Developmental Biology 329 (2009) 191–200

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

The bHLH transcription factor *Hand2* is essential for the maintenance of noradrenergic properties in differentiated sympathetic neurons

Mirko Schmidt [a], Shengyin Lin [b], Manuela Pape [a], Uwe Ernsberger [c], Matthias Stanke [a,1], Kazuto Kobayashi [d], Marthe J. Howard [b], Hermann Rohrer [a,*]

[a] RG Developmental Neurobiology, Department of Neurochemistry, MPI for Brain Research, Deutschordenstr. 46, 60528 Frankfurt/M, Germany
[b] Department of Neurosciences, Program in Neurosciences and Degenerative Disease, University of Toledo Health Sciences Center, Toledo, OH 43614, USA
[c] Interdisciplinary Center for Neurosciences (IZN), University of Heidelberg, INF 307, 69120 Heidelberg, Germany
[d] Department of Molecular Genetics, Institute of Biomedical Sciences, Fukushima University School of Medicine, Fukushima, Japan

ARTICLE INFO

Article history:
Received for publication 21 October 2008
Revised 17 February 2009
Accepted 18 February 2009
Available online 27 February 2009

Keywords:
Hand2
siRNA knockdown
TH
DBH
ChAT
VAChT
Sympathetic neuron survival
Proliferation
Sympathetic ganglia

ABSTRACT

The basic helix–loop–helix transcription factor *Hand2* is essential for the proliferation and noradrenergic differentiation of sympathetic neuron precursors during development. Here we address the function of *Hand2* in postmitotic, differentiated sympathetic neurons. Knockdown of endogenous *Hand2* in cultured E12 chick sympathetic neurons by siRNA results in a significant (about 60%) decrease in the expression of the noradrenergic marker genes *dopamine-β-hydroxylase* (*DBH*) and *tyrosine hydroxylase* (*TH*). In contrast, expression of the pan-neuronal genes TuJ1, HuC and *SCG10* was not affected. To analyze the in vivo role of *Hand2* in differentiated sympathetic neurons we used mice harboring a conditional *Hand2*-null allele and excised the gene by expression of Cre recombinase under control of the *DBH* promotor. Mouse embryos homozygous for *Hand2* gene deletion showed decreased sympathetic neuron number and TH expression was strongly reduced in the residual neuron population. The in vitro *Hand2* knockdown also enhances the CNTF-induced expression of the cholinergic marker genes *vesicular acetylcholine transporter* (*VAChT*) and *choline acetyltransferase* (*ChAT*). Taken together, these findings demonstrate that the *Hand2* transcription factor plays a key role in maintaining noradrenergic properties in differentiated neurons.

Introduction

The development of sympathetic neurons is initiated by bone morphogenetic proteins (BMPs) acting on neural crest cells that have migrated to the dorsal aorta. BMPs control the expression of a group of transcription factors, including *Ascl1*, *Phox2b*, *Phox2a*, *Hand2* and *Gata2/3* that play important roles in sympathetic neuron specification and differentiation (reviewed in Goridis and Rohrer, 2002; Howard, 2005; Rohrer, 2003). Sympathetic neuron development is completely prevented in the absence of *Phox2b*, which is essential for development of the autonomic nervous system (Pattyn et al., 1999). *Ascl1* mainly affects the timing of sympathetic neuron development, as shown by delayed sympathetic neuron differentiation in *Ascl1*-deficient mouse embryos (Pattyn et al., 2006). A similar role has been demonstrated for the transcription factor *Insm1*, which in addition also affects sympathetic neuron proliferation (Wildner et al., 2008). Whereas the knockouts of *Phox2b*, *Ascl1* and *Insm1* displayed pleiotropic functions on noradrenergic and generic neuronal differentiation, more selective effects on the expression of noradrenergic marker genes were observed in the absence of *Gata2/3* and *Hand2*, in particular during early development. In the absence of *Gata3* a selective decrease of *TH* expression was found at E10.5, followed by impaired neuronal differentiation and cell death (Lim et al., 2000; Tsarovina et al., 2004). *TH* and *DBH* are strongly reduced in developing sympathetic ganglia of the *Hand2* zebrafish mutant *hands off* (Hendershot et al., 2008; Lucas et al., 2006) and in conditional *Hand2* mouse knockouts where the *Hand2* gene was excised in neural crest cells, using *Wnt1-Cre* (*Hand2*^{Wnt1Cre}) (Hendershot et al., 2008; Morikawa et al., 2007). HuC, TuJ1 and NF160 are less affected and reduced to a variable extent in different model sytems analyzed (Hendershot et al., 2008; Morikawa et al., 2007; Lucas et al., 2006). Interestingly, *Hand2* is also required for the proliferation of sympathetic neuron precursors and immature sympathetic neurons, resulting in a massive decrease in neuron numbers in the conditional *Hand2*^{Wnt1Cre} knockout (Hendershot et al., 2008).

