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Forkheadbox N4 (FoxN4) triggers context-dependent differentiation in the developing chick retina and neural tube

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ABSTRACT

FoxN4, a forkhead box transcription factor, is expressed in the chicken eye field and in retinal progenitor cells (RPCs) throughout development. FoxN4 labelling overlapped with that of Pax6 and Sox2, two crucial transcription factors for RPCs. Later, during neurogenesis in the retina, some cells were intensely and transiently labelled for FoxN4. These cells co-labelled for Lim1, a transcription factor expressed in early-born horizontal cells. The result suggests that high levels of FoxN4 combined with expression of Lim1 define a population of RPCs committed to the horizontal cell fate prior to their last apical mitosis. As these prospective horizontal cells develop, their FoxN4 expression is down-regulated. Previous results suggested that FoxN4 is important for the generation of horizontal and amacrine cells but that it is not sufficient for the generation of horizontal cells (Li et al., 2004). We found that over-expression of FoxN4 in embryonic day 3 chicken retina could activate horizontal cell markers Prox1 and Lim1, and that it generated numerous and ectopically located horizontal cells of both main subtypes. However, genes expressed in photoreceptors, amacrine and ganglion cells were also activated, indicating that FoxN4 triggered the expression of several differentiation factors. This effect was not exclusive for the retina but was also seen when FoxN4 was over-expressed in the mesencephalic neural tube. Combining the results from over-expression and wild-type expression data we suggest a model where a low level of FoxN4 is maintained in RPCs and that increased levels during a restricted period trigger neurogenesis and commitment of RPCs to the horizontal cell fate.

1. Introduction

Retinal neurons are generated in a conserved temporal order during development. Combinations of transcription factors, such as Ath3, NeuroD1, Pax6, Pax2, and Otx2 regulate the cell fates of retinal progenitor cells (RPCs) (Hatakeyama et al., 2001; Inoue et al., 2002; Akagi et al., 2004; Ohsawa and Kageyama, 2008).

Forkhead box N4 (FoxN4) belongs to the winged helix/forkhead box family, a subgroup of the helix-turn-helix class of transcription factors that regulate many aspects of cell growth, proliferation and differentiation (Kaestner et al., 2000). In chicken, the expression of FoxN4 is initiated in the eye field and it continues to be expressed in the developing retina until embryonic day (E) 10–12 (Boije et al., 2008). The mouse homologue, Foxn4, has been shown to act as a cell fate determiner of RPCs. The specific phenotype of the Foxn4 knock-out mice includes a complete loss of horizontal cells (HCs) and a reduction of amacrine cells (ACs) in favour of rod photoreceptor cells (Li et al., 2004). Loss of Foxn4 also results in a reduced proliferation of the RPCs.

Even though Foxn4 knock-out mice lose all their HCs, over-expression of Foxn4 in mouse E17.5 or P0 retina generated exclusively ACs. This led to the conclusion that Foxn4 is not sufficient to generate HCs (Li et al., 2004). This is somewhat unexpected as lineage analysis suggests that HCs and ACs are derived from a common pool of progenitors (Jusuf et al., 2011). However, developmental stage is an important factor and the E17.5 or P0 RPCs, which were targeted by the over-expression, may have lost the capacity to generate HCs. A difference between early and late RPCs is the transcription factor Ikaros, which is expressed in early but not late RPCs. Mis-expression of Ikaros is sufficient to confer late RPCs the competence to generate early born neurons (Elliott et al., 2008). Thus the role of FoxN4 could be associated to the intrinsic properties of RPCs.

The aim of this work was to further clarify the role of FoxN4 in RPCs and to analyse if FoxN4 is sufficient to commit cells to the HC fate in the chicken retina. Analysis of the endogenous expression was performed. Low levels of FoxN4 were maintained in the entire RPC pool throughout development. During initial retinogenesis, cells with

Abbreviations: AC, Amacrine cell; E, Embryonic day; FoxN4, Forkhead box N4; GC, Ganglion cell; HC, Horizontal cell; PR, Photoreceptor cell; RPC, Retinal Progenitor Cell; st, developmental stage

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transient high levels of FoxN4 co-labelled for Lim1 suggesting a commitment to the HC fate. Over-expression of FoxN4 in the developing chick retina activated the HC-marker genes Prox1 and Lim1 resulting in an increase of HCs. In addition, FoxN4 over-expression initiated expressions of genes associated with photoreceptor cells (PRs) and retinal ganglion cells (GCs). The effects of FoxN4 were not exclusive to the retinal neuroepithelium but were also seen when FoxN4 was over-expressed in the mesencephalic neural tube. Our results imply that low levels of FoxN4 are maintained in the RPCs and that high levels of FoxN4 induce a general push of cells towards differentiation based on the intrinsic competence of the RPC.

