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Promotion of embryonic cortico-cerebral neuronogenesis by miR-124

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Abstract

Background: Glutamatergic neurons of the murine cerebral cortex are generated within periventricular proliferative layers of the embryonic pallium, directly from apical precursors or indirectly via their basal progenies. Cortical neuronogenesis is the result of different morphogenetic subroutines, including precursor proliferation and death, changes in histogenetic potencies, and post-mitotic neuronal differentiation. Control of these processes is extremely complex, involving numerous polypeptide-encoding genes. Moreover, many so-called 'non-coding genes' are also expressed in the developing cortex. Currently, their implication in corticogenesis is the subject of intensive functional studies. A subset of them encodes microRNAs (miRNAs), a class of small RNAs with complex biogenesis that regulate gene expression at multiple levels and modulate histogenetic progression and are implicated in refinement of positional information. Among the cortical miRNAs, miR-124 has been consistently shown to promote neuronogenesis progression in a variety of experimental contexts. Some aspects of its activity, however, are still controversial, and some have to be clarified. An in depth *in vivo* characterization of its function in the embryonic mammalian cortex is still missing.

Results: By integrating locked nucleic acid (LNA)-oligo *in situ* hybridization, electroporation of stage-specific reporters and immunofluorescence, we reconstructed the cortico-cerebral miR-124 expression pattern during direct neuronogenesis from apical precursors and indirect neuronogenesis via basal progenitors. The miR-124 expression profile in the developing embryonic cortex includes an abrupt upregulation in apical precursors undergoing direct neuronogenesis as well as a two-step upregulation in basal progenitors during indirect neuronogenesis. Differential post-transcriptional processing seems to contribute to this pattern. Moreover, we investigated the role of miR-124 in embryonic corticogenesis by gain-of-function approaches, both *in vitro*, by lentivirus-based gene transfer, and *in vivo*, by *in utero* electroporation. Following overexpression of miR-124, both direct neuronogenesis and progression of neural precursors from the apical to the basal compartment were stimulated.

Conclusion: We show that miR-124 expression is progressively up-regulated in the mouse embryonic neocortex during the apical to basal transition of neural precursor cells and upon their exit from cell cycle, and that miR-124 is involved in the fine regulation of these processes.

Background

The glutamatergic neuronal complement of the mouse cerebral cortex is generated from neural precursors within periventricular proliferative layers of the embryonic pallium from embryonic day 11 (E11) onward [1,2]. Neural precursors include apical elements undergoing interkinetic nuclear migration (self-renewing neural stem cells and neuronally committed short neural precursors, also termed 'pin-like cells') as well as basal elements dividing far from the ventricle (neuronally committed intermediate precursor cells) [3-7]. Neurons originate from apical precursors directly or via their intermediate precursor cell progenies [8-10]. Throughout cortical development, indirect neuronogenesis is usually much more frequent than direct neuronogenesis [11].

Kinetics of neuronal generation emerges as a result of different basic morphogenetic subroutines, such as precursor proliferation and death, transitions among distinct proliferative compartments, cell cycle exit, and post-mitotic neuronal differentiation. Control of these subroutines is extremely complex, involving a large number of polypeptide-encoding genes belonging to distinct structural and functional families [9,12-19]. In addition to polypeptide-encoding mRNAs, a huge number of so-called non-coding RNAs are expressed in the developing central nervous system (CNS). Their expression patterns and their functions are presently the subject of intense investigation [20-22].

MicroRNAs (miRNAs) are a class of small non-coding RNA that are mainly transcribed by either RNA polymerase II or III as long precursors, processed by the sequential activities of the RNAse III enzymes Drosha and Dicer and eventually incorporated into bioactive RISC complexes [23,24]. miRNA functions include promotion of mRNA degradation and sequestration as well as inhibition of mRNA translation [25]. A huge number of miRNAs are specifically expressed in the developing embryo, where they are implicated in regulating histogenetic progression [26-29] as well as in refining positional information [30].

