Molecular and Cellular Neuroscience 45 (2010) 121–131

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

TLX activates MASH1 for induction of neuronal lineage commitment of adult hippocampal neuroprogenitors

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ARTICLE INFO

Article history:
Received 30 January 2010
Revised 1 June 2010
Accepted 9 June 2010
Available online 18 June 2010

Keywords:
TLX
FGF
Neuroprogenitor
MASH1

ABSTRACT

The orphan nuclear receptor TLX has been proposed to act as a repressor of cell cycle inhibitors to maintain the neural stem cells in an undifferentiated state, and prevents commitment into astrocyte lineages. However, little is known about the mechanism of TLX in neuronal lineage commitment and differentiation. A majority of adult rat hippocampus-derived progenitors (AHPs) cultured in the presence of FGF express a high level of TLX and a fraction of these cells also express the proneural gene MASH1. Upon FGF withdrawal, TLX rapidly decreased, while MASH1 was intensely expressed within 1 h, decreasing gradually to disappear at 24 h. Adenoviral transduction of TLX in AHP cells in the absence of FGF transiently increased cell proliferation, however, later resulted in neuronal differentiation by inducing MASH1, Neurogenin1, DCX, and MAP2ab. Furthermore, TLX directly targets and activates the MASH1 promoter through interaction with Sp1, recruiting co-activators whereas dismissing the co-repressor HDAC4. Conversely, silencing of TLX in AHPs decreased β-III tubulin and DCX expression and promoted glial differentiation. Our results thus suggest that TLX not only acts as a repressor of cell cycle and glial differentiation but also activates neuronal lineage commitment in AHPs.

Introduction

Neural stem cells are currently used in drug screening and its use in cell therapy will soon be a real option. In order to develop the optimal method to utilize neural stem cells in therapy of neurodegenerative diseases by either activating endogenous stem cells or grafting in vitro manipulated stem cells, it is essential to understand mechanisms involved in proliferation, lineage commitment, differentiation, and apoptosis of neural stem cells. The orphan nuclear receptor TLX, also known as NR2E1 or Mtll, has recently gained attention for its role in self-renewal of neural stem cells (Shi et al., 2004; Zhang et al., 2008).

Nuclear receptors are transcription factors that regulate the expression of several genes important for biological processes, such as cell proliferation, differentiation, and cellular homeostasis. TLX is a member of the *tailless* class of orphan nuclear receptors, a highly conserved family in both vertebrates and invertebrates, suggesting the importance of this protein family during evolution (Mangelsdorf et al., 1995).

Expression of TLX in the mouse starts at embryonic day 8 (E8), and its expression peaks at E13.5 in the ventricular and subventricular zone (SVZ), and decreases by E16 with barely detectable levels in the perinatal brain (Monaghan et al., 1995). However, it increases again after birth in particular, in the dentate gyrus and SVZ of the adult brain where neurogenesis continues in adulthood (Shi et al., 2004). Although TLX-knockout mice are viable and appear normal at birth, mature mice suffer from severe limbic defects, aggressiveness in both sexes, and retinal degeneration (Monaghan et al., 1995; Yu et al., 2000). Anatomically, adult mutant brains manifest reduced neurogenic areas in the hippocampal dentate gyri, expanded lateral ventricles, and reduced volumes of olfactory bulbs (Shi et al., 2004). An accumulated body of evidence suggests that TLX is expressed in adult neural stem cells (NSC), maintaining the cells in an undifferentiated proliferative state by its role as a transcriptional repressor (Shi et al., 2004; Sun et al., 2007; Li et al., 2008). TLX represses cell cycle inhibitors, such as p21 and PTEN genes, as well as astrocytic genes, GFAP and aquaporin, by recruitment of co-repressors, LSD1, HDACs and Atrophin1, and prevents commitment of progenitors into

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doi:10.1016/j.mcn.2010.06.003

lineages (Sun et al., 2010; Yokoyama et al., 2008; Zhang et al., 2006). Although the role of TLX in adult NSC has begun to emerge, the exact role and mechanism of TLX in neuronal differentiation remain to be elucidated. The fact that neurogenesis persists in the subgranular zone of the dentate gyrus of adult hippocampus points to an association of these progenitor status with several neuropsychiatric diseases such as mood disorders, dementia, and cognitive deficits (Kempermann, 2008).

Neural specification and differentiation are preceded by expression of sequential proneural transcription factors. The family of basic helix–loop–helix (bHLH) transcription factors is important in neurogenesis as well as in gliogenesis. MASH1, also named *Ascl1*, an early proneural gene broadly expressed by brain and spinal cord progenitors, has been shown to give rise to specific neurons and oligodendrocytes in different neurogenic areas, including the hippocampus (Guillemot and Joyner, 1993; Kim et al., 2007; Parras et al., 2004). Herein, we demonstrate that TLX will target and activate MASH1, which appears to be involved in the neuronal induction of AHPs in culture systems.

Results

Overexpression of TLX helps differentiate AHP cells in FGF-depleted medium

Previous reports had suggested that TLX is required for maintaining neural stem cells in an undifferentiated state (Shi et al., 2004). Continued maintenance of neural stem cells and progenitor cells as proliferating undifferentiated cells requires presence of bFGF in culture medium. We therefore examined whether the absence of bFGF in AHP cultures was sufficient to induce differentiation by immunostaining. Prominin1, a membrane protein that was originally identified to be expressed on hematopoietic stem and progenitor cells, has recently been shown to be expressed on neural stem cells, and used as their marker (Marzesco et al., 2005; Miraglia et al., 1997; Weigmann et al., 1997). It is advantageous to use a membrane marker in order to separate viable neuroprogenitors by cell sorters, since the expression of prominin1 on rat hippocampal neuroprogenitors has not been characterized.