2. Results

2.1. FoxN4 is expressed in the early neural retina

We analysed FoxN4 expression in relation to Pax6, Pax2 and Sox2 expression in embryonic stage (st, embryonic stage according to Hamburger and Hamilton (1951)) 12 optic vesicle, st14–15 optic cup, st21, st24, st35 (E9) and st42 (E16) neural retina. Pax2 contributes to the spatial organisation of the optic vesicle and cup (Schwarz et al., 2000), Sox2 maintains self-renewal of undifferentiated neural progenitors in the retina (Fantes et al., 2003; Wegner and Stolt, 2005) and Pax6 is required for the multipotent potential of RPCs (Marquardt et al., 2001). Labelling for FoxN4 mRNA in the optic vesicle overlapped with the Pax2 immunoreactive region in the st12 and st14 prospective neural retina but not with Pax2 labelling in the optic stalk or any of the intensely Pax6 labelled regions, such as the lens and prospective pigment epithelium (Fig. 1A–D). Labelling for FoxN4 mRNA has been seen from st12 to st35 in the neural retina (Boije et al., 2008). In the st12 and st14 eye, FoxN4, overlapped with weak but not strong Pax6 expression (Fig. 1). The inset in Fig. 1C depicts the distinction between weak and strong Pax6 labelling in the neural retina. In order to further characterise the FoxN4 expressing cells we made an anti-chicken FoxN4 antiserum that reproduced the known patterns of FoxN4 in situ hybridisation analysis (Danilova et al., 2004; Boije et al., 2008) both in the retina and in the developing spinal cord (Fig. 1E–H).

2.2. Co-expression of FoxN4, Sox2 and weak Pax6 in early RPCs

FoxN4 immunoreactivity overlapped with that of Sox2 and with weakly labelled Pax6 cells in the prospective st15 neural retina (Fig. 2A and B). Neither FoxN4 nor Sox2 labelling extended into the most dorsal part of the optic cup where intense Pax6 was observed (Fig. 2A and B). Sox2 and strong Pax6 labelling was seen in the lens while only Pax6 was seen in the prospective pigment epithelium (Fig. 2A). In the st24 retina weakly FoxN4 positive (+) cells were Pax6+ (Fig. 2C) and Sox2+ (Fig. 2D). However, the most intensely FoxN4+cells were Pax6 negative (Fig. 2C, single arrow, 400 cells inspected). Intensely FoxN4+ cells were Sox2+ (Fig. 2D, single arrow). Among the intensely FoxN4+ cells, some had condensed chromatin and mitotic figures (Fig. 2C and D, double arrows). Conversely, cells with intense Pax6 labelling, were FoxN4 negative (Fig. 2C, arrow head, 400 cells inspected). At st35 this pattern remained; weak FoxN4 labelling overlapped with weak Pax6+ cells (Fig. 2E) and Sox2+ cells (Fig. 2F), while the intensely FoxN4+ cells were Pax6 negative (Fig. 2E, single arrow) and Sox2+ (Fig. 2F, single arrow). Strong Pax6+cells in HCs, ACs and GCs in their respective layer did not overlap with FoxN4 labelling. FoxN4+ cells with mitotic figures were Sox2+ (Fig. 2F, double arrows), but not all Sox2+ cells with mitotic figures were FoxN4+ (Fig. 2F, arrowhead). At st42 (E16), FoxN4+, Sox2+ and weak Pax6+ cells were only seen in the most peripheral retina (Fig. 2G and H).

