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MECHANISMS OF DEVELOPMENT

Fibronectin and integrin alpha 5 play essential roles in the development of the cardiac neural crest

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ABSTRACT

Cardiac neural crest (CNC) plays a requisite role during cardiovascular development and defects in the formation of CNC-derived structures underlie several common forms of human congenital birth defects. Migration of the CNC cells to their destinations as well as expansion and maintenance of these cells are important for the normal development of the cardiac outflow tract and aortic arch arteries; however, molecular mechanisms regulating these processes are not well-understood. Fibronectin (FN) protein is present along neural crest migration paths and neural crest cells migrate when plated on FN *in vitro*; therefore, we tested the role of FN during the development of the CNC *in vivo*. Our analysis of the fate of the neural crest shows that CNC cells reach their destinations in the branchial arches and the cardiac outflow tract in the absence of FN or its cellular receptor integrin α5β1. However, we found that FN and integrin α5 modulate CNC proliferation and survival, and are required for the presence of normal numbers of CNC cells at their destinations.

1. Introduction

Neural crest cells (NCCs) are a population of multipotent progenitor cells that originate in the dorsal neural tube during embryogenesis (Crane and Trainor, 2006). In the dorsal neural tube, NCC progenitors undergo the epithelial-to-mesenchymal transition (EMT), detach from the neural tube and migrate to their diverse destinations. Depending on the axial position, NCCs give rise to a wide array of cell types in the embryo, including neurons, glia, vascular smooth muscle cells (VSMCs), cranial bones and cartilage (Le Douarin, 1982).

In chick, a subpopulation of the cranial NC originating from the region of the dorsal neural tube between the mid-otic placode and the posterior boundary of the third somite is termed the cardiac neural crest (CNC). CNC gives rise to VSMCs around aortic arch arteries and the distal portions of aorta and pulmonary artery (Hutson and Kirby, 2007). The importance of the CNC in cardiovascular development was first recognized in seminal studies performed in chick, showing that the ablation of a portion of the dorsal neural tube containing CNC progenitors resulted in aberrant septation and positioning of the cardiac outflow tract, absence of CNC-derived VSMCs and defective remodeling of pharyngeal arch arteries into their canonical mature asymmetric arrangement. These defects resemble some of the most common and severe forms of human congenital cardiovascular disorders, including those found in patients with DiGeorge syndrome (Hutson and Kirby, 2007). Similar to the chick, mouse NC progenitors that give rise

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to the CNC originate between the pro-rhombomere c and the fourth somite (Chan et al., 2004).

In vitro experiments performed with NCCs isolated from different axial levels of the neural tube showed that depending on their site of origin, NCCs exhibit different migratory responses to extracellular matrix (ECM). These experiments also showed that components of the ECM and levels and repertoire of integrins expressed by NCCs modulate NCC migratory behavior (Delannet et al., 1994; Strachan and Condic, 2003; Testaz et al., 1999; Xu et al., 2006). While there are examples indicating that some ECM components (e.g. laminin alpha 5 and fibulin-1) modulate NC migratory paths *in vivo* (Coles et al., 2006; Cooley et al., 2008), the functions of many other components of the ECM during the development of the NC are not completely understood.

Fibronectin (FN) is an essential component of the ECM present along the paths of NCC transit (Duband and Thiery, 1982; George et al., 1997; Mayer et al., 1981; Rovasio et al., 1983). The appreciation that both the *FN* gene and the neural crest are unique to vertebrates led to the hypothesis that FN could have evolved to play an important role during the development of the neural crest and its derived lineages (Hynes and Zhao, 2000; Whittaker et al., 2006). While experiments *in vitro* indicate that FN serves as a permissive substratum for NCC migration (Rovasio et al., 1983) the function of FN during NC development *in vivo* is not known.

In order to determine the role of FN in the development of the neural crest, we performed lineage-tracing experiments to follow the fate of CNC precursors in embryos that lack FN or its cellular receptor, integrin α5. While we observed extensive migration of cranial NCCs, including CNCCs in the absence of FN or integrin α5, our experiments also indicate that there is a significant deficiency in the number of CNCCs in the mutant embryos. Our experiments show that this deficiency is not due to defective formation of the CNC or defective exit of CNC precursors from the neural tube, but rather to the depletion of the CNC progenitor pool within the neural tube and decreased proliferation and survival of CNCCs. Our studies are the first to demonstrate the requisite role of FN and integrin α5 during the ontogeny of the CNC.

