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Different downstream pathways for Notch signaling are required for gliogenic and chondrogenic specification of mouse mesencephalic neural crest cells

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ABSTRACT

We examined the roles of Notch signaling and fibroblast growth factors (FGFs) in the gliogenesis of mouse mesencephalic neural crest cells. The present study demonstrated that Notch activation or FGF treatment promotes the differentiation of glia expressing glial fibrillary acidic protein. Notch activation or FGF2 exposure during the first 48 h in culture was critical for glial differentiation. The promotion of gliogenesis by FGF2 was significantly suppressed by the inhibition of Notch signaling using *Notch-1* siRNA. These data suggest that FGFs activate Notch signaling and that this activation promotes the gliogenic specification of mouse mesencephalic neural crest cells. Notch activation and FGF treatment have been shown to participate in the chondrogenic specification of these cells [Nakanishi, K., Chan, Y.S., Ito, K., 2007. Notch signaling is required for the chondrogenic specification of mouse mesencephalic neural crest cells. Mech. Dev. 124, 190–203]. Therefore, we analyzed whether or not there were differences between gliogenic and chondrogenic specifications in the downstream pathway of the Notch receptor. Whereas the activation of only the Deltex-mediated pathway was sufficient to promote glial specification, the activation of both RBP-J- and Deltex-dependent pathways was required for chondrogenic specification. These results suggest that the different downstream pathways of the Notch receptor participate in the gliogenic and chondrogenic specification of mouse mesencephalic neural crest cells.

1. Introduction

The neural crest is a transient structure in vertebrate embryos. Neural crest cells arise within the neural tube, migrate ventrally and laterally, and contribute significantly to a wide variety of cell types, including melanocytes, peripheral neurons and their glial cells, smooth muscle cells, and skeletal derivatives (Le Douarin and Kalcheim, 1999; Hall, 1999). Cranial neural crest cells migrate beneath the ectoderm and populate cranial ganglia of the peripheral nervous system (PNS) to differentiate into glia and neurons. In contrast with the mechanisms of neurogenesis, those of glial differentiation in the PNS remain largely unknown.

Notch activation and fibroblast growth factor (FGF) 2 treatment promote the gliogenesis of mouse trunk neural crest cells (Morrison et al., 2000; Ota and Ito, 2006). Since trunk neural crest cells differ from cranial neural crest cells in how they respond to specific extracellular signals (Abzhanov et al., 2003), we analyzed the roles of Notch signaling and FGFs in the gliogenesis of mouse mesencephalic neural crest cells. We demonstrated that Notch signaling activated by FGFs promotes the gliogenesis of mesence-

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phalic neural crest cells, similar to the case with trunk neural crest cells.

Notch signaling is an evolutionarily conserved mechanism that influences various biological processes, such as cell fate specification, differentiation, proliferation, and apoptosis (Artavanis-Tsakonas et al., 1999; Miele and Osborne, 1999). Notch signaling is known to have different Notch receptor downstream pathways: RBP-J-dependent and Deltex-dependent pathways (Kato et al., 1997; Matsuno et al., 1995; Yamamoto et al., 2001). Both types of pathways regulate various cellular behaviors independently or interactively (Kato et al., 1997; Yamamoto et al., 2001; Patten et al., 2006). We have shown that Notch signaling activated by FGFs promotes the chondrogenic specification of mouse mesencephalic neural crest cells (Nakanishi et al., 2007). Therefore, we focused on the differences in the downstream Notch receptor pathways between gliogenesis and chondrogenesis. The present data show that the activation of the Deltex-mediated pathway by means of FGFs promotes the gliogenesis of mesencephalic neural crest cells and that chondrogenesis requires the activation of both RBP-J-dependent and Deltex-dependent pathways.

2. Results

2.1. *Effects of Notch signaling and FGFs on the differentiation of glia*

When mouse mesencephalic neural crest cells were cultured in the presence of a fusion protein containing the extracellular domain of a Notch ligand, Delta-1, and the Fc region of human immunoglobulin IgG (Delta-Fc) or FGFs, a large number of small and stellate cells appeared within 24 h in culture. In the absence of Delta-Fc and FGFs or in the cultures containing the Fc region of human immunoglobulin IgG (Fc) only, on the other hand, many flattened cells were found. To examine the effects of Notch signaling and FGFs on the gliogenesis of mesencephalic neural crest cells, we detected glia by means of immunocytochemistry using anti-glial fibrillary acidic protein (GFAP) antibodies and counted the number of cells containing GFAP on culture day 4. The percentage of cells expressing GFAP per neural crest cell colony (each colony was derived from a primary explant) dramatically increased in the presence of Delta-Fc and FGF2 (Fig. 1A–D). Treatment with FGF4 or FGF8 also promoted GFAP expression, but the effect of each was weaker than that of FGF2 (Fig. 1D). Anti-GFAP-positive cells displayed a small and dendritic morphology and were first observed after 2 days in culture.