Among the best characterized miRNAs specifically expressed in the CNS is miR-124 [31]. Its expression goes up during neuronal differention, both prenatal and postnatal [32,33]. miR-124 over-expression channels non-neural HeLa cells to neuron-specific molecular profiles [34], inhibits proliferation in medulloblastomas and adult neural precursors [35,36] and promotes neuronal differentiation of committed neural precursors [36,37]. The molecular mechanisms underlying its action have been the subject of intensive investigation and include stimulation of neuron-specific transcriptome splicing [38], cross-talk with the general anti-neuronal REST/SCP1 transcriptional machinery [39,40], modulation of neuron-specific chromatin remodeling [41], down-regulation of the neuronogenesis-inhibitor *Sox9* [36], and modulation of β1-integrin-dependent attachment of neural stem cells to the basal membrane [42]. So far, however, the role of miR-124 in mammalian embryonic corticogenesis has been determined *in vivo* only partially. Makeyev *et al* [38] performed cross-correlation studies on the expression of miR-124 and selected targets of it. Both Makeyev *et al* [38] and De Pietri-Tonelli *et al* [43] analyzed the consequences of cortico-cerebral ablation of the whole miR machinery following conditional *Dicer* knock-out. A reduction in proliferation has recently been reported to occur in the mammalian embryonic spinal cord upon combined miR-9*/miR-124 overexpression in neural precursors [41]. miR-124 functions have also been studied *in vivo* in the developing chick spinal cord [40,42], however, these studies led to some contrasting conclusions.

By integrated use of *in utero* electroporation of stage-specific reporter genes, locked nucleic acid (LNA)-oligo *in situ* hybridization and immunofluorescence, we investigated miR-124 expression in the developing mouse cortex. Then, by *in vitro* lentivirus-based gene transfer and *in utero* electroporation of miR-124-expressing plasmids, we addressed the roles played by this molecule in the regulation of embryonic cortico-cerebral neuronogenesis.

Results

Expression pattern

We systematically studied the miR-124 expression pattern in the developing mouse cerebral cortex by LNA-oligo *in situ* hybridization [44]. No miR-124 signal was detectable in the cortex at E10.5, although it was strongly expressed by post-mitotic neurons of the ganglionic eminence at this time (Figure 1A). At E12.5, a light and diffuse signal was found throughout the cortical ventricular zone (VZ) and a stronger one within postmitotic neurons of the cortical preplate (Figure 1B). The pattern became more complex at E14.5, when three distinct radial expression domains could be distinguished. A faint signal was still detectable within the VZ, an intermediate signal appeared in the subventricular zone (SVZ), and a strong signal demarcated presumptive subplate, cortical plate and marginal zone. Remarkably, a few intensely labeled cells could be seen at the VZ-SVZ border (Figure 1C). This profile was basically retained at E16.5 and E18.5. At both these ages, miR-124 staining could distinguish the subplate from the cortical plate. Moreover, heavily labeled cells at the VZ-SVZ border were much more common. Finally, scattered cells expressing miR-124 at high levels also appeared in the VZ (Figure 1D, E). The miR-124 expression pattern became simplified at post-natal day 4 (P4), when the signal was restricted to the grey matter and was more intense in recently generated layer 2 to 4 neurons (Figure 1F). Remarkably, at all ages investigated, miR-124 expression was tightly restricted to the CNS,