2. Results

2.1. *Fibronectin synthesis is upregulated in CNC progenitors at the time corresponding with their expansion and the onset of migration*

Prior studies in chick and frog showed that NCC progenitors within the dorsal neural tube as well as NCCs exiting the neural tube are surrounded by FN protein (Alfandari et al., 2003; Duband and Thiery, 1982; Le Douarin, 1982; Mayer et al., 1981). However, the cellular source(s) of FN during NC ontogeny are not known. Therefore, we used *in situ* hybridization to determine the spatio-temporal localization of mouse FN mRNA and immunofluorescence (IF) to examine FN protein expression at different embryonic stages corresponding with induction, expansion and migration of CNCCs.

We first assayed the presence of FN mRNA and protein in embryos with five somites, a time-point when the first known markers (e.g. Pax3) are already expressed by the CNC progenitors in the dorsal neural folds (Goulding et al., 1991). At this stage, we did not detect FN mRNA within the neural folds in wild-type embryos ($n = 6$ embryos), while FN was abundantly transcribed in other embryonic locations such as the foregut endoderm, splanchnic mesoderm and endocardium (Fig. 1A and B). Since FN is a secreted protein, we used IF microscopy to assay its spatio-temporal distribution. Concordant with the FN mRNA expression data, we did not detect FN protein in the dorsal neural tube in embryos with 5 somites, even though other embryonic locations were abundant with FN protein (Fig. 1C). Taken together, these experiments show that FN mRNA and protein are undetectable in the dorsal neural tube prior to CNC formation.

By the 10th somite stage, CNCCs have begun exiting from the dorsal neural tube (Chan et al., 2004; Serbedzija et al., 1992; Stottmann et al., 2004). Examination of FN synthesis in 10–11 somite embryos showed that FN mRNA is upregulated in the top few cell layers of the dorsal neural tube as well as in the cells of apposing surface ectoderm (Fig. 1D and E). Concomitantly, FN protein is localized between the surface ectoderm and the neural epithelium containing NC progenitors (Fig. 1F). This localization pattern of FN protein is similar with that observed during NC development in chick and frog (Alfandari et al., 2003; Mayer et al., 1981). FN protein is also localized within the embryonic mesenchyme where it could interact with migrating CNCCs (Supplementary Fig. S1) (Duband and Thiery, 1982; Mayer et al., 1981; Peters and Hynes, 1996).

FN synthesis by NC progenitors is maintained in embryos at E9.5 (23 somites) (Fig. 1G, black arrow) and NCCs exiting the neural tube also synthesize FN (Supplementary Fig. S2A). FN synthesis is downregulated within the arch mesenchyme while within the cardiac outflow tract, mesenchymal cells (presumptive CNCCs) and endocardial cells express FN mRNA (Supplementary Fig. S2B and B'). Taken together, our studies indicate that FN synthesis is induced in CNC precursors within the dorsal neural tube at the time corresponding with their expansion and exit (Conway et al., 2000; Serbedzija et al., 1992) and suggest a potential role for FN during these processes.

In mice, CNCCs destined for the cardiac outflow tract, migrate mainly through the fourth pharyngeal arch (Chan et al., 2004) and CNCCs remaining within the third, fourth and sixth pharyngeal arches give rise to VSMCs of aortic arch arteries (Jiang et al., 2000). In embryos with 6–23 somites, FN mRNA is highly enriched in the pharyngeal pocket endoderm and pharyngeal ectoderm, the areas of future pharyngeal arches 3, 4 and 6 (Fig. 1E and G and Supplementary Fig. S2B). In order to determine whether enrichment of FN mRNA within this region corresponds with enrichment in FN protein, we performed IF microscopy and quantified fluorescent pixel intensity within the pharyngeal arches of E9.5 embryos (Fig. 1H). This analysis showed that similar to FN mRNA, FN protein is enriched in the mesenchyme of pharyngeal arches 3 and 4 compared with arches 1 and 2 (Fig. 1H and I). Taken together our studies show that FN protein is expressed by and is localized to tissues important for the development of the CNC.