We examined the effects of Delta-Fc and FGF2 on the proliferation of the neural crest cells using the 5-bromo-2′-deoxyuridine (BrdU) incorporation method. Whereas the percentage of BrdU-incorporated cells significantly increased by FGF2 treatment at 4 days in culture (Fig. 1H), neither Delta-Fc nor FGF2 had any effects on the proliferation of GFAP-expressing cells as shown by double-labeling experiments using anti-GFAP and anti-BrdU (Fig. 1E–G and I).

2.2. *Critical Notch-sensitive period for gliogenesis*

To establish whether or not there is a critical period during which Notch signaling is required for gliogenesis, mouse mesencephalic neural crest cells were exposed to Delta-Fc during several distinct periods of the cultures. Exposure to Delta-Fc during the period 0–24 h or the period 24–48 h in culture promoted GFAP expression (Fig. 2A). Delta-Fc treatment for the first 48 h was the most critical for the stimulation of GFAP expression (Fig. 2A). However, exposure during the period 48–72 h or the period 72–96 h did not promote GFAP expression (Fig. 2A). Furthermore, the overexpression of the mouse Notch-1 intracellular domain (Notch-IC) by transfection with the expression vector encoding Notch-IC during the first 48 h stimulated GFAP expression (Fig. 2A). The most critical exposure period of FGF2 for GFAP expression was also the first 48 h in culture (Fig. 2B). These observations show that the critical exposure period of Delta-Fc or FGF2 for GFAP expression differs from that for chondrogenesis, which was the first 24 h in culture (Nakanishi et al., 2007).

The first 48 h in culture were both Notch-sensitive and FGF2-sensitive periods for GFAP expression. Therefore, we analyzed the functional relationship between FGF2 and Notch signaling using *Notch-1* siRNA to block Notch-1 expression. The effect of *Notch-1* siRNA was examined by immunostaining with anti-Notch-1. Anti-Notch-1-positive cells were observed in the absence of siRNA (Fig. 2D) or in the presence of a negative control of *Notch-1* siRNA (Fig. 2E). Cells immunoreactive to anti-Notch-1 were hardly observed when *Notch-1* siRNA was transfected (Fig. 2F). Thus, Notch-1 expression was effectively prevented by *Notch-1* siRNA (Nakanishi et al., 2007). When mouse mesencephalic neural crest cell cultures were treated with *Notch-1* siRNA during the first 48 h in the presence of FGF2, the percentage of GFAP-expressing cells significantly decreased (Fig. 2C). This suppression of GFAP expression by *Notch-1* siRNA was recovered by treatment with Notch-IC expression vector (Fig. 2C). Moreover, GFAP expression was prevented by the treatment with SU5402, which is an inhibitor of FGF signaling (Wang et al., 2006), during the first 48 h despite the presence of FGF2 (Fig. 2C). The treatment with Delta-Fc or Notch-IC expression vector recovered this prevention (Fig. 2C).

2.3. *Expression patterns of Delta, Notch-1 and p75 in vivo*

We investigated the expression patterns of Delta, Notch-1, and p75, which is a marker for undifferentiated neural crest cells (Rao and Anderson, 1997), in the presumptive trigeminal ganglionic region of the mouse embryo, where mesencephalic neural crest cells colonize and undergo gliogenesis. At embryonic day (E) 8.5 (12-somite stage), intense immunoreactivities against anti-Delta, anti-Notch-1, and anti-p75 were observed throughout this region (Fig. 3A–E). Most of the anti-Delta-positive cells expressed Notch-1 (Fig. 3D). The expression of Delta and Notch-1 was also observed at E9.5 (25-somite stage, Fig. 3F–J). However, the expression of p75 slightly decreased from E8.5 to E9.5 (Fig. 3J). The expression levels of Delta, Notch-1, and p75 declined at E10.5 (39-somite stage, Fig. 3K–O). Furthermore, we examined the expression patterns of p75 and Notch-1 in mouse mesencephalic neural crest cell cultures to confirm that these are co-expressed in the neural crest cells. When the expression of p75 and Notch-1 was analyzed on culture day 1, eighty percent (79.8(±3.7)%) of the cells immunoreactive against anti-Notch-1 expressed p75 (Fig. 3P–S).