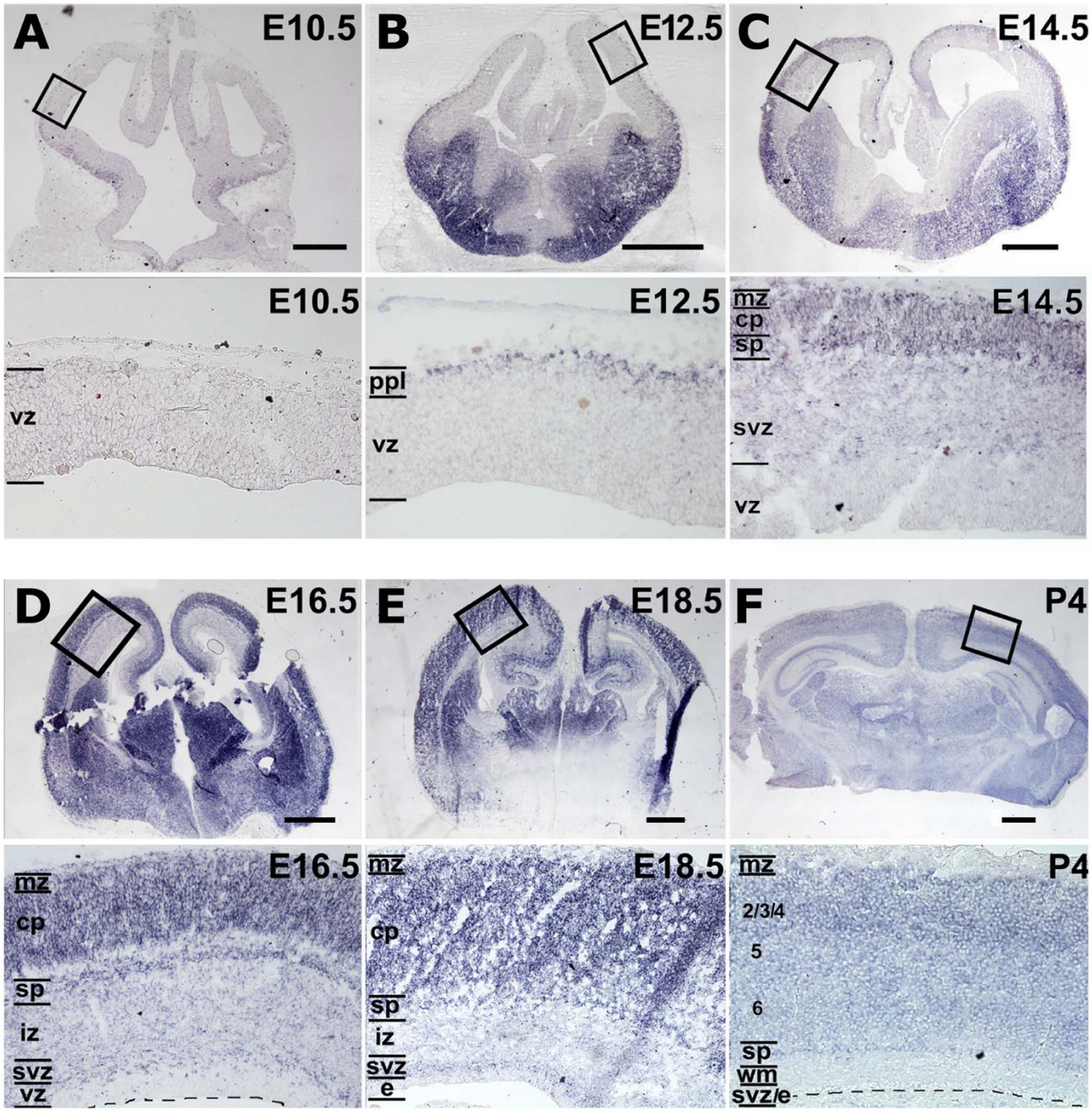

Figure 1

Time course analysis of miR-124 expression. (A-F) *In situ* hybridization of miR-124 on midfrontal E10.5 to post-natal day 4 (P4) mouse telencephalic sections. Magnifications of boxed areas are shown below each panel. Scale bars = 400 μm. Abbreviations: cp, cortical plate; e, ependyma; iz, intermediate zone; mz, marginal zone; ppl, preplate; sp, subplate; svz, subventricular zone; vz, ventricular zone; wm, white matter; 2/3/4, 5 and 6 are cortical layers.

being completely absent in the surrounding mesenchymal tissue (Figure 1; Additional file 1) [45,46].

