, 19240–19245. doi:10.1073/pnas.1113746108 [PubMed: 22084120]

Mica Y, Lee G, Chambers SM, Tomishima MJ, Studer L, 2013 Modeling neural crest induction, melanocyte specification, and disease-related pigmentation defects in hESCs and patient-specific iPSCs. Cell Reports 3, 1140–1152. doi:10.1016/j.celrep.2013.03.025 [PubMed: 23583175]

Mohammadi M, McMahon G, Sun L, Tang C, Hirth P, Yeh BK, Hubbard SR, Schlessinger J, 1997 Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science 276, 955–960. [PubMed: 9139660]

Monsoro-Burq AH, Fletcher RB, Harland RM, 2003 Neural crest induction by paraxial mesoderm in Xenopus embryos requires FGF signals. Development 130, 3111–3124. doi:10.1242/dev.00531 [PubMed: 12783784]

Morrison SJ, White PM, Zock C, Anderson DJ, 1999 Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. Cell 96, 737–749. [PubMed: 10089888]

Murray SA, Gridley T, 2006 Snail family genes are required for left-right asymmetry determination, but not neural crest formation, in mice. Proceedings of the National Academy of Sciences 103, 10300–10304. doi:10.1073/pnas.0602234103

Nichols DH, 1981 Neural crest formation in the head of the mouse embryo as observed using a new histological technique. J Embryol Exp Morphol 64, 105–120. [PubMed: 7031165]

Nikitina N, Sauka-Spengler T, Bronner-Fraser M, 2008 Dissecting early regulatory relationships in the lamprey neural crest gene network. Proceedings of the National Academy of Sciences 105, 20083–20088. doi:10.1073/pnas.0806009105

O'Rahilly R, Müller F, 2007 The development of the neural crest in the human. J. Anat. 211, 335–351. doi:10.1111/j.1469-7580.2007.00773.x [PubMed: 17848161]

Ota KG, Kuraku S, Kuratani S, 2007 Hagfish embryology with reference to the evolution of the neural crest. Nature 446, 672–675. doi:10.1038/nature05633 [PubMed: 17377535]

Patthey C, Edlund T, Gunhaga L, 2009 Wnt-regulated temporal control of BMP exposure directs the choice between neural plate border and epidermal fate. Development 136, 73–83. doi:10.1242/dev.025890 [PubMed: 19060333]

Patthey C, Gunhaga L, 2011 Specification and regionalisation of the neural plate border. Eur. J. Neurosci. 34, 1516–1528. doi:10.1111/j.1460-9568.2011.07871.x [PubMed: 22103410]

Patthey C, Gunhaga L, Edlund T, 2008 Early development of the central and peripheral nervous systems is coordinated by Wnt and BMP signals. PLoS ONE 3, e1625. doi:10.1371/journal.pone.0001625 [PubMed: 18286182]

Pearn JH, Vickers TH, 1966 The rabbit thalidomide embryology. British Journal of Experimental Pathology 47, 186–192. [PubMed: 4957599]

Peeters MC, Viebahn C, Hekking JW, van Straaten HW, 1998 Neurulation in the rabbit embryo. Anat Embryol 197, 167–175. [PubMed: 9543335]

Piliszek A, Madeja ZE, Plusa B, 2017 Suppression of ERK signalling abolishes primitive endoderm formation but does not promote pluripotency in rabbit embryo. Development 144, 3719–3730. doi:10.1242/dev.156406 [PubMed: 28935706]

Prasad MS, Sauka-Spengler T, LaBonne C, 2012 Induction of the neural crest state: Control of stem cell attributes by gene regulatory, post-transcriptional and epigenetic interactions. Developmental Biology 366, 10–21. doi:10.1016/j.ydbio.2012.03.014 [PubMed: 22583479]

Plöger R, Viebahn C, in press. PITX2 and NODAL expression during axis formation in the early rabbit embryo. Ann Anat. doi:10.1016/j.aanat.2018.02.016

Püschel B, Bitzer E, Viebahn C, 2010a Live rabbit embryo culture. Cold Spring Harb Protoc 2010, pdb.prot5352. doi:10.1101/pdb.prot5352

Püschel B, Daniel N, Bitzer E, Blum M, Renard J-P, Viebahn C, 2010b The rabbit (Oryctolagus cuniculus): a model for mammalian reproduction and early embryology. Cold Spring Harb Protoc 2010, pdb.emo139. doi:10.1101/pdb.emo139

