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Research Article

PHOX2A and PHOX2B are differentially regulated during retinoic acid-driven differentiation of SK-N-BE(2)C neuroblastoma cell line

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ABSTRACT

PHOX2B and its paralogue gene PHOX2A are two homeodomain proteins in the network regulating the development of autonomic ganglia that have been associated with the pathogenesis of neuroblastoma (NB), because of their over-expression in different NB cell lines and tumour samples. We used the SK-N-BE(2)C cell line to show that all-*trans* retinoic acid (ATRA), a drug that is widely used to inhibit growth and induce differentiation in NBs, regulates both PHOX2A and PHOX2B expression, albeit by means of different mechanisms: it up-regulates PHOX2A and down-regulates PHOX2B. Both mechanisms act at transcriptional level, but prolonged ATRA treatment selectively degrades the PHOX2A protein, whereas the corresponding mRNA remains up-regulated. Further, we show that PHOX2A is capable of modulating PHOX2B expression, but this mechanism is not involved in the PHOX2B down-regulation induced by retinoic acid. Our findings demonstrate that PHOX2A expression is finely controlled during retinoic acid differentiation and this, together with PHOX2B down-regulation, reinforces the idea that they may be useful biomarkers for NB staging, prognosis and treatment decision making.

1. Introduction

A neuroblastoma, one of the most frequent tumours of childhood, is caused by the arrested differentiation of neural crest sympatho-adrenal progenitor cells [1]. PHOX2B, and its paralogue PHOX2A, are two homeodomain transcription factors that play a pivotal role in the development of the autonomic nervous system and specification of the neurotransmitter phenotype by controlling the expression of the two enzymes responsible for noradrenaline biosynthesis (tyrosine hydroxylase [*TH*] and dopamine-β-hydroxylase [*DβH*]), and thus directing neurons towards their terminal noradrenergic differentiation [2,3]. PHOX2B also modulates its own expression by means of an auto-regulatory mechanism [4] and the expression of *PHOX2A* [5,6], whereas PHOX2A regulates the expression of the human *α3* nAChR subunit gene [7]. Both therefore play a primary role in controlling a number of the molecular determinants of autonomic neurons.

PHOX2A and PHOX2B are also involved in coordinating cell cycle exit and the differentiation of neural progenitors during sympathetic neuronal differentiation [8] as a result of their ability to induce the transcription of $p27^{Kip1}$ [9–11], a cyclin-dependent kinase inhibitor (CKI) whose expression is also regulated by retinoic acid (RA) at post-translational level [12], followed by PHOX2B down-regulation during final neuronal differentiation [13].

Recently, the *PHOX2A* gene has been localised to near the deletion breakpoint of a number of 11q-deleted NB specimens [14], and microarray expression analysis has shown that it is one of nine noradrenaline biosynthesis pathway genes whose expression is reduced in unfavourable NB tumours [14]. However, the possible contribution of PHOX2A to the pathogenesis of NB is not univocal as it is over-expressed in a number of NB tumours and cell lines [15]. As no mutations have been observed in the *PHOX2A* regulatory or coding regions of tumour samples [14,16], it is likely that this gene is involved in the pathogenesis of NB when its

Abbreviations: α3 nAChR, alpha 3 nicotinic Acetylcholine Receptor; ATRA, *all-trans* Retinoic Acid; BMP-2, Bone morphogenetic protein-2; CCHS, Congenital Central Hypoventilation Syndrome; Cdk, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; CKI, Cdk inhibitor; DβH, dopamine-β-hydroxylase; DR, directed repeat; GDNF, glial derived neurotrophic factor; HSCR, Hirschprung's disease; NB, neuroblastoma; NT3, neurotrophin 3; RAR, retinoic acid receptor; RARE, retinoic acid responsive element; RXR, retinoid X receptor; TH, tyrosine hydroxylase; TH-MYCN, tyrosine hydroxylase-*v*-myc avian myelocytomatosis viral oncogene

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expression is deregulated in either direction.

The pathogenetic role of PHOX2B in NB is supported by the presence of heterozygous mutations in familial, sporadic and syndromic cases of NB, and its over-expression in tumour samples and NB cell lines, sometimes associated with other neurochristopathies such as Congenital Central Hypoventilation Syndrome (CCHS) and Hirschprung's disease (HSCR) [16–23], but the underlying mechanisms are still largely unknown. *In vitro* and *in vivo* studies have linked the PHOX2B mutations associated with NB with the impaired differentiation of immature sympathetic neurons that can proliferate, and aberrant differentiation towards the glial lineage [10,24]. PHOX2B over-expression leads to contradictory results as some studies indicate that it inhibits the proliferation of motoneuron progenitors and of immature sympathetic neurons [8,10,16] and promotes the differentiation of human NB cells after treatment with RA [16], whereas conditional Phox2b knockout studies have revealed that Phox2b is required for the proliferation of immature sympathetic neurons [25], and Alam et al. [13], and Ke et al. [23] have shown that a high level of PHOX2B promotes neuroblastoma cell proliferation and xenograft tumour growth in the TH-MYCN murine model, and that this correlates with a high level of MYCN expression. Furthermore, the presence of aberrant Phox2b expression in a zebrafish model has shown that the correct amount of the *Phox2b* gene is important for the differentiation of sympathetic neurons [26].