Glial fibrillary acid protein (GFAP) is expressed by adult neural progenitor cells (Doetsch et al., 1997). In earlier passages, AHPs rarely stains for GFAP. However, during serial cultures of AHP cells, increasing numbers of GFAP-positive cells were observed in cells passaged more than 18 times. Thus, AHPs cultured with or without bFGF were immunostained and analyzed for the proliferation markers BrdU and KI67 as well as for TLX, prominin1, and GFAP. As shown in Fig. 1Aa–d, almost all of BrdU- and/or KI67-labeled cells express TLX in both conditions (Suppl. Fig. 1). In FGF-containing cultures, a significantly larger number of AHPs express TLX, prominin1, and GFAP as compared to cells cultured in the absence of bFGF (Fig. 1Ae–j, B). A higher fraction of cells expressing both prominin1 and KI67 could be seen in the presence of FGF than in its absence (20% vs 13%, Figs. 1Ag, h). Furthermore, in the proliferative condition, 74% of the prominin1-positive cells co-expressed GFAP (Figs. 1Ai and B). In the differentiating condition, the number of prominin1/GFAP-positive cells was significantly reduced, and some of them showed more matured GFAP-positive fibers, suggesting early astrocyte differentiation (Fig. 1Aj and B). These results show that removal of bFGF is indeed sufficient in order to reduce the number of progenitors, promoting cellular differentiation.

We then investigated the role of exogenously overexpressed TLX in AHP cells. We infected AHP cells in the differentiating condition with either Ad-LacZ or Ad-TLX at DIV2. Our adenoviral expression system provided infection efficiency at 70%, as determined by staining for green fluorescence protein (GFP) coupled with TLX. After another 2 days in culture, cells were fixed and immunostained for prominin1. However, the expression of exogenous TLX could not compensate the effect of FGF, resulting in a significant decrease of prominin1-expressing AHPs as compared to control Ad-LacZ-infected cells (Fig. 1C and Suppl. Fig. 2). In conclusion, our results suggest that adenoviral-transduced exogenous TLX, in the absence of FGF, does not help maintaining AHP cells in an undifferentiated state. On the contrary, TLX was able to induce differentiation of the progenitors. In order to confirm this, thymidine incorporation was done following transfection of TLX in differentiation condition at various time points. Cells were thus transfected at 8, 24, or 48 h prior to the addition of [^{3}H]thymidine and harvested for analysis 24 h later. Short-term expression of TLX (8 h prior to addition of [^{3}H]thymidine) resulted in a small increase of proliferation compared with a vector control (Fig. 1D). Longer expression of TLX significantly induced more cells to proliferate as compared to the control. However, this difference was reduced at 48 h, when the level of TLX slightly declined (Suppl. Fig. 3).

These data demonstrate that TLX induces proliferation of AHP cells, however transiently, suggesting that the expression level of TLX must be maintained at a high level for continued renewal. In agreement, when AHPs were infected with adeno-TLX and after 48 h were double-stained for KI67 and TLX, there were significantly more KI67+ cells than KI67-cells coexpressing TLX (24.2% vs 17.3%, respectively, $n = 10$, $p = 0.03$; Suppl. Fig. 4). This supports an effect of TLX in stimulating cell proliferation.

In order to further determine whether the withdrawal of FGF affected TLX overexpression, expression of nestin and Sox2 was examined in cells after infection of AHPs with Ad-LacZ or TLX in both conditions with and without FGF (Suppl. Fig. 5). Depletion of FGF decreased the expression of nestin only slightly, whereas TLX infection, but not LacZ, down-regulated nestin. This was not observed in the presence of FGF where TLX was expressed. No matter if FGF was present or absent, Sox2 was expressed in almost all AHPs after Ad-LacZ infection. In contrast, after Ad-TLX infection the number of Sox2-expressing cells clearly decreased, although, in the cells expressing TLX strongly, Sox-2 expression was enhanced. These results indicate that TLX overexpression yields a marginal effect on AHPs in proliferation condition, while in the absence of FGF, it gives a clearly stronger but heterogeneous TLX expression, which could maintain proliferation but also trigger induction of differentiation.

TLX overexpression represses GFAP and induces MASH1 and neuronal differentiation

TLX has been shown to repress GFAP expression in adult neural stem cells isolated from mice forebrains (Shi et al., 2004). We examined whether TLX acts in this way on AHP cells in differentiating conditions. AHPs were transfected with the GFAP-Luc promoter together with either control- or TLX-expression vector on DIV2. After 24 h, cells were treated with two well-known glial inducers, LIF or BMP6, or the vehicle DMSO, and 24 h later cells were harvested for luciferase assay. The result shows that TLX abolishes LIF- and BMP6-induced GFAP promoter activity (Fig. 2A). Furthermore, AHP cells in differentiation condition were infected with either Ad-LacZ or Ad-TLX on DIV2 and after 4 days in culture processed for western blot analysis. Consistent with the promoter-luciferase assay result, expression of TLX reduced the GFAP protein expression in AHP cells (Fig. 2B).

It has been reported that MASH1-expressing progenitors are able to differentiate into neurons and oligodendrocytes, but not astrocytes (Torii et al., 1999; Parras et al., 2004, Battiste et al., 2007). In order to see whether AHPs express MASH1, AHPs were cultured in the presence of bFGF. Following this, MASH1 expression was registered before and after withdrawal of FGF at various time intervals. TLX is expressed strongly in almost all of the cells in the presence of FGF but only around 30% of the TLX-expressing cells also expressed MASH1 in the nuclei (Fig . 3A and B, Suppl. Fig. 6). At 30 min and 1 h after FGF