Quinlan GA, Williams EA, Tan SS, Tam PP, 1995 Neuroectodermal fate of epiblast cells in the distal region of the mouse egg cylinder: implication for body plan organization during early embryogenesis. Development 121, 87–98. [PubMed: 7867510]

Rex M, Orme A, Uwanogho D, Tointon K, Wigmore PM, Sharpe PT, Scotting PJ, 1997 Dynamic expression of chicken Sox2 and Sox3 genes in ectoderm induced to form neural tissue. Dev. Dyn. 209, 323–332. [PubMed: 9215646]

Rogel-Gaillard C, Ferrand N, Hayes H, 2009 Rabbit, in: Genome Mapping and Genomics in Domestic Animals. Springer, Berlin, Heidelberg, Berlin, Heidelberg, pp. 165–230. doi:10.1007/978-3-540-73835-0_7

Rossant J, 2011 Developmental biology: A mouse is not a cow. Nature 471, 457–458. doi:10.1038/471457a [PubMed: 21430771]

Ruffins S, Artinger KB, Bronner-Fraser M, 1998 Early migrating neural crest cells can form ventral neural tube derivatives when challenged by transplantation. Developmental Biology 203, 295–304. [PubMed: 9808781]

Samuel CA, Jack PMB, Nathanielsz PW, 1975 Ultrastructural studies of the rabbit placenta in the last third of gestation. J. Reprod. Fertil. 45, 9–14. [PubMed: 1238566]

Sauka-Spengler T, Bronner-Fraser M, 2008 A gene regulatory network orchestrates neural crest formation. Nat. Rev. Mol. Cell Biol. 9, 557–568. doi:10.1038/nrm2428 [PubMed: 18523435]

Schumacher H, Blake DA, Gurian JM, Gillette JR, 1968 A comparison of the teratogenic activity of thalidomide in rabbits and rats. J. Pharmacol. Exp. Ther. 160, 189–200. [PubMed: 5639104]

Serbedzija GN, Bronner-Fraser M, Fraser SE, 1992 Vital dye analysis of cranial neural crest cell migration in the mouse embryo. Development 116, 297–307. [PubMed: 1283734]

Shen H, Wilke T, Ashique AM, Narvey M, Zerucha T, Savino E, Williams T, Richman JM, 1997 Chicken transcription factor AP-2: cloning, expression and its role in outgrowth of facial prominences and limb buds. Developmental Biology 188, 248–266. doi:10.1006/dbio.1997.8617 [PubMed: 9268573]

Shi W, Dirim F, Wolf E, Zakhartchenko V, Haaf T, 2004 Methylation reprogramming and chromosomal aneuploidy in in vivo fertilized and cloned rabbit preimplantation embryos. Biol. Reprod. 71, 340–347. doi:10.1095/biolreprod.103.024554 [PubMed: 15028628]

Shiomi M, 2009 Rabbit as a model for the study of human diseases, in: Rabbit Biotechnology: Rabbit Genomics, Transgenesis, Cloning and Models. Springer Netherlands, Dordrecht, pp. 49–63. doi:10.1007/978-90-481-2227-1_7

Simoes-Costa M, Bronner ME, 2015a Establishing neural crest identity: a gene regulatory recipe. Development 142, 242–257. doi:10.1242/dev.105445 [PubMed: 25564621]

Steventon B, Araya C, Linker C, Kuriyama S, Mayor R, 2009 Differential requirements of BMP and Wnt signalling during gastrulation and neurulation define two steps in neural crest induction. Development 136, 771–779. doi:10.1242/dev.029017 [PubMed: 19176585]

Stottmann RW, Klingensmith J, 2011 Bone morphogenetic protein signaling is required in the dorsal neural folds before neurulation for the induction of spinal neural crest cells and dorsal neurons. Dev. Dyn. 240, 755–765. doi:10.1002/dvdy.22579 [PubMed: 21394823]

Stuhlmiller TJ, García-Castro MI, 2012a Current perspectives of the signaling pathways directing neural crest induction. Cell. Mol. Life Sci. 69, 3715–3737. doi:10.1007/s00018-012-0991-8 [PubMed: 22547091]