The knockouts demonstrate that *Hand2* is important for the proliferation and noradrenergic differentiation in sympathetic ganglia. The marked decrease in *TH* and *DBH* expression implies an

* Corresponding author. Fax: +49 69 96769 441.
E-mail address: rohrer@mpih-frankfurt.mpg.de (H. Rohrer).
[1] Present address: Institute of Cell Biology and Neuroscience, J.W. Goethe-University, Max-von Laue-Str. 9, 60438 Frankfurt am Main, Germany.

0012-1606/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2009.02.020

essential function for *Hand2* in the initial onset of noradrenergic differentiation (Hendershot et al., 2008; Morikawa et al., 2007; Lucas et al., 2006). However, *Hand2* is not required for the initial expression of *TH* and *DBH* in young parasympathetic ciliary ganglion neurons that are devoid of *Hand2* (Müller and Rohrer, 2002). *TH* and *DBH* are expressed during normal ciliary neuron development only transiently, but the loss of *TH* and *DBH* could be prevented by ectopic *Hand2* expression. We thus proposed that the function of *Hand2* in sympathetic neurons includes the maintenance of noradrenergic differentiation (Müller and Rohrer, 2002).

To address the role of *Hand2* in sustaining noradrenergic gene expression, a siRNA knockdown approach was used in cultures of postmitotic, differentiated neurons from E12 chick sympathetic ganglia. The *Hand2* knockdown resulted in a strongly decreased expression of *TH* and *DBH*, without affecting neuron number and the expression of pan-neuronal genes like TuJ1, HuC and *SCG10*. In parallel, we analyzed sympathetic ganglia of a mouse line where *Hand2* has been conditionally eliminated in differentiated, *DBH*-expressing neurons (*Hand2*^{DBHCre}). Sympathetic ganglia of E11.5 and E14.5 *Hand2*^{DBHCre} mouse embryos show decreased neuron numbers and a significantly diminished TH expression in the residual neuron population, confirming the results of the in vitro *Hand2* knockdown. Thus, we conclude that *Hand2* is essential for the maintenance of the noradrenergic phenotype of sympathetic neurons.

Materials and methods

Construction of plasmids

pCAGGS-mHand2 and pCAGGS-zHand2 PCR technology was used to insert a Kozak sequence linked to a NheI site and a ClaI site flanking the coding sequence of *pcDNA3-mus musculus Hand2* (kindly provided by E. Doxakis) and *pCS2+-zebrafish Hand2* (kindly provided by P. D. Henion). *mHand2*-Primer: sense: 5′-AA**G CTA GC**A CCA CCA **TGA** GTC TGG TGG GGG GC-3′; antisense: 5′-TT**A TCG AT**T CAC TGC TTG AGC TCC AGG G-3′; *zHand2*-Primer: sense: 5′-AA**G CTA GC**A CCA CCA **TGA** GTT TGA TTG GAG GGT TTC-3′; antisense: 5′-TT**A TCG AT**T CAT TGC TTC AGT TCC AAT GCC-3′ (sense primer: bold, NheI site; underlined, Kozak sequence; bold + underlined, Start) (antisense primer: bold, ClaI site; bold + underlined, Stop). The PCR product was then cloned directly into the *pCAGGS* vector.

Primary culture preparation and electroporation

Chick sympathetic neurons were prepared by dissociation of paravertebral lumbosacral sympathetic chain ganglia, dissected at E7/E12 (Ernsberger et al., 1989a,b). Cells were cultivated in the presence of either nerve growth factor (NGF, 15 ng/ml, (Peprotech)) or of both NGF and ciliary neurotrophic factor (CNTF, 30 ng/ml, (Peprotech)). For *Hand2* knockdown experiments, 200,000 cells were mixed with either 3 μg siRNA against *Hand2* or control siRNA and electroporated with the Amaxa Nucleofector device according to the Amaxa nucleofection protocol for primary chicken neurons (Program G13). For rescue experiments, cells were transfected by electroporation as described above with 3 μg siRNA plus expression plasmids (1 μg control siRNA plus 1 μg *pCAGGS-eGFP* (*GFP*) for controls, 1 μg control siRNA plus 1 μg *pCAGGS-eGFP* and 1 μg *pCAGGS-z/mHand2* for overexpression, 1 μg siRNA against *Hand2* plus 1 μg *pCAGGS-eGFP* for knockdown and 1 μg siRNA against *Hand2* plus 1 μg *pCAGGS-eGFP* and 1 μg *pCAGGS-z/mHand2* for rescue). 200,000 cells per electroporation were plated on a 35 mm 4-well dish or 35 mm dish precoated with poly-DL-ornithine (Sigma) and Laminin (Invitrogen). The cultures were incubated for 2 days in MEM media containing 5% FCS, 10% HS, 1% glutamine and 1% Penicillin/Streptomycin at 37 °C and 5% CO_2. Cells were harvested after 4 days and total RNA was isolated using the QIAGEN RNeasy Mini Kit, following the manufacturer's protocol. The siRNAs against gg*Hand2* target the following sequence: 5′-CACAGTTAGCAGCAGCGATAA-3′ (*Hand2* siRNA1; Qiagen) and 5′-GAAGAGGAAGAAGGAGCTGAA-3′ (*Hand2* siRNA2; Qiagen). *Hand2* siRNA1 does not target mouse and zebrafish *Hand2* due to 3 and 5 nucleotide mismatches, respectively (Dahlgren et al., 2008). The control siRNA against *GFP* (*siGFP*; Qiagen) targets the sequence 5′-GCAAGCTGACCCTGAGTTC-3′. The control siRNA against *NP25* is non-functional and targets the sequence 5′GCACCTCTGTCTGTAGAGA.

