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The importance of basonuclin 2 in adult mice and its relation to basonuclin 1

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ABSTRACT

BNC2 is an extremely conserved zinc finger protein with important functions in the development of craniofacial bones and male germ cells. Because disruption of the *Bnc2* gene in mice causes neonatal lethality, the function of the protein in adult animals has not been studied. Until now BNC2 was considered to have a wider tissue distribution than its paralog, BNC1, but the precise cell types expressing *Bnc2* are largely unknown. We identify here the cell types containing BNC2 in the mouse and we show the unexpected presence of BNC1 in many BNC2-containing cells. BNC1 and BNC2 are colocalized in male and female germ cells, ovarian epithelial cells, sensory neurons, hair follicle keratinocytes and connective cells of organ capsules. In many cell lineages, the two basonuclins appear and disappear synchronously. Within the male germ cell lineage, BNC1 and BNC2 are found in prospermatogonia and undifferentiated spermatogonia, and disappear abruptly from differentiating spermatogonia. During oogenesis, the two basonuclins accumulate specifically in maturing oocytes. During the development of hair follicles, BNC1 and BNC2 concentrate in the primary hair germs. As follicle morphogenesis proceeds, cells possessing BNC1 and BNC2 invade the dermis and surround the papilla. During anagen, BNC1 and BNC2 are largely restricted to the basal layer of the outer root sheath and the matrix. During catagen, the compartment of cells possessing BNC1 and BNC2 regresses, and in telogen, the two basonuclins are confined to the secondary hair germ. During the next anagen, the BNC1/BNC2-containing cell population regenerates the hair follicle. By examining *Bnc2*$^{-/-}$ mice that have escaped the neonatal lethality usually associated with lack of BNC2, we demonstrate that BNC2 possesses important functions in many of the cell types where it resides. Hair follicles of postnatal *Bnc2*$^{-/-}$ mice do not fully develop during the first cycle and thereafter remain blocked in telogen. It is concluded that the presence of BNC2 in the secondary hair germ is required to regenerate the transient segment of the follicle. Postnatal *Bnc2*$^{-/-}$ mice also show severe dwarfism, defects in oogenesis and alterations of palatal rugae. Although the two basonuclins possess very similar zinc fingers and are largely coexpressed, BNC1 cannot substitute for BNC2. This is shown incontrovertibly in knockin mice expressing *Bnc1* instead of *Bnc2* as these mice invariably die at birth with craniofacial abnormalities undistinguishable from those of *Bnc2*$^{-/-}$ mice. The function of the basonuclins in the secondary hair germ is of particular interest.

1. Introduction

Basonuclin 1 (BNC1) possesses three pairs of zinc fingers and a nuclear localization signal (NLS) (Tseng and Green, 1992). BNC1 was hitherto thought to be very restricted in its tissue distribution as it had been found only in basal keratinocytes of stratified squamous epithelium and in certain keratinocytes of hair follicles. It was later detected in lens and corneal epithelia, and in reproductive germ cells (Mahoney et al., 1998; Tseng and Green, 1994; Tseng et al., 1999; Yang et al., 1997). The only known function of BNC1 is that of a transcription factor in the synthesis of ribosomal RNA (Iuchi and Green, 1999). BNC1 is essential for oogenesis and spermatogenesis (Ma et al., 2006; Zhang et al., 2012). No function of BNC1 in hair follicles has been reported.

A gene encoding a second basonuclin (BNC2) has been discovered independently by us (Vanhoutteghem and Djian, 2004) and by Romano et al. (2004). The deduced amino acid sequences of BNC1 and BNC2 of the mouse are only about 44% identical, but BNC2 possesses zinc fingers and an NLS very similar to those of BNC1. The *Bnc2* mRNA is thought to have a wider tissue distribution than BNC1 as it has been found not only in skin and testis, but also in tissues that were considered to be devoid of BNC1 (Vanhoutteghem and Djian, 2004). Disruption of the *Bnc2* gene in mice causes neonatal death associated with cleft palate

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and craniofacial abnormalities. This phenotype results from a direct effect of BNC2 on the multiplication of craniofacial mesenchymal cells (Hervé et al., 2012; Vanhoutteghem et al., 2009, 2011). BNC2 is also found in prospermatogonia (gonocytes) and spermatogonial stem cells, where it acts as a repressor of both meiosis and mitosis (Vanhoutteghem et al., 2014). In the human, BNC2 has been associated with susceptibility to ovarian cancer (Goode et al., 2010; Song et al., 2009; Winham et al., 2014), urethral abnormalities (Bhoj et al., 2011) and adolescent idiopathic scoliosis (Ogura et al., 2015).