Stuhlmiller TJ, García-Castro MI, 2012b FGF/MAPK signaling is required in the gastrula epiblast for avian neural crest induction. Development 139, 289–300. doi:10.1242/dev.070276 [PubMed: 22129830]

Suzuki T, Sakai D, Osumi N, Wada H, Wakamatsu Y, 2006 Sox genes regulate type 2 collagen expression in avian neural crest cells. Develop. Growth Differ. 48, 477–486. doi:10.1111/j.1440-169X.2006.00886.x

Tam PP, 1989 Regionalisation of the mouse embryonic ectoderm: allocation of prospective ectodermal tissues during gastrulation. Development 107, 55–67. [PubMed: 2627894]

Tam PPL, Gad JM, 2004 Gastrulation in the mouse embryo, in: Stern CD (Ed.), Gastrulation. pp. 233–264.

Tucker GC, Aoyama H, Lipinski M, Tursz T, Thiery JP, 1984 Identical reactivity of monoclonal antibodies HNK-1 and NC-1: conservation in vertebrates on cells derived from the neural primordium and on some leukocytes. Cell Differ. 14, 223–230. [PubMed: 6207939]

Tucker GC, Delarue M, Zada S, Boucaut JC, Thiery JP, 1988 Expression of the HNK-1/NC-1 epitope in early vertebrate neurogenesis. Cell Tissue Res. 251, 457–465. [PubMed: 2449968]

Umeda K, Oda H, Yan Q, Matthias N, Zhao J, Davis BR, Nakayama N, 2015a Long-term expandable SOX9+ chondrogenic ectomesenchymal cells from human pluripotent stem cells. Stem Cell Reports 4, 712–726. doi:10.1016/j.stemcr.2015.02.012 [PubMed: 25818812]

Viebahn C, 2004 Gastrulation in the rabbit, in: Gastrulation. Cold Spring Harbor Laboratory Press, pp. 263–287.

Viebahn C, Mayer B, de Angelis MH, 1995 Signs of the principle body axes prior to primitive streak formation in the rabbit embryo. Anat Embryol 192, 159–169. doi:10.1007/BF00186004 [PubMed: 7486012]

Viebahn C, Stortz C, Mitchell SA, Blum M, 2002 Low proliferative and high migratory activity in the area of Brachyury expressing mesoderm progenitor cells in the gastrulating rabbit embryo. Development 129, 2355–2365. [PubMed: 11973268]

Waddington CH, Waterman AJ, 1933 The Development in vitro of Young Rabbit Embryos. J. Anat. 67, 355–370. [PubMed: 17104430]

Watt KEN, Trainor PA, 2014 Neurocristopathies, Neural Crest Cells. doi:10.1016/b978-0-12-401730-6.00018-1

Wilson SI, Graziano E, Harland R, Jessell TM, Edlund T, 2000 An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. Current Biology 10, 421–429. [PubMed: 10801412]

Wilson YM, Richards KL, Ford-Perriss ML, Panthier J-J, Murphy M, 2004 Neural crest cell lineage segregation in the mouse neural tube. Development 131, 6153–6162. doi:10.1242/dev.01533 [PubMed: 15548576]

Highlights

- Molecular characterization of neural crest formation in the rabbit embryo.
- First evidence of early neural crest specification in a mammalian embryo.
- FGF signaling is required for rabbit neural crest formation.