Periventricular neural precursors fall into two distinct compartments: an apical, self-renewing compartment that includes cells with interkinetic nuclear migration and lying entirely within the VZ; and a basal compartment derived from the former, including non-motile cells which lie in both the VZ and SVZ [8-10]. To finely map the previously described different miR-124 expression levels to these compartments, these levels were compared with the distribution of specific protein markers at E14.5. Apical precursors, characterized by high Pax6 expression [7], generally displayed faint miR-124 staining (Figure 2D, box 2). This also specifically applies to a subset of these precursors - the short neural precursors [4] or 'pin-like cells' [5] - that are committed to neuronogenesis and distinguishable at E12.5 by *in utero* electroporation using enhanced green fluorescent protein (EGFP) immunoreactivity driven by the tubulin α1 promoter [4] and by the retention of a process connecting them with the ventricle (Figure 2G, boxes 1 and 2). Heterogeneous miR-124 expression was conversely detectable in basal precursors, characterized by weak Pax6 staining and robust Tbr2 immunoreactivity (Figure 2J) [7]. Among $Tbr2^+$ cells, presumptively younger elements, lying at more ventricular levels, showed weak miR-124 staining, like apical precursors (Figure 2J, box 2). On other hand, older elements, lying more marginally (and including basal progenitors as well as newborn neurons), displayed enhanced, intermediate miR-124 staining (Figure 2J, box 3). Furthermore, isolated $Pax6^-$ and $Tbr2^-$ cells expressed miR-124 at the highest level (Figure 2D, J, arrowheads). Their positions corresponded to those of VZ and SVZ cells, respectively, which co-expressed β-tubulin and abundant miR-124 (Figure 2M, arrowheads); they were thus considered to be newborn neurons. Remarkably, high miR-124 staining was not tightly restricted to post-mitotic neurons, but could also be detected in some $\beta\text{-tubulin}^-$ elements within the SVZ (Figure 2M, asterisks); these were considered to be basal progenitors [47]. On the other hand, not all $\beta\text{-tubulin}^+$ neurons in the outer SVZ expressed miR-124 at the highest levels (Figure 2M, arrows), suggesting a remarkable variability in miR-124 upregulation along the neuronogenic lineage.

Overexpression of miR-124

To cast light on the role played by miR-124, we developed a set of molecular tools for gain-of-function analysis. We cloned the Pri-miR-124(2) cDNA fragment [48,49] into the BLOCK-iT™ expression vector (Invitrogen) in-between the pCMV-EmGFP and TKpA modules in place of Pri-miR-155 derivative cDNA sequences (plasmid pPri-miR-124(2)). BLOCK-iT was used as a negative control expression vector (pPri-miR-155neg_control). To assess the effectiveness of pPri-miR-124(2) to over-express mature miR-124, we built up a dedicated sensor plasmid, cloning a *Lhx2*_3' untranslated region cDNA fragment harboring two miR-124 responsive elements [48-50] into the pDsRed2-N1 plasmid (Clontech) in-between the pCMV-DsRed2 and SV40pA modules (plasmid pmiR-124-sensor) (Figure 3A). Compared with pPri-miR-155neg_control/pmiR-124-sensor control, cotransfection of pPri-miR-124(2) and pmiR-124-sensor in HeLa cells specifically reduced the fraction of fluorescent cells expressing DsRed2 by about 60% (Figure 3B, C). To over-express miR-124 in primary cortical precursor cells, we transferred the Pri-miR-124(2) cDNA fragment into the DsRed2 derivative of the constitutive lentiviral expressor pCCLsin.PPT.prom.EGFP.Wpre [51]. Transduction of primary cortical precursor cells with the resulting LV_Pri-miR-124(2) promoted neuronal generation, as shown by the increase in β-tubulin-expressing cells at 72 hours post-infection (Figure 3F, G). Enhancement of neuronal differentiation induced by LV_Pri-miR-124(2) was further confirmed by the increase in average total neurite length, calculated at 72 hours post-infection on low density cultures by NeuriteTracer® (Figure 3H, I) [52]. Remarkably, these effects took place specifically when using 2.5% fetal calf serum (FCS) (Figure 3F-I). *In vivo* electroporation of pPri-miR-124(2) into the E12.5 lateral cortex resulted in specific upregulation of miR-124 in periventricular layers (Figure 3J, K). However, a more detailed analysis showed that the amplitude of this upregulation was moderate and only strongly electroporated periventricular cells expressed miR-124 at levels above the local natural range (Additional file 2). This might depend on poor processing of Pri-miR sequences to mature miRNAs that is peculiar to undifferentiated cells [53-55]. Consistent with this hypothesis, neuroblasts infected by LV_Pri-miR-124(2) and allowed to differentiate under FCS specifically and progressively down-regulated DsRed2 (Additional file 3) concurrent with enhanced maturation of their primary DsRed2/miR-124(2) transcripts to Pre-miR-124.

