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Post-transcriptional regulation of the Brn-3b transcription factor in differentiating neuroblastoma cells

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Abstract The post-transcriptional control of mRNA levels is a very powerful mechanism which allows cells to quickly change the amount of specific proteins. In this study, we wanted to analyze whether the Brn-3b transcription factor, essential for the proper development of mouse retinal ganglion cells, is subjected to such post-transcriptional regulation. In particular, due to its conservation amongst different species, we wanted to study the role of its 3′ untranslated region (3′UTR).

We show that the 3′UTR of the Brn-3b mRNA does indeed contain regulatory sequences that mediate mRNA degradation upon serum starvation-induced differentiation of ND7 neuroblastoma cells. The specific region mediating this effect has been characterized and two different microRNAs that potentially regulate the stability of Brn-3b have been identified. Moreover we show that Dicer, one of the key enzymes in the production of microRNAs, is strongly up-regulated in ND7 cells subjected to differentiation.

Keywords: 3′UTR; Brn-3b; microRNA; Differentiation; Dicer

1. Introduction

Brn-3a and Brn-3b, members of the POU IV class of transcription factors are expressed in different parts of the central and peripheral nervous system [1–5]. While mice lacking Brn-3a die a few days after birth, Brn-3b knock-out mice are viable but exhibit a specific and dramatic loss of up to 70% of retinal ganglion cells (RGC) [6].

Our laboratory has previously shown that when the ND7 neuroblastoma cell line is induced to differentiate via serum removal, Brn-3b levels decrease while Brn-3a is increased [7]. The switch between Brn-3a and Brn-3b is extremely important and allows neuronal cells to activate different subset of genes involved in the cellular routing toward differentiation (Brn-3a) [8–12], or proliferation (Brn-3b) [13–16]. A stringent regulation of Brn-3a and Brn-3b expression is thus essential for cellular fate and development.

The control of gene expression and overall protein synthesis is a key process in the metabolism of a cell. Post-transcriptional regulation of mRNA half-life or the regulation of its translation allows cells to have a very fine and rapid control over protein synthesis. One of the known mechanisms involved in the modulation of mRNA stability resides in their 3′UTR, a region containing motifs, such as the AU-rich regions (AREs) which regulate mRNA half-life [17,18].

In this work, we wanted to understand whether Brn-3b mRNA is subjected to a post-transcriptional regulation in ND7 cells induced to differentiate and whether this process is mediated by its 3′UTR. This region, in fact, exhibits between 99% and 97% nucleotide identity between human and mouse and mouse and rhesus monkey, respectively (our unpublished observation), suggesting a possible important role due to its conservation.

The data presented herein show that the 3′UTR of Brn-3b mediates the degradation of a chimaeric reporter mRNA. This process is induced by differentiation stimuli applied to ND7 cells and is likely to be mediated by two different microRNAs. Furthermore we also present evidence that Dicer, one of the key enzymes in the production of microRNAs, is strongly up-regulated in ND7 cells induced to differentiate.

2. Materials and methods

2.1. Constructs and vectors

The 3′UTR of Brn-3b (NM_138944) was amplified from mouse genomic DNA with the following primers: 3bUTR-SacI: 5′gagctctagggaccctctccagggatggccc3′ and 3bUTR-BamHI: 5′ggaacccgggtgccagtaaaacattaaaatcatctc3′. The resulting circa product was sub-cloned in the multiple cloning site of pEGFP-C1, downstream of GFP. The mutants in the microRNA target sequence were obtained via the QuickChange site directed mutagenesis (Stratagene) following manufacturer's instruction with the following primers: mir-23(upstream)-Fw-5′aacaattccggtaaacgggcacccagaccaagccag3′, mir-23(upstream)-Rw-5′ctggcttggtctgggtgcccgtttaccggaattgtt3′, mir-23(downstream)-Fw5′gagttgatgcttaacgggctatgatagagacatctc3′, mir23(downstream)Rw5′gagatgtctctatcatagcccgttaagcatcaactc3′. mir214-Fw5′ggaagttgcgacggttgcgttcactg3′; mir214-Rw5′cagtgaacgcaaccgtcgcaacttcc3′, mir128-Fw5′ccgtgtagaccagatgcgctggcggaagtc3′; mir128-Rw5′caagacttccgccagcgcatctggtctaca3′. The sequential deletion of Brn-3b was performed with the Bal-31 nuclease (New England Biolabs). All constructs have been checked by sequencing.