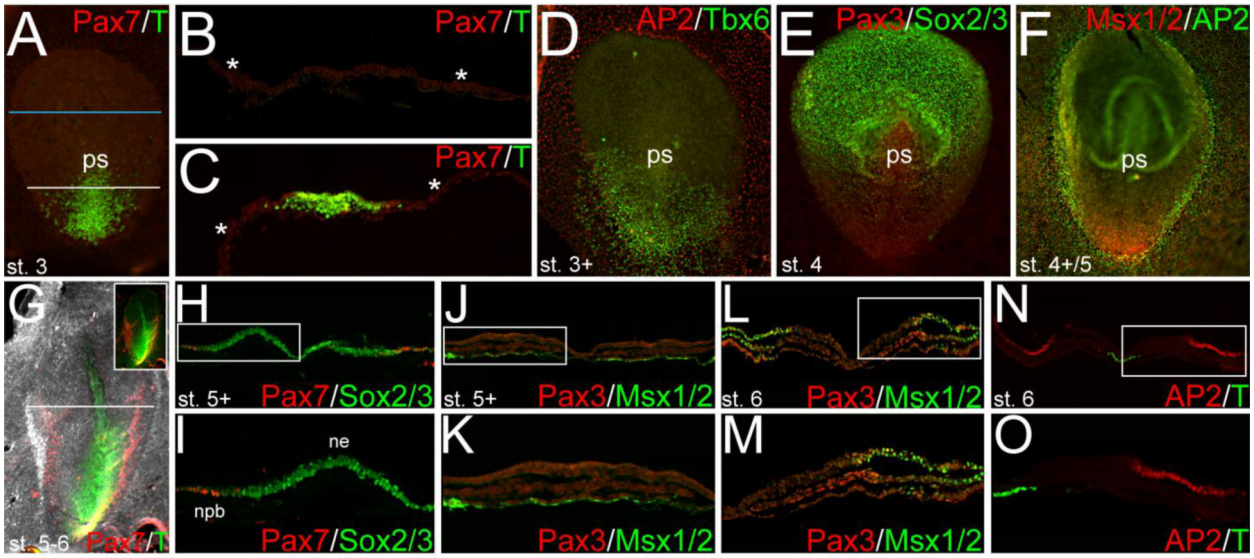

Figure 1.

Expression of neural crest markers in early rabbit embryos. (A) Stage 3 rabbit embryo reveals posterior expression of T/Brachyury (mesoderm), but no Pax7. Blue and white lines indicate levels of sections in (B) and (C), respectively. (B-C) Sections from a stage 3 rabbit embryo stained for Pax7 and T. Anterior sections do not display Pax7 nor T expression (B), while T expression is observed in the ingressing mesoderm of posterior sections taken through the primitive streak (C). T-positive cells are also observed in the epiblast overlying this region. Asterisks in panels (B) and (C) indicate the border of the embryonic/extraembryonic tissue. (D) Stage 3+ embryo stained for AP2α and the mesodermal marker Tbx6. While AP2α is not detected in prospective NC territories at this stage, expression is detected in the extraembryonic tissue surrounding the entire embryo. Tbx6 is detected in the posterior epiblast. (E) Stage 4 embryo stained for Pax3 and the neural marker Sox2. While no Pax3 expression is detected, Sox2 is robustly expressed in the anterior epiblast, marking the prospective neural tissue. (F) Stage 4+/5 embryo stained for Msx½ and AP2α. Msx½ is detected in the posterior epiblast and in the extraembryonic tissue. AP2α signal is detected in the extraembryonic tissue, in particular surrounding the embryo at the border of the area pellucida and area opaca. (G) Whole mount early neurula stage 5–6 rabbit embryo reveals Pax7 and T expression. White line indicates the level of sections presented in panels (H-O). (H-I) Anterior sections reveal Pax7 expression in the early neural plate border and Sox2 in the neuroectoderm. Panel (I) is a magnified image of the region boxed in (H). (J-K) Pax3 and Msx½ expression is not detected in the neural plate border in sections from a st. 5+ embryo. Panel (K) is a magnified image of the region boxed in (J). (L-M) Msx½ expression is detected in the neural plate border, in addition to a small number of Pax3-positive cells. Panel (M) is a magnified image of the region boxed in (L). (N-O) AP2α expression is detected in the neural plate border, with some expression extending into the non-neural ectoderm. T expression is detected in the underlying mesoderm. Panel (O) is a magnified image of the region boxed in (N). ps, primitive streak; npb, neural plate border; ne, neuroectoderm