In vivo *promotion of neuronogenesis by miR-124*

The miR-124 expression pattern reported above suggested active involvement in promotion of cortico-cerebral neuronogenesis. To confirm this and to clarify the cellular mechanisms underlying such promotion, we electroporated pPri-miR-124(2) or pPri-miR-155neg_control into the lateral ventricle of E12.5 mouse embryos and, 2 days later, immunoprofiled emerald green fluorescent protein $(EmGFP)^+$ electroporated cells and their progenies for molecular markers of neural precursors and newborn neurons. For each marker, at least three embryos electroporated with pPri-miR-124(2) and three electroporated with pPri-miR-155neg_control constructs ($N \geq 3+3$) were analyzed and, for every embryo, at least 400 $EmGFP^+$ cells were scored. Upon pPri-miR-124(2) electroporation, the

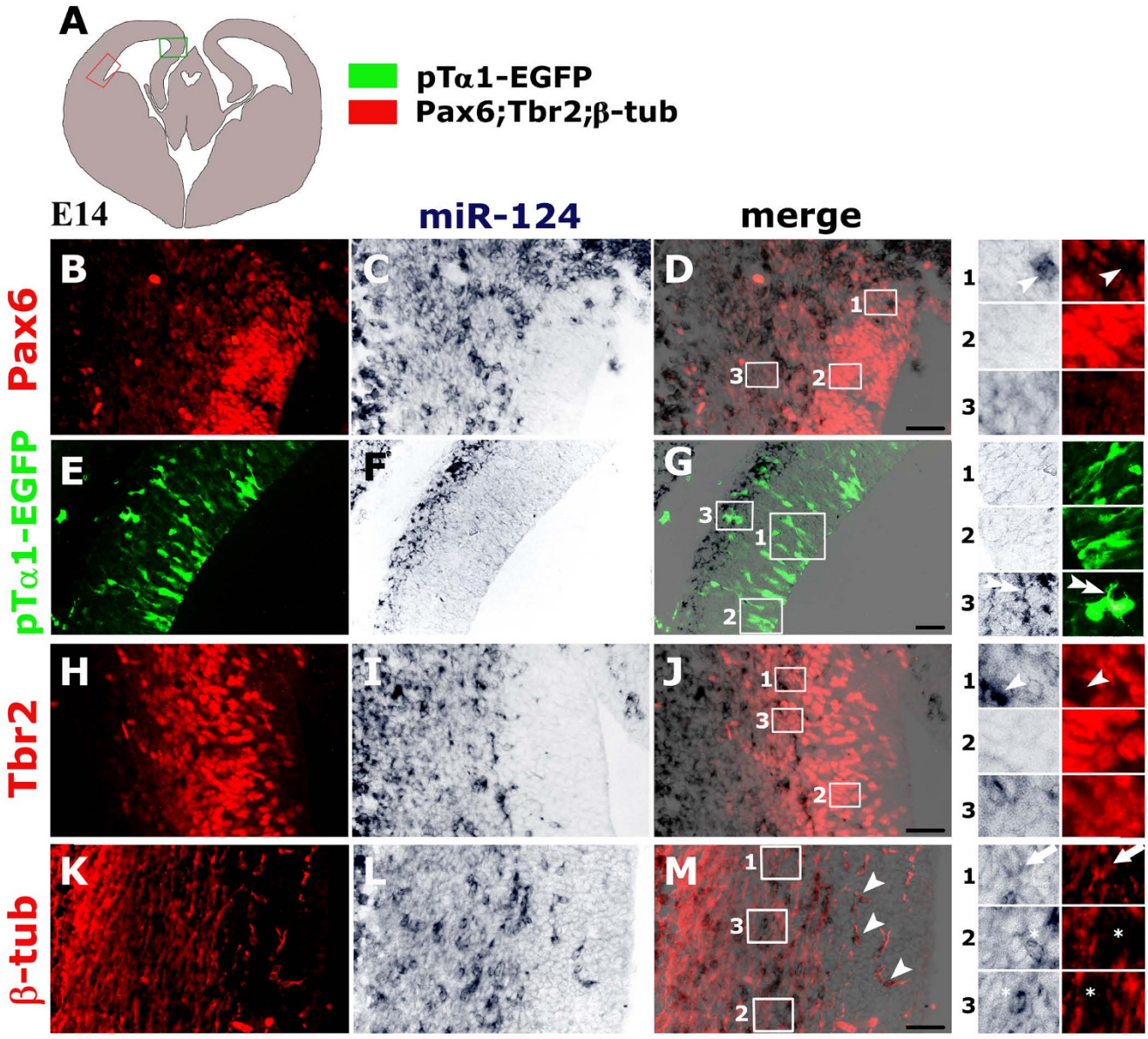

Figure 2