Using newly developed antibodies and the Ayu21-18 mice, whose *Bnc2* gene is disrupted by *lacz* (Vanhoutteghem et al., 2009), we identify the cell types containing BNC2 in the mouse and we show that most of these cell types also contain BNC1. Taking advantage of a small number of $Bnc2^{-/-}$ mice that have survived into adulthood because their palates have closed, we show that adult mice lacking BNC2 present a complex syndrome associating dwarfism, infertility, developmental abnormalities of palatal rugae and inability of hair follicles to cycle. We also demonstrate that expression of *Bnc1* instead of *Bnc2* does not correct the cleft palate and the lethality caused by lack of BNC2.

2. Material and methods

2.1. Mice

All mice were housed and used at the central animal facility of the Neuroscience Institute of Université Paris Descartes under an approved institutional animal care and use committee protocol following the national guidelines for the care and use of laboratory animals. We used the Ayu21-18 mouse line whose *Bnc2* gene is disrupted by a *lacz*-containing vector.

2.2. Histological analysis

For indirect immunofluorescence staining, samples were snap frozen in OCT (Miles) and isopentane, and sectioned (6 μm) using a cryomicrotome. Sections were fixed in 4% formaldehyde for 5 min on ice, permeabilized in PBS containing 0.2% Triton X-100. Nonspecific sites were blocked with 5% BSA in PBS and sections were stained with primary antibodies as described earlier (Vanhoutteghem and Djian, 2007). Antibodies used are listed in Table S1.

For both histochemical detection of β-galactosidase activity and hematoxylin/eosin staining, samples were successively incubated in 4% formaldehyde for 1 h, in PBS containing 15% sucrose for 3 h, in PBS containing 30% sucrose overnight and finally snap frozen in OCT and isopentane. Samples were cut with a cryomicrotome at −27 °C and sections (7 μm) were stained in the presence of X-gal overnight at 37 °C or in the presence of hematoxylin and eosin. For examination of palate and teeth, newborn heads were fixed in Bouin's solution for 24 h. Photographs were then acquired on 20 consecutive planes and reconstituted using Adobe Photoshop CS4.

2.3. Measurement of GH and IGF levels

Circulating growth hormone and IGF1 levels were measured by ELISA on two one-month-old $Bnc2^{-/-}$ mice and two wt mice of the same age. Measurements were carried out at the Clinique de la Souris, Illkirch, France.

2.4. Production of Bnc1 knockin mice ($Bnc2^{KIBnc1/KIBnc1}$)

2.4.1. Generation of mouse Bnc1 cDNA

For producing *Bnc1* knockin mice, it was first necessary to generate the complete cDNA for mouse BNC1. This cDNA was produced in two fragments that were then joined together. The very GC-rich 5′ region (220 bp) was synthesized chemically while the rest of the cDNA (about 3 kb) was amplified by RT-PCR.

RNA was prepared from mouse keratinocytes propagated in low calcium medium, on collagen-coated Petri dishes, in the presence of 3T3-J2 feeder cells. 3T3-J2 cells were maintained and treated with mitomycin C, as described previously (Vanhoutteghem et al., 2004). Skin fragments of newborn mice were rinsed several times in PBS and floated (epidermal side up) in HEPES buffered saline containing 0.5 mg/ml thermolysin (Sigma) for 45 min at 37 °C. The epidermis was then peeled off with forceps and disaggregated in a trypsinizing flask for 15 min at 37 °C in the presence of 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid. Then cells were sedimented by centrifugation for 5 min at 800 × *g* and plated on collagen-coated Petri dishes containing mitomycin-treated 3T3-J2F cells (2.5×10^4 3T3 cells/cm^2) and cultivated as previously described (Allen-Hoffmann and Rheinwald, 1984; Simon and Green, 1985) except that the medium was a 3:1 mixture of Dulbecco-Vogt and F12 medium (Life Technologies, low calcium DMEM).