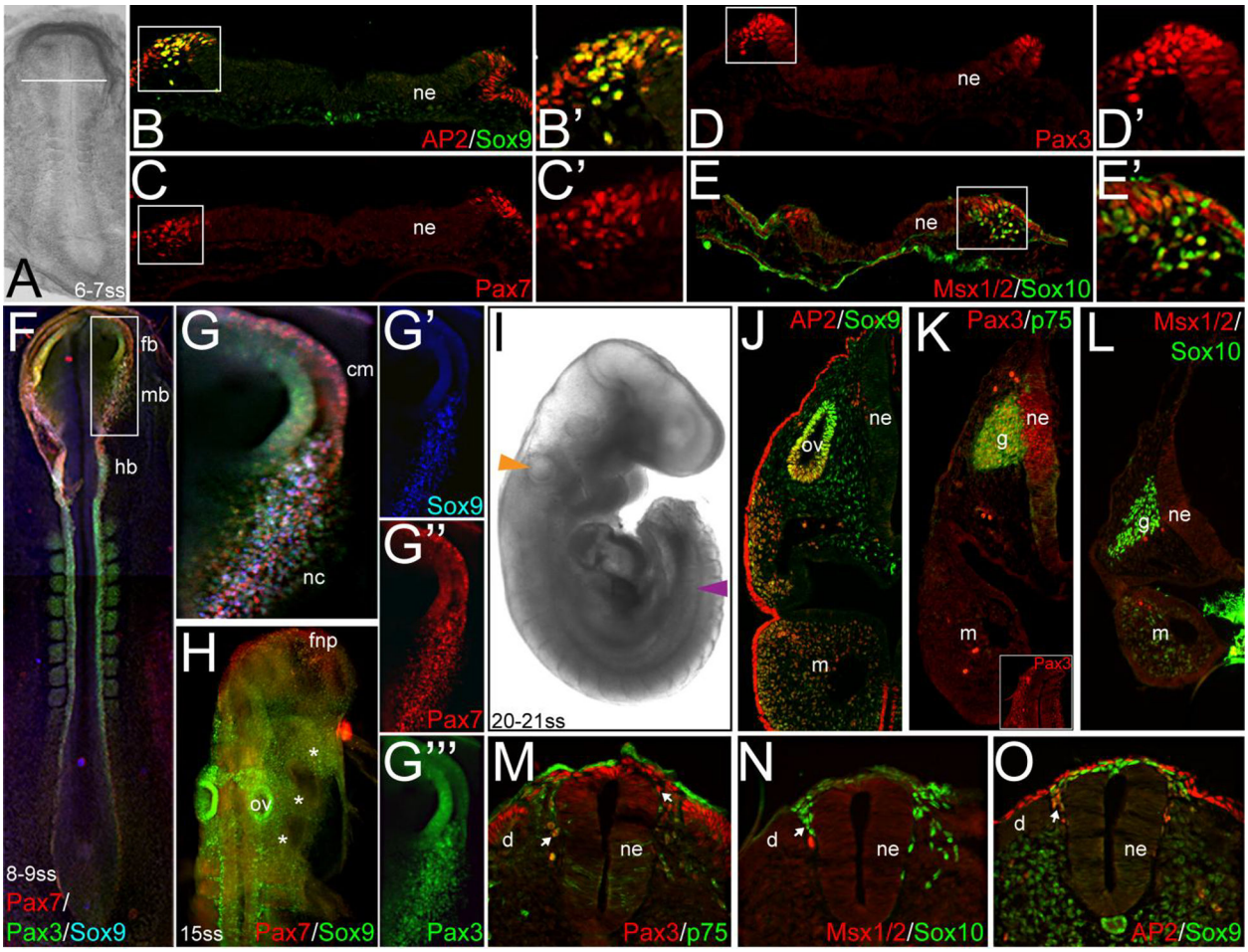

Figure 2.

Markers of pre-migratory and migratory rabbit neural crest cells. (A) Bright field image of a whole 6–7ss rabbit embryo. White line indicates axial level of sections presented in (B-E). (B) Cranial section of 6–7ss embryo reveals AP2α and Sox9 expression in migratory NC cells, as well as in the tips of the unfused neural folds. A number of double-positive cells are observed. AP2α expression also extends out into the non-neural ectoderm. A magnified image of the boxed region is presented in (B'). (C) Pax7 expression is detected in both pre-migratory and migratory cranial NC cells. A magnified image of the boxed region is presented in (C'). (D) Pax3 labels both pre-migratory and migratory cranial NC cells at the 6–7ss. A magnified image of the boxed region is presented in (D'). (E) Staining for Msx½ and Sox10 reveals Msx½ expression predominantly in the neural plate border/pre-migratory NC, with a few Msx½-positive migratory NC cells. By contrast, Sox10 expression is most frequently observed in migratory NC cells at this stage. A magnified image of the boxed region is presented in (E'). (F) Whole 8–9ss rabbit embryo stained for Pax7, Pax3, and Sox9, presented as a tiled compilation of two images. Migratory NC cells are observed emerging from the level of the midbrain. A magnified image of the boxed region is shown in (G), and single-channel images in (G'-G'''). (H) Migratory cranial NC cells observed at the 15ss, with Pax7-positive cells populating the frontonasal process, and Sox9-positive cells migrating towards the branchial arches (asterisks). (I) Brightfield image of a whole 20–21ss