Comparative profiling of cortical periventricular layers for miR-124 and markers of apical progenitors (Pax6 and pTα1-driven EGFP), basal progenitors (Tbr2) and post-mitotic neurons (β-tubulin). **(A)** Schematic of an E14 midfrontal telencephalic section showing the areas analyzed. **(B, E, H, K)** Immunofluorescence of Pax6, pTα1-driven EGFP, Tbr2 and neuron-specific β-tubulin, respectively. **(C, F, I, L)** *In situ* hybridization of miR-124. **(D, G, J, M)** Electronic merging of (B, C), (E, F) (H, I) and (K, K), respectively. Numbered magnifications of boxed areas in (D, G, J, M) show Pax6, pTα1-EGFP, Tbr2 and β-tubulin in cells expressing different levels of miR-124 Arrowheads in (D1), (J1) and (M) point to Pax6^{-}/miR-124^{high}, Tbr2^{-}/miR-124^{high} and β-tubulin^{-}/miR-124^{high} elements, respectively. Double arrowheads in (G3) denote pTα1-EGFP^{+}/miR-124^{high} cells that no longer contact the ventricular cavity. Arrows in (M1) indicate SVZ β-tubulin^{+} cells expressing intermediate levels of miR-124. Asterisks in (M2, M3) demarcate SVZ β-tubulin^{-} cells expressing high levels of miR-124. Scale bars = 100 μm.