2.2. Cell culture

The neuroblastoma ND7 and the breast cancer MCF-7 cell line were routinely cultured in DMEM supplemented with 10% Foetal Calf Serum (FCS) at 37°C, 95% humidity and 5% CO_2. Transfection was performed with GeneJuice (Novagen) according to the manufacturer's instruction. SH-SY5Y cells were cultured in 50% MEM, 50% Hams F12 supplemented with 15% FCS. For all the serum starvation experiments, cells were transfected with the indicated constructs and the following day (day 0) incubated in full medium with or without FCS in order to induce differentiation for the indicated days.

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2.3. RNA extraction, real-time RT-PCR, Northern blot

For mRNA turnover experiment ND7 cells were transfected and treated as described above. Post-transfection cells (48 h) were treated with actinomycin D (7.5 μg/ml) for 0, 4 and 8 h [19]. Total RNA, extracted with Trizol (Invitrogen), has been subjected to real-time RT-PCR reaction using SYBR I Green technology on the DNA Engine Opticon System (MJ Research) as previously described [20] with primers specific for GFP and for neomycin for normalization purposes.

For Northern blot analysis of small RNA, 30 μg of total RNA were loaded and run on a 15% polyacrilamide, 7 M UREA gel. The prehybridized membrane was incubated overnight with the appropriate probes at 42°. Probes: U6-gcagggccatgctaatcttctctgtatcg; mir-214-ctgcctgtctgtgcctgctgt; mir-128-aaaagagaccggttcac tgtga; mir-23-ggaaatccctggcaatgtgat, were end labeled with T4 Kinase in the presence of γ^{32}P-ATP. Washes were done at room temperature in 2× SSC, 0.1% SDS. Membranes were then exposed to a phosphor-imager screen.

2.4. Western blot

Transfected cells were lysed with RIPA buffer (10 mM TRIS pH 7.5, 150 mM NaCl, 0.2% NP-40) supplemented with protease inhibitors (mini-cocktail, Roche). Membranes were probed with an anti-GFP antibody (Sigma G-1544), stripped and reprobed with anti-neomycin antibody (Upstate) for normalization purposes. For Dicer, an anti-Dicer antibody (St. Cruz) was used and data normalized with anti-p85 (Upstate). Western blots were scanned and bands analyzed via the QuantityOne software (Biorad). All experiments were repeated at least in triplicate. Error bars indicate S.D.

3. Results

3.1. The 3′UTR of Brn-3b mediates the down-regulation of a chimaeric reporter construct

The 3′UTR of Brn-3b was sub-cloned downstream of GFP in the peGFP expression vector. The peGFP::3bUTR and the empty peGFP control constructs were transfected into neuroblastoma ND7 cells which were then induced to differentiate via serum removal and harvested at different time points. After 1, 3 or 5 days, GFP levels were assessed by Western blot and normalized with neomycin, which is expressed from the same vector. As shown in Fig. 1A serum removal induces a progressive and strong reduction of GFP expression in the construct containing the 3′UTR of Brn-3b compared to the 'empty' vector.

In order to confirm the result, we repeated the experiment in the SH-SY5Y human neuroblastoma and in the MCF-7 human breast cancer cell line. As shown in Fig. 1B, C, the levels of the chimaeric mRNA are reduced in the SH-SY5Y but not in the MCF-7 cell line following serum removal thus suggesting that the chimaeric mRNA is not intrinsically unstable and that its down-regulation seems to be specific for neuronal cell lines and to occur in both human and mouse cells.