rabbit embryo. Arrowheads indicate the axial levels of the sections presented in (J-L) (cranial; orange) and (M-O) (trunk; purple). (J) AP2α and Sox9 staining of cranial sections from a 20–21ss embryo. AP2α and Sox9-positive cells are observed in the otic vesicle as well as the NC-populated maxillary process. (K) Pax3 and p75 staining of cranial sections reveals Pax3 and p75 expression in the NC-derived cranial ganglia. Pax3 expression is also observed in the neuroepithelium, and in the cranial mesenchyme of more rostral sections (inset). (L) Sox10 expression is observed in the cranial ganglia, and sparse Sox10-positive cells are found in the maxillary process. Definitive Msx½ signal is not observed. (M-O) Trunk sections generated from 20–21ss embryos at the axial level marked by the purple arrowhead in panel (I). (M) Pax3 and p75NTR expression is detected in migratory NC cells, with Pax3 further marking pre-migratory NC. (N) Sox10 marks rabbit migratory NC cells. Msx½ expression is not detected. (O) AP2α and Sox9 expression marks migratory NC cells. Sox9 expression, but not AP2α, is detected in trunk pre-migratory NC. Arrows mark examples of migratory NC cells. ne, neuroepithelium; fb, forebrain; mb, midbrain; hb, hindbrain; cm, cranial mesenchyme; fnp, frontonasal process; ov, otic vesicle; ss, somite stage; d, dermomyotome.

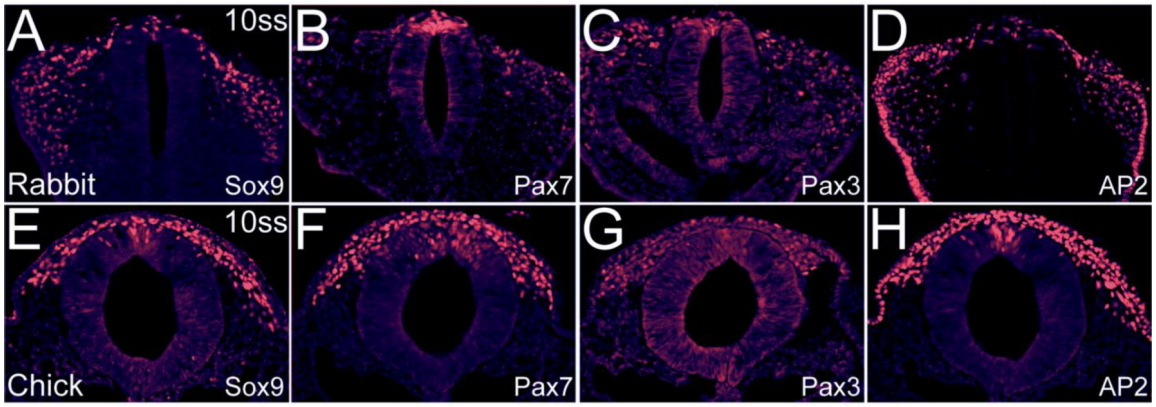

Figure 3.

Comparison of cranial neural crest cell markers in 10ss rabbit and chick embryos. Rabbit (A-D) and chick (E-H) midbrain cranial sections from 10ss embryos stained for the NC markers Sox9 (A, E), Pax7 (B, F), Pax3 (C, G), and AP2α (D, H). At this stage and axial level, broad conservation is observed in NC marker expression between the two species, with subtle differences in the expression of Pax7 and Pax3.