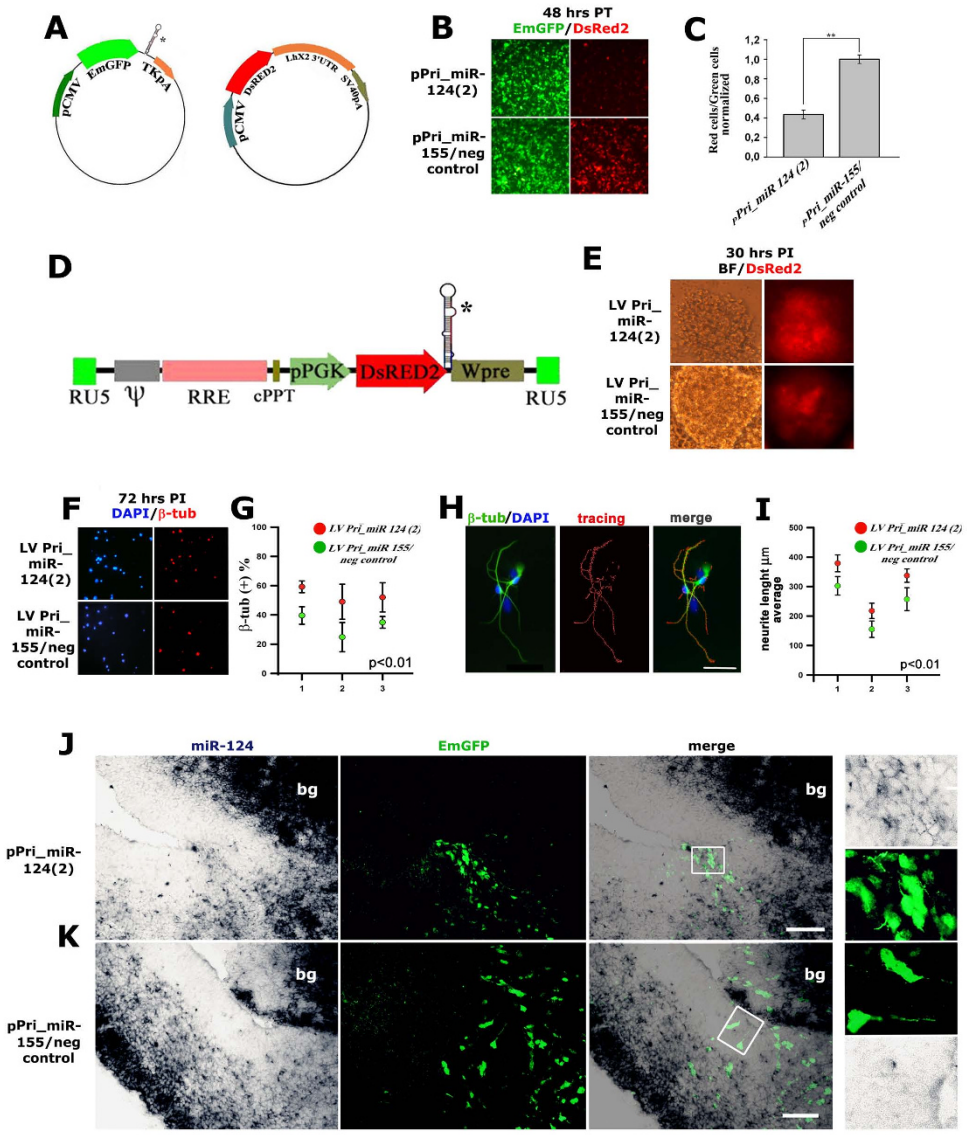

Figure 3

Overexpression of miR-124 *in vitro* and *in vivo*. (A) Backbone of the expression plasmids pPri-miR-124(2) and pPri-miR-155/neg_control; miR-124-responsive sensor plasmid pCMV-DsRed2/*Lhx2*_3'UTR. The asterisk indicates the position of Pri-miR cDNA fragments. **(B, C)** Specific attenuation of DsRed2 expression in HeLa cells cotransfected with pPri-miR-124(2) and pCMV-DsRed2/*Lhx2*_3'UTR. PT, post-transfection. **(D)** Backbone of lentivectors LV_Pri-miR-124(2) and LV_Pri-miR-155/neg_control. The asterisk indicates the position of Pri-miR cDNA fragments. **(E)** DsRed2 expression in E12.5 primary cortico-cerebral progenitors infected by lentiviruses LV_Pri-miR-124(2) and LV_Pri-miR-155/neg_control at a multiplicity of infection of 40 and kept for 30 h in DMEM:F12:N2 medium supplemented with 2.5% fetal calf serum. PI, post-infection. BF, bright field. **(F, G)** Differential β-tubulin immunoprofiling of acutely infected, E12.5 dissociated cortical progenitor cells, at 72 h after infection. **(H)** Example of neurite outgrowth evaluation by immunostaining and subsequent NeuriteTracer® analysis. **(I)** Differential neurite outgrowth in low density cortical progenitor cells 72 h after infection at E12.5, calculated for three different experiments by NeuriteTracer®. **(J, K)** *In vivo* overexpression of miR-124 in lateral neocortex. Distribution of miR-124 and pCMV-driven EmGFP on E14.5 midfrontal telencephalic sections from brains electroporated at E12.5 with the plasmids pPri-miR-124(2) and pPri-miR-155/neg_control, respectively. Magnifications of boxed insets of electronic merges are shown to the right. Abbreviations: bg, basal ganglia. In (C) error bars represent the standard error of the mean calculated among the means of each experiment; $^{**}P < 0.01$, as evaluated by ANOVA test; N = 3+3. In (G, I) error bars represent the standard error of the mean calculated within each experiment; the $P < 0.01$ value was evaluated by *t*-test (one-tail, paired); N = 3+3. Scale bars = 40 μm (H) and 100 μm (I).

