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Stabilization of ATF4 protein is required for the regulation of epithelial–mesenchymal transition of the avian neural crest

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ABSTRACT

Epithelial–mesenchymal transition (EMT) permits neural crest cells to delaminate from the epithelial ectoderm and to migrate extensively in the embryonic environment. In this study, we have identified ATF4, a basic-leucine-zipper transcription factor, as one of the neural crest EMT regulators. Although *ATF4* alone was not sufficient to drive the formation of migratory neural crest cells, *ATF4* cooperated with *Sox9* to induce neural crest EMT by controlling the expression of cell–cell and cell–extracellular matrix adhesion molecules. This was likely, at least in part, by inducing the expression of *Foxd3*, which encodes another neural crest transcription factor. We also found that the ATF4 protein level was strictly regulated by proteasomal degradation and p300-mediated stabilization, allowing ATF4 protein to accumulate in the nuclei of neural crest cells undergoing EMT. Thus, our results emphasize the importance of the regulation of protein stability in the neural crest EMT.

Introduction

Epithelial–mesenchymal transition (EMT) is an important process in the tissue morphogenesis and organogenesis to generate motile cells (Acloque et al., 2009; Kalluri and Weeinberg, 2009 for reviews). During EMT, epithelial cells lose tight cell–cell adhesion, remodel their cytoskeleton so as to lose apicobasal polarity, degrade the basal membrane, and increase the association with the extracellular matrix. The neural crest is one of the model systems to study EMT in development. The neural crest is initially induced at the boundary of neural plate and non-neural ectoderm in vertebrate embryos by BMP and Wnt signalings (Kalcheim and Burstyn-Cohen, 2005; Sakai and Wakamatsu, 2005; Sauka-Spengler and Bronner-Fraser, 2008 for reviews), and crest cells subsequently undergo EMT. Highly motile crest cells eventually give rise to various tissues including neurons, glial cells, melanocytes, and cranial mesenchymal tissues in distinct locations (Le Douarin and Kalcheim, 1999).

In recent years, several transcription factors have been identified as important regulators of the neural crest EMT. Group E *Sox* genes such as *Sox8*, *9*, and *10*, which encode HMG-box transcription factors, appear to be important for formation of neural crest cells. In particular, *Sox9* seems to be essential for the regulation of EMT both in mouse and in chick (Cheung and Briscoe, 2003; Cheung et al., 2005; Sakai et al. 2006). Another EMT-related transcription factor gene, *Snail2*, is also essential for the neural crest EMT in avian embryos (Neito et al., 1994; Sakai et al, 2006), and Sox9 directly activates the transcription of *Snail2* (Sakai et al., 2006). *Foxd3*, a zinc-finger transcription factor gene, is also involved in the neural crest EMT phenotype (Dottori et al., 2001; Cheung et al., 2005). Thus, misexpression of *Foxd3* in the neural tube results in the induction of neural crest markers and in the reduction of *N-cadherin* and the induction of *Integrin-β1* (Cheung et al., 2005), but no (Kos et al., 2001; Suzuki et al., 2006) or limited (Dottori et al., 2001) induction of EMT was observed. Cotransfection experiments revealed that the combination of such transcription factors, *Sox9*, *Snail2*, and *Foxd3*, effectively induces ectopic EMT, along with other traits of neural crest cells, in the transfected neural tube (Cheung et al., 2005).

Posttranslational modifications of these transcription factors appear to be deeply involved in the neural crest development. For example, SOMOylation of *Xenopus* Sox9 modulates its function in neural crest induction and following differentiation (Taylor and LaBonne, 2006). Direct phosphorylation by PKA is essential for chick Sox9 to promote EMT, although this phosphorylation of Sox9 is not required for the activation of *Snail2* transcription (Sakai et al., 2006). Ubiquitination of *Xenopus* Snail2 by Ppa/Fbxl-14, one of the target-recognition subunits of Skp1-CDC53/Cullin-1-F-box protein (SCF) complex, limits the Snail2 protein level via proteasomal degradation (Vernon and LaBonne, 2006). Consistently, *Xenopus*

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Cullin-1 seems to be important for the allocation of the neural crest domain in the embryonic ectoderm (Voigt and Papalopulu, 2005).