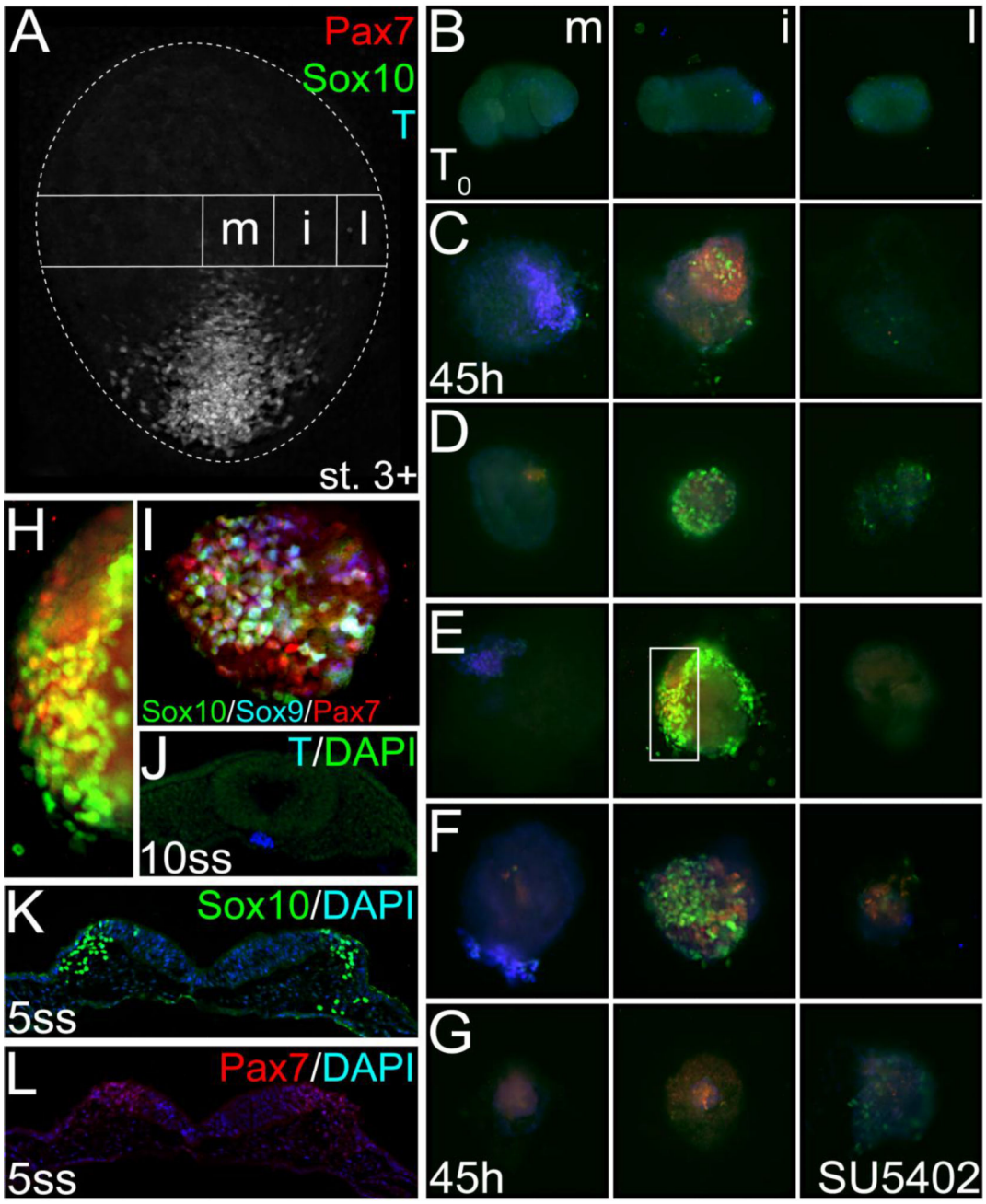

Figure 4.

Rabbit neural crest is specified in the gastrula embryo and requires FGF signaling. (A) Representative schematic of explant generation from stage 3+ rabbit gastrula embryos. All explants were co-stained for NC markers Pax7 and Sox10, and the mesodermal marker T. (B) Uncultured (T0) explants generated from stage 3+ rabbit embryos do not express Pax7 or Sox10. (C-F) Explants generated from stage 3+ were cultured for 45h and stained for Pax7, Sox10, and T. Explants from four representative embryos are shown (n=5 total embryos). After culture, “intermediate” embryos express Pax7 and Sox10. (G) Explants generated from stage 3+ embryos and cultured for 45h in the presence of 10 ⌠M SU5402 to

inhibit FGF signaling. After treatment, intermediate explants do not express Pax7 and Sox10. Explants from one representative embryo shown (n=5 total embryos). (H) A magnified image of Pax7 and Sox10 positive cells in the intermediate explant presented in (E). (I) The intermediate explant presented in (F) was re-stained for Sox9 (blue). (J-L) Endogenous Sox10, Pax7, and T expression in cranial sections from 5ss and 10ss embryos were used as comparative controls. m, medial/primitive streak; i, intermediate; l, lateral.