In situ hybridization in cell culture

Non-radioactive in situ hybridization in cell culture and preparation of digoxigenin-labeled riboprobes for *SCG10*, *DBH*, *TH* and *Hand2* were carried out as described previously (Ernsberger et al., 1997; Stanke et al., 1999).

Immunocytochemistry in cell culture

Cell culture dishes were washed once with PBS and cells were fixed using 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 15 min. Cells were washed with PBS and incubated for 15 min in staining buffer (PBS, 5% FCS, 0.2% Triton-X 100). Primary antibody for Hand2 (Santa Cruz) was diluted 1/100, for TH (Rohrer et al., 1986), for βIII-Tubulin (Tuj1 antigen, HISS Diagnostics, MMS-435P), for HuC/HuD (Molecular Probes) were diluted 1/1000 in staining buffer and incubated for 1 h at RT. Cell culture dishes were washed twice with PBST (PBS, 0.2% Triton-X 100). Fluorophore coupled secondary antibody (in PBST + DAPI (1 μg/ml)) was incubated for 30 min at RT and washed off twice with PBS. Sections were covered with AquaPolyMount covering media (Polysciences, Inc.) and glass cover slips. Neurons counts were obtained by examining phase-bright or fluorescent neurons in randomly selected fields of the culture dish. At least 10 visual fields were counted for each well. Two to four wells of a dish were examined for each parameter in each experiment. The number of independent experiments analyzed is indicated by *n*. The results are given as the mean number per dish ± s.e.m. of at least three experiments analyzed. Student's *t*-test was used for statistical analysis.

Semiquantitative RT-PCR

cDNA synthesis on total RNA from sympathetic ganglia cells was performed using the M-MLV Reverse Transcriptase Kit (Invitrogen). For detection of *ChAT*, *VAChT*, *SCG10* and *GAPDH* mRNA levels, the following primer combinations and the Taq Polymerase Kit (Invitrogen) for PCR reaction were used: gg*ChAT*-forward (for) 5′-CAACATTAGGTCTGCTACGGCG-3′, gg*ChAT*-reverse (rev) 5′-GCAACTGTGTGGCTTCTTCTTG-3′, gg*VAChT*-for 5′-TTTCTGGCAGGTCATCATCCC-3′, gg*VAChT*-rev 5′-GGTGTCGTAGAGTCCCTTAGGTCC-3′; gg*SCG10*-for 5′-TTTAATGCCCGGAGATTCTG-3′, gg*SCG10*-rev 5′-TCAGCTTTTCCTCTGCCATT-3′ gg*GAPDH*-for 5′-CAGAGGTGCTGCCCAGAA-3′, gg*GAPDH*-rev 5′-GCAGGGGCTCCAACAAAG-3′.

All PCR reactions were performed within the linear range of amplification; this was determined empirically for each primer pair and cell preparation. *GAPDH* was analyzed after 20 and 22 cycles, *SCG10* after 27 and 29 cycles, *VAChT* after 29 and 31 cycles and *ChAT* after 31 und 33 cycles. The PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide. Quantification was done with the ScionImage Software version 1.63 (Scion Corporation). *ChAT*, *VAChT*, and *SCG10* mRNA levels were normalized to *GAPDH* mRNA level. In some cases *ChAT* and *VAChT* mRNA levels were referred to *SCG10* mRNA levels. Quantification for each experiment and gene was done in triplicate. Data shown represent mean ± s.e.m. of at least 4 independent experiments. Statistical analysis was done using paired Student's *t*-test.