In this study, we focus on the function of *Activation transcription factor 4* (*ATF4*, also known as *TAXCREB67*, *CREB-2*, *mTR67*, and *C/ATF*. See Ameri and Harris, 2008 for nomenclatures.), which appears to be expressed at high levels in the neural folds and the migrating neural crest cells of mouse embryos at least at the mRNA level (Murphy and Kolstø, 2000). *ATF4* gene encodes a basic-leucine-zipper-type transcription factor of the ATF/Creb family (Hai and Hartman, 2001, for a review). Numerous reports revealed the *ATF4* functions, such as modulation of metabolic and oxidative stress, regulations of eye development, hematopoiesis, bone morphogenesis, fertility, long-term memory storage, and synaptic plasticity (Ameri and Harris, 2008, for a review), but no function in neural crest development has been reported so far.

In this study, we have identified an avian homolog of *ATF4*, generated by an anti-ATF4 antibody, and found the ATF4 protein rapidly accumulates in the neural crest cells undergoing EMT. Subsequently, our gain- and loss-of-function experiments revealed that *ATF4* is one of the EMT regulating components of neural crest cells. We also show that the nuclear accumulation of ATF4 protein is facilitated in the neural crest cells undergoing EMT by p300-mediated stabilization.

Materials and methods

Experimental animals

Japanese quail (*Coturnix japonica*) eggs were obtained from Sendai Jun-ran, Sendai. Embryos were staged according to Hamburger and Hamilton (1951; HH stage).

In situ hybridization

Whole-mount and section *in situ* hybridizations were performed as described previously (Wakamatsu and Weston, 1997). The coding sequences of quail *ATF4* and *p300* were PCR-amplified from oligo (dT) primed E2 embryo cDNA pool, and were subcloned into *pBluescriptII* (Stratagene). The identity of avian *ATF4* cDNA was confirmed by an amino acid sequence comparison with CREB, ATF4, and ATF5 sequences of other species (see Supplemental Figure 1). The sequences of primers were as follows: ATF4F, GGAAGACACTGGTGATCTCC, ATF4R, CTACTCAGGGACTCTAGCTC; p300F, ATCCTCAGGCACAGCAGATG, p300R, CTAGTGTATGTCTAGTGTAC. Quail *Snail2*, *Sox2*, *Sox9*, and chicken *Sox10* cDNAs for cRNA probes were described previously (Cheng et al., 2000; Endo et al., 2002; Wakamatsu et al., 2004; Sakai et al., 2006). Chicken cDNAs of *Foxd3* (Kos et al., 2001), *Integrin-β1* (Cao et al., 2007), *N-cadherin* (Matsumata et al., 2005), *Cadherin7* and *Cadherin6B* (Nakagawa and Takeichi, 1995) were kind gift from Drs. C. Erickson, F. Gage, M. Uchikawa, and S. Nakagawa, respectively.

Antibodies and immunostaining

Anti-quail ATF4 polyclonal antiserum was raised by immunizing rabbits with a GST-tagged recombinant quail ATF4 protein (aa 1–296, ATF4$^{\Delta C}$). Thus, the corresponding *ATF4* sequence was inserted into *pET41a* (Novagen) for GST-fusion and bacterial expression. Recombinant GST-ATF4 fusion protein was purified from bacterial lysates with B-PER GST-fusion protein purification kit (Pierce). HNK1 mouse IgM and anti-Pax6 rabbit antibodies were described previously (Tucker et al., 1988; Inoue et al., 2000). PAX7 anti-Pax7, 3H11 anti-Laminin, and 6B3 anti-N-cadherin mouse IgG antibodies were obtained from Developmental Study Hybridoma Bank (University of Iowa). M2 anti-FLAG (mouse IgG1; Sigma), anti-GFP (rabbit polyclonal; Chemicon), 12CA5 anti-HA (mouse IgG; Roche), anti-ZO1 (rabbit polyclonal; Zymed), N15 anti-p300 (rabbit polyclonal; Santa Cruz), anti-phospho-Histone H3 (rabbit polyclonal; Upstate), and anti-active caspase 3 (rabbit monoclonal; BD Pharmingen) antibodies were commercially obtained. T8-754 anti-ZO1 mouse IgG was kindly provided by Dr. M. Furuse (Kitajiri et al., 2004). Fluorochrome-conjugated secondary antibodies were purchased from Jackson Immuno Research. Phalloidin conjugated with Texas Red-X or Oregon Green was obtained from Molecular Probes.