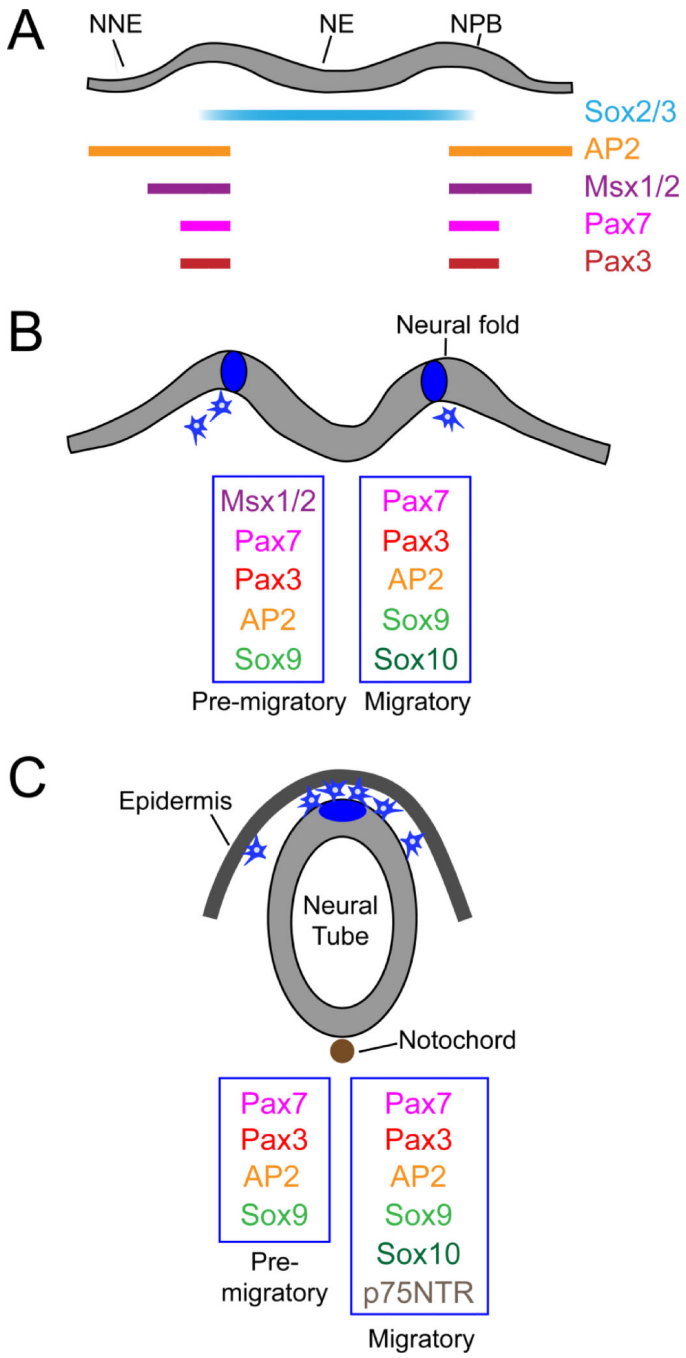

Figure 5.

Molecular markers of early rabbit NC formation and migration. Summary of molecular markers of rabbit NC investigated in this report. (A) Expression patterns of NC markers at the early neural plate. This study indicates that Pax7 is the first marker with expression restricted to the NPB. Very few Pax3-positive cells are detected in at the NPB in stage 6 embryos. (B) NC markers are expressed in pre-migratory NC cells at the tips of the unfused neural folds, as well as in freshly migratory NC cells. (C) Rabbit NC cells robustly migrate from the closed neural tube at anterior and posterior axial levels. We note that Pax7

expression was not detected in pre-migratory nor migratory NC in trunk sections at 20–21ss. The HNK-1 antibody failed to recognize rabbit NC cells. npb, neural plate border; ne, neuroectoderm; nne, non-neural ectoderm.