Research Article

Transcription factor c-Myb is involved in the regulation of the epithelial-mesenchymal transition in the avian neural crest

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Abstract. Multipotential neural crest cells (NCCs) originate by an epithelial-mesenchymal transition (EMT) during vertebrate embryogenesis. We show for the first time that the key hematopoietic factor c-Myb is synthesized in early chick embryos including the neural tissue and participates in the regulation of the trunk NCCs. A reduction of endogenous c-Myb protein both in tissue explants in vitro and in embryos in ovo, prevented the formation of migratory NCCs. A moderate over-expression of c-myb in naive intermediate neural plates triggered the EMT and NCC migration probably through cooperation with BMP4 signaling because (i) BMP4 activated c-myb expression, (ii) elevated c-Myb caused accumulation of transcripts of the BMP4 target genes msx1 and slug, and (iii) the reduction of c-Myb prevented the BMP4-induced formation of NCCs. The data show that in chicken embryos, the c-myb gene is expressed prior to the onset of hematopoiesis and participates in the formation and migration of the trunk neural crest.

Key words. c-myb; chick embryo; neural crest; epithelial-mesenchymal transition; antisense morpholino oligonucleotides; BMP4; slug; msx1.

Epithelial and mesenchymal cells represent two principal cell phenotypes, which differ both in their intracellular characteristics and in their relationships to neighboring cells. While epithelial cells, owing to their cytoskeletal architecture and surface receptors for cell-to-cell interactions, form well-organized sheets of tightly connected cells, the distinct cytoskeletal organization and receptors for extracellular matrix (ECM) enable mesenchymal cells to locomote as individuals and invade their surroundings, directed by cues from the ECM and distant cells. Epithelial and mesenchymal phenotypes are inter-convertible. This provides important means for the formation of complex body structures in metazoans because epithelial cells, upon turning into the mesenchyme, can migrate out of primitive epithelia into cell-free spaces and again adopt epithelial characteristics to give rise to new tissues. These phenotypical transformations are referred to as epithelial-mesenchymal and mesenchymal-epithelial transitions [reviewed in ref. 1]. The epithelial-mesenchymal transition (EMT) is a crucial morphogenetic event for vertebrate embryogenesis. This process is of eminent interest since it also participates in tissue repair...
and regeneration and during tumor growth and metastasis [reviewed in ref. 2].

Neural crest cells (NCCs) arise in early vertebrate embryos in neural tube folds through the EMT [reviewed in refs. 3, 4]. This is a complex process programmed by a series of inductive events triggered by gradients of principal inductors, such as bone morphogenetic proteins (BMPs) and members of the Wnt family of secreted glycoproteins and fibroblast growth factor (FGF) family of growth factors [reviewed in ref. 5]. In chick embryos, BMPs appear to contribute to both early and late inductive events. Chicken BMP4 is expressed in neural folds by a series of inductive events triggered by gradients of BMPs and members of the Wnt family of secreted glycoproteins and fibroblast growth factor (FGF) family of growth factors [reviewed in ref. 5]. BMP4 (R&D Systems) was used at 300 ng/ml. Retroviral stocks were prepared from the media of virus-producing chick embryo fibroblasts (CEF) essentially as described elsewhere [18] and concentrated tenfold on Omega 300K filters (Pall Gelman) to reach titers of 10⁸/ml. The c-myb retrovirus arose from the pneo-ccc vector [31] rescued by MAV1 helper virus; the control myb-less construct was pneo-ccc with the c-myb coding sequence deleted.

**Morpholino oligonucleotides.** antimyb1: 5'-GTCTC-GGGGCACTCTCGCGGC (antisense); antimyb1m: 5'-GTCTGCGGcCCATCcGCGGC (lowercase letters denote the mismatched bases) and Standard control morpholino oligonucleotidese (MO) (GeneTools) were introduced (20 µM) into neural explants by passive transfer as described elsewhere [32]. Delivery into CEFs was achieved mainly by the scraping technique (20 µM) or by EPEI transfer (1.4 µM) [33]. Fluorescein-labeled anti-myb MO and Standard control MO were often used to monitor the oligonucleotide uptake. The cultures were analyzed after 1–6 days.

**In ovo electroporation.** The electroporation of chick embryos was performed as follows: 400 µM MO duplexes with DNA oligonucleotides (GeneTools, special delivery) in water and the green fluorescent protein (GFP) expression vector pCLGFP (kindly provided by M. Scacchi) [34]; 10 mg/ml in phosphate-buffered saline) were mixed in the ratio 3:1. The mixture was injected into the neural tube of HH stage 10–12 embryos and electroporated using a BTX electroporator ECM 830, with the following parameters: five times 40-V square pulses of 20 ms. Efficient transfer of MOs into cells by electroporation was checked using special-delivery fluorescein-labeled MOs and pCLGFP. Twenty-four hours after electroporation, GFP-positive embryos, identified with UV light under a dissection microscope (Olympus SZX12), were isolated and analyzed by immunohistochemistry. At least nine electroporated embryos were analyzed in each experiment.

**Antibodies and immunofluorescence analysis.** The rabbit polyclonal α-Myb antibody was raised against the purified full-length AMV v-Myb protein. Monoclonal antibody G3G4 (against BrdU) was from Dev. Studies Hybridoma Bank; anti-CD57 TB01 antibody against the melanocytic lineage in chicken embryo cultures [27; Karafi at et al., unpublished data]. Since melanocytes descend from the trunk neural crest, we asked whether the c-myb protooncogene, the parental gene of both v-myb oncogenes, plays any role in neural crest biology. In this paper, we show that the c-Myb protein is likely a relevant regulator of the neural crest. We demonstrate that graded intracellular levels of the c-Myb protein are critical for the formation of migratory NCCs both in vitro and in vivo. This observation defines a novel role for the c-myb gene in vertebrates.

**Materials and methods**

**Embryos, cell cultures and viruses.** Brown or Barred Leghorn eggs (from the hatchery at the Institute of Molecular Genetics) were incubated until embryos reached required developmental stages. Neural fold (NF) and neural plate (NP) explants were isolated [28], collected in F12 medium supplemented with 10% horse serum, and transferred to culture dishes pre-coated with collagen type I (Sigma), 1 mg/ml. Explants were cultivated in DMEM (Sigma) with supplements [29], containing 12% fetal calf serum and 5% chick embryo extract. NP s were cultured in F12-N2 medium [30]. BMP4 (R&D Systems) was used at 300 ng/ml. Retroviral stocks were prepared...