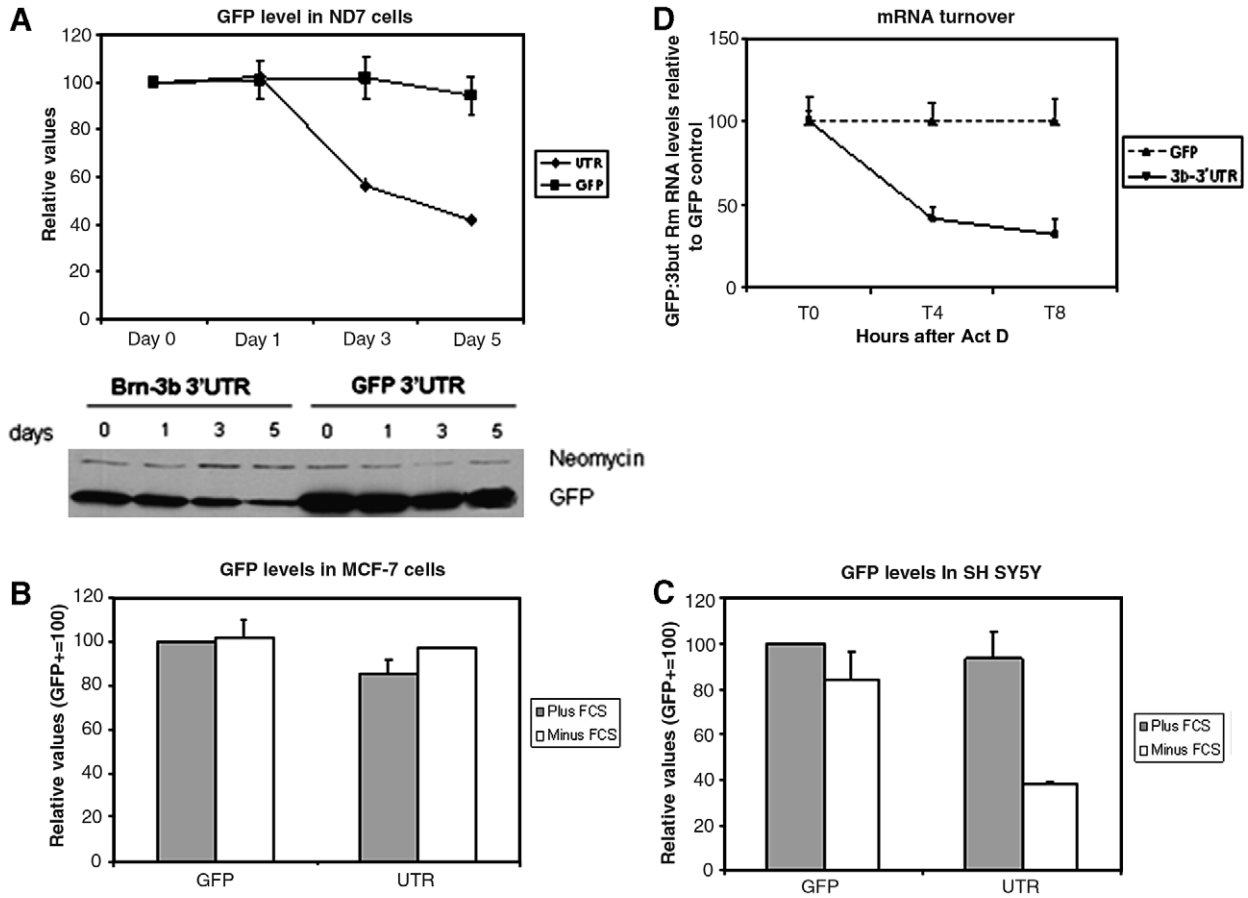

Fig. 1. Effect of the 3′UTR of Brn-3b on the stability of a chimaeric construct. (A) Western blot analysis of GFP levels in ND7 cells transfected with the GFP::3b-3′UTR but not with the GFP::UTR control vector and subjected to differentiation via serum removal for the indicated days (upper panel). A typical Western blot is represented in the lower panel. GFP levels in the SH-SY5Y neuronal cells (B) but not in MCF-7 breast cancer cell line (C) serum starved for two days after transfection. (D) Realtime RT-PCR analysis of GFP:3b-3′UTR mRNA levels after Actinomycin D treatment for the indicated hours. Data have been normalized with neomycin and expressed relative to peGFP control. All experiments have been repeated at least in triplicate. Error bars indicate S.D.