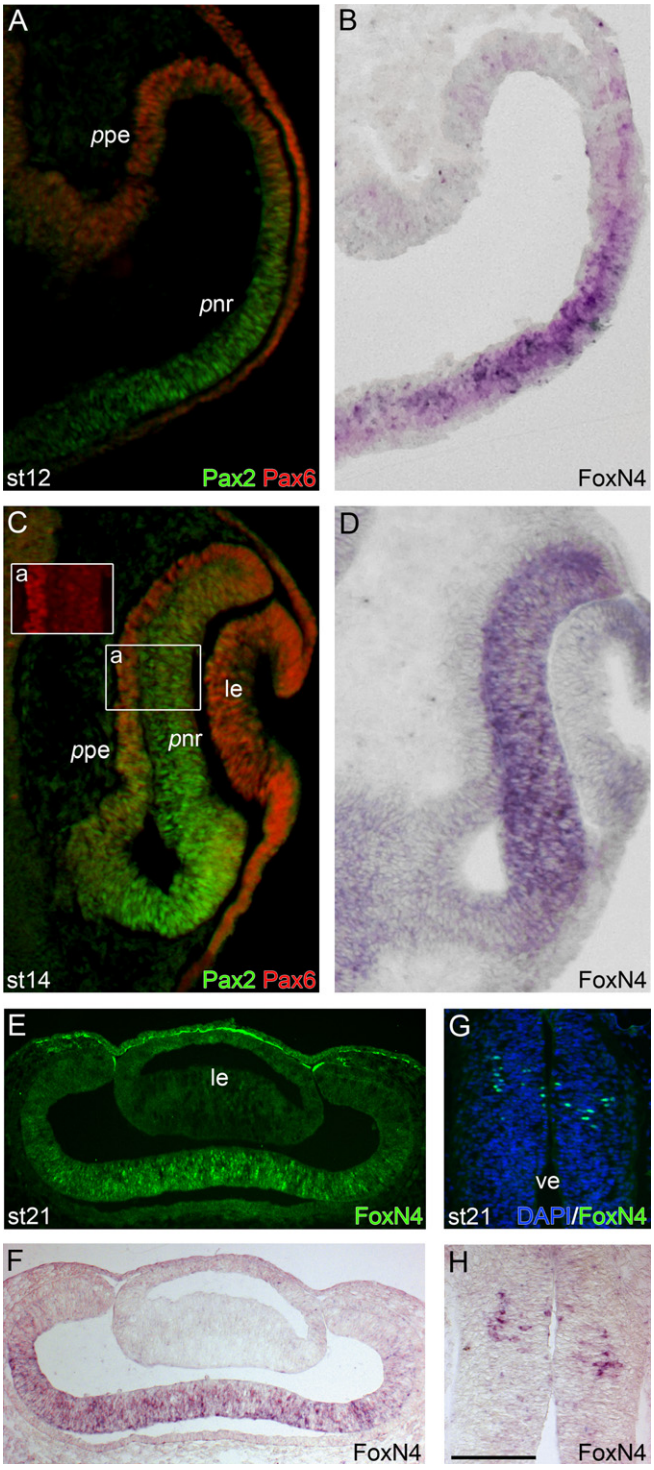

Fig. 1. FoxN4 expression in optic vesicle and cup. Fluorescence and bright-field micrographs of (A, B) st12 optic vesicle, (C, D) st14 optic cup, (E, F) st21 embryonic eye and (G, H) st21 spinal cord. Micrographs in (A, C) show Pax6 and Pax2, and (E, G) FoxN4 immunoreactivity. (B, D, F, H) Non-radioactive in situ hybridisation analysis of FoxN4 mRNA expression in consecutive sections to sections shown in A, C, E and G. Note the overlap of FoxN4 immunoreactivity with the in situ hybridization signal in retina and spinal cord. *p*pe, prospective pigment epithelium; *p*nr, prospective neural retina; le, lens; ve, ventricle. Scale bar in H equals 50 μm for A–D, G and H and 100 μm in E and F.

2.3. Strong FoxN4 labelling in Lim1+ progenitors but not in mature Lim1+ HCs

In the st24 retina, cells with weak Lim1 immunoreactivity and a position on the ventricular/apical side (Fig. 3A, double arrows)