Vitamin A (retinol) profoundly affects various biological processes during development and adulthood. Most of its actions are mediated by its metabolic product, retinoic acid, which binds to specific nuclear receptors: heterodimers of retinoic acid receptors (RARs) α, β and γ, and retinoid X receptors (RXRs) α, β and γ. These ligand-activated receptors regulate gene transcription by binding to retinoic acid responsive elements (RAREs) in the promoter regions of responsive genes [27]. At embryological level, retinoids control the proliferation, migration and differentiation of neural crest-derived progenitors and, in developing sympathetic neurons, RA cooperates with Bone morphogenetic protein-2 (BMP-2) to make cells responsive to neurotrophic factors such as glial derived neurotrophic factor (GDNF) and neurotrophin 3 (NT3) [28,29]. The pleiotropic effects of RA on the regulatory network governing sympathetic neuron differentiation are well known, but very little is known about its effect on the transcription factors (such as MASH1, PHOX2A and PHOX2B) that play a fundamental role in this process. *In vitro*, retinoids arrest cell growth in the G1 phase of the cell cycle, and induce differentiation in human NB cell lines [30,31] along neuronal- or glial-like lineages depending on the cell line [32] by regulating, for example, the expression of $p27^{Kip1}$, a target gene of PHOX2A and PHOX2B that has major functions in controlling the cell cycle.

As the target genes mediating retinoid-induced differentiation are largely unknown, and the molecular mechanisms by which RA regulates the different signalling pathways necessary for retinoid-induced cellular differentiation in various tissues and at different times are poorly understood, we tested the hypothesis that there may be a direct regulatory link between RA and PHOX2A and PHOX2B expression/activity in the SK-N-BE(2)C NB cell line. The findings show that the retinoic-acid induced differentiation of SK-N-BE(2)C cells is accompanied by a differential regulation of *PHOX2A* and *PHOX2B* expression, with up-regulation of *PHOX2A* mRNA followed by the disappearance of PHOX2A protein (the mRNA remains stably expressed), and a marked decrease in the expression of *PHOX2B* mRNA and protein, thus suggesting that their expression must be finely controlled during RA-induced differentiation, reinforcing the idea that they may be useful biomarkers for NB staging, prognosis and treatment decision making.

2. Material and methods

2.1. Cell lines and cultures

The SK-N-BE(2)C and IMR32 human neuroblastoma cell lines were grown in RPMI 1640, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Lonza). All-*trans* retinoic acid (ATRA; Sigma-Aldrich, St.Louis, Missouri, USA), dissolved in 100% EtOH, was added at a final concentration of 10 μM for the times described in the and the medium was changed every day. Each treatment was carried out in duplicate and repeated at least three times in independent experiments using different batches of ATRA. Cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) was added at a final concentration of 10 μg/ml before or after ATRA for the times described in the The proteasome inhibitor MG-132 (8 μM; Calbiochem, Darmstadt, Germany) was added for eight and 24 h after initial treatment with ATRA for 24 and 48 h.

2.2. Total RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA was extracted and reverse transcribed, and gene expression was quantitatively analysed as described by Benfante et al. [33] with minimal modifications. The TaqMan® primer and probe assays (Life Technologies, Inc., Carlsbad, CA, USA) were *PHOX2A* (ID #Hs00605931_mH) and *PHOX2B* (ID #Hs00243679_m1), and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*; ID# Hs99999905_m1) was used as an endogenous controls after its compatible with the other assays had been confirmed. The results were calculated using the $2^{-\Delta CT}$ and the $2^{-\Delta\Delta CT}$ methods in order to allow the normalisation of each sample to the endogenous control, and comparison with the calibrator of each experiment (set to a value of 1) as described in the figure legends.

2.3. Nuclear run-on

Nuclear run-on transcription was performed in accordance with the protocol described by Patrone et al. [34]. The nuclei (5×10^7) were prepared from SK-N-BE(2)C cells treated for 24 h with ATRA or vehicle. RNA was synthesised *in vitro* by adding an equal volume of transcription buffer containing 0.4 mM biotin-16-UTP (Roche Diagnostics SpA, Monza, Milan, Italy), and the biotin-labelled RNA was isolated by means of streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal Biotech ASA, Oslo, Norway). Gene expression was quantitatively analysed by reverse transcribing 8 μl of the nuclear RNA sample and 1 μg of the total RNA sample and using real-time PCR.

2.4. Chromatin immunoprecipitation and qPCR

Chromatin immunoprecipitation was carried out as previously described [4]. Chromatin was incubated overnight at 4 °C with 5 μg of anti-PHOX2A antibody (Davids Biotechnologie, Regensburg, Germany), and chicken pre-immune IgY (Davids Biotechnologie), and the immunocomplexes were collected on monoclonal anti-chicken IgY-agarose beads or protein G/agarose bead slurry (InVitrogen, Carlsbad, CA, USA) pre-adsorbed with 20 μg/μl tRNA and 10 μg/μl salmon sperm DNA (Sigma-Aldrich). After washes and elution, the cross-linking was reversed by heating to 65 °C overnight, and the samples were purified on columns (High Pure PCR product purification kit, Roche Diagnostics SpA, Italy). For the PCR detection of the immunoprecipitated chromatin, 5% of the purified DNA was used as a template to amplify the PHOX2B promoter using the primers ChIP[2 bprom] UP, 5′-CAA GCT TAT TTC CAA GTA GTG TGA TTG AAT-3′, and ChIP[2bprom] LOW, 5′-GCC TCC TAT