Immunological staining on sections and cultured cells was performed as described previously (Wakamatsu et al., 1993, 1997). Immunostaining for ATF4 and p300 required a short fixation condition (in 4% paraformaldehyde/PBS for 30 min at 4 °C). Sections treated with antibodies were also exposed to DAPI (Sigma) to visualize nuclei.

Expression vectors

PCR-amplified coding sequences of quail *ATF4* and *p300* were inserted into expression vectors *pyDF30* and *pyDF-HA* for N-terminal FLAG and HA tagging, respectively. Substitutions of β-TrCP1 binding site (aspartic acid 218 and serine 219) to alanine and the nuclear localization signal (lysine 281, 282, 284, 285; Cibeli et al., 1999) to asparagine were introduced to wild type *ATF4* sequence by using in vitro mutagenesis kit (Stratagene). For generating a repressor form of *ATF4* (*En-ATF4$^{\Delta N}$*), recombinant PCR was performed to generate N-terminal deletion of *ATF4* cDNA lacking the corresponding sequence (aa 1–180), and the deletion mutant cDNA was subsequently fused to the repression domain of *Engrailed2* (a gift from Dr. H. Nakamura; Matsunaga et al., 2000) to construct *pyDF-HA-En-ATF4$^{\Delta N}$*. The dominant-negative action of *En-ATF4$^{\Delta N}$* was confirmed by the fact that the transcriptional activation of *pCRE-luc*, a *Luciferase* reporter gene under the control of cAMP responsive element (Clontech), was activated by wild type *ATF4*, and that *En-ATF4$^{\Delta N}$* cotransfection attenuated the activation (Supplemental Figure 2). An expression vector of quail *Sox9* was previously described (Sakai et al., 2006). *GFP*-tagged chicken *Foxd3* expression construct (Kos et al., 2001) was kindly provided by C. Erickson, and GFP-tag was removed (*pyDF-HA-Foxd3*). Expression of these transgenes was confirmed by immunostaining of transfected cells by anti-epitope tag antibodies and/or Western blotting (data not shown). *pEGFP-N1* was purchased from Clontech. When HA-tagged *ATF4* was transfected into the neural tube along with *pEGFP-N1*, more than 70% of EGFP-positive cells coexpressed HA-ATF4 protein in their nuclei, revealed by immunostaining of sections. In contrast, only 17% of EGFP-positive cells coexpressed FLAG-tagged ATF4 protein, when cotransfected (see text and Fig. 6). Thus, for studying the misexpression phenotype, HA-tagged *ATF4* vector was used, while for testing the protein expression efficiency, FLAG-tagged versions were used. In addition, FLAG-tagged *ATF4* cDNAs were subcloned into *pCMS-EGFP* dual promoter vector (Clontech) to ensure coexpression of *EGFP* and *ATF4* transgenes in individual transfected cell.

Neural plate explant culture

Cultures of neural plate explants were performed as described previously (Wakamatsu et al., 2004, Sakai et al., 2005). In brief, intermediate fragments of the neural plate at the level of forebrain and midbrain were surgically dissected with a tungsten needle along with underlying mesoderm and endoderm. To remove the mesoderm and the endoderm, the dissected tissues were treated with Pancreatin (Wako). The isolated neural plates were cultured in F12-based medium containing 3% FCS on fibronectin (Sigma)-coated dishes. N2-supplement (1:100 dilution, Invitrogen) and recombinant human BMP4 (20 ng/ml; R&D Systems) were added in culture to induce neural crest formation. Ten micromolars of MG132 (Wako) was added in culture as a proteasome inhibitor, when indicated.