First strand synthesis was initiated with oligo(dT) from 5 μg of total RNA prepared from cultured mouse keratinocytes. The *Bnc1* cDNA was then amplified with an *Eco*RI-site containing sense primer in the 3' region of exon 1 (5'-GGAATTCCAGCGGCTGCAGGATGGC-3') and an *Xba*I-site-containing antisense primer in exon 5 (5'-GCTCTAGATGGCTTGTTACTGGAGGTG-3'). PCR conditions were 30 cycles at 95 °C for 1 min, 59 °C for 1 min and 68 °C for 4 min in the presence of *Pfu Turbo* DNA polymerase (Stratagene). After being digested with *Eco*RI and *Xba*I, the PCR product was ligated to pGEM-3Zf(-) (Promega). The resulting plasmid (pGEM-Bnc1) was then digested with *Eco*RI and *Bam*HI (internal site in the *Bnc1* cDNA). The 5′ GC-rich region of the cDNA (226 bp, containing the *Bam*HI internal site) was synthesized by Eurofins MWG Operon (Ebersberg, Germany) and cloned into pCR2.1 (5′ and 3′ cloning sites were *Eco*RI and *Bam*HI). The resulting plasmid was digested with *Eco*RI and *Bam*HI and the insert was cloned into pGEM-Bnc1 previously digested with *Eco*RI and *Bam*HI, thus generating a plasmid containing the entire mouse *Bnc1* cDNA coding region but neither the 5′ nor the 3′ untranslated regions. The sequence of the cDNA was verified by Eurofins MWG Operon.

2.4.2. Plasmids used for knockin

We used the Cre expression vector pCAGGS-Cre (Araki et al., 1995). The replacement vector was assembled from components of pSP73 (Promega), the *lox* KR3 sequence (Araki et al., 2010), the splice acceptor (SA) sequence of the mouse *En-2* gene, the cDNA for BNC1, the *FRT* sequence, the mouse *phosphoglycerate kinase-1* (*Pgk*) gene promoter, the *puromycin N-acetyltransferase* (*Pac*) gene and the *lox*P sequence.

2.4.3. ES cell cultures

ES cells were cultivated in KSR-GMEM medium consisting of Glasgow Minimum Essential Medium (GMEM) (Sigma) with 1× MEM non-essential amino acids (Gibco Invitrogen), 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1% FBS (Hyclone), 14% Knockout™ Serum Replacement (KSR; Gibco), and 1100 U/ml leukemia inhibitory factor (ESGRO, Chemicon). For neutralization of trypsin, FBS-GMEM in which the KSR in KSR-GMEM was replaced with FBS (final concentration, 15% FBS) was used (Taniwaki et al., 2005). Replacement by Cre-mediated recombination in ES cells was performed as described previously (Araki et al., 1999, 2010).

2.4.4. Analysis of DNA

ES cells were lysed with sodium dodecyl sulfate/proteinase K, treated with 1:1 (vol/vol) phenol/chloroform, precipitated with ethanol, and dissolved in 10 mM Tris–HCl, pH 7.5 containing 1 mM ethylenediamine tetraacetic acid. Genomic DNA (6 μg) was digested with appropriate restriction enzymes, resolved by electrophoresis in a 0.9% agarose gel, and blotted onto a nylon membrane (Roche). Hybridization was performed using a DIG DNA Labeling Kit (Roche). For PCR detection of the 5′ junction, DNA (50 ng) was subjected to 30 cycles of amplification (30 s at 94 °C, 60 s at 60 °C and 60 s at 72 °C, using KOD-FX DNA polymerase (Toyobo Life Science). The primer sequences were as follows: SA-125