fraction of intermitotic EmGFP+ cells was specifically reduced by 20% (N = 5+5; $P < 0.05$) when assessed by the S-phase marker terminally administered bromodeoxyuridine (BrdU). However, it did not display any statistically relevant change when evaluated by the M-phase marker phospho-histone3 (N = 3+3). EmGFP+ cells expressing the apical precursor marker Pax6 decreased by 20% (N = 4+4; $P < 0.05$); conversely, those positive for the basal precursor marker Tbr2 increased by a similar percentage (N = 5+5; $P < 0.05$) (Figure 4). Electroporation of both pPri-miR-124(2) and pPri-miR-155neg_control also led to displacement of apical Pax6+ and basal Tbr2+ precursors towards the cortical plate (in six out of eight and eight out of ten embryos, respectively; Figure 4A, B, arrowheads). This phenomenon was not restricted to electroporated cells, but occurred mainly in their close surroundings. Since the two plasmids share a similar stem-and-loop Pri-miR moiety, to assess if such displacement were specifically linked to over-expression of this structure, we performed a further electroporation with the control plasmid pEGFP-C1™ (Clontech), which harbors the same pCMV-GFP module but is missing the Pri-miR element. Remarkably, even in this case, Pax6 and Tbr2 displacement was evident (three out of three electroporated embryos for both), ruling out that it was due to the Pri-miR moiety and indicating it was a consequence of the *in utero* electroporation protocol used (Additional file 4, arrowheads). In two out of five analyzed embryos, pPri-miR-124(2) electroporation specifically elicited strong activation of the post-mitotic markers β-tubulin (Figure 5A, B, arrowheads) and Tbr1 [7] (Figure 5D, E, arrowheads) as well as neurite outgrowth (Additional file 5, arrowheads) in the VZ. These effects were associated with strong over-expression of miR-124 in this zone (Figure 5C, arrows). In summary, overexpression of miR-124 seems to promote precursor transition from the apical to the basal compartment and to stimulate direct differentiation of apical progenitors to post-mitotic neurons. As suggested by activated-Caspase3 immunoprofiling of electroporated tissues (N = 4+4), such shifts seem not to arise as a consequence of differential cell death (Additional file 6). Finally, high-level miR-124 expression elicited in apical progenitors (including pin-like cells; Figure 6, arrowheads) upon *in vivo* electroporation may support these phenomena.

Looking for mechanisms linking miR-124 overexpression with promotion of apical-to-basal transition, we assayed expression of β1-integrin. This protein is necessary for integrity of adherens junctions among radial glial cells and the subpial basal membrane [56] and is an established target of miR-124 in chicken [42]. The cortical expression profile of β1-integrin normally includes a strong signal in the VZ, transitional field and marginal zone and a palisade-like pattern in the cortical plate, possibly corresponding to pial processes of radial glia and migrating neurons super-imposed on a weaker signal from resident neurons (Additional file 7). This domain is quite complementary to that expressing miR-124 at high levels. Unexpectedly, however, electroporation of Pri-miR-124(2) in the mouse cerebral cortex did not elicit any detectable down-regulation of β1-integrin. This may mean that miR-124-dependent regulation of β1-integrin is peculiar to the chicken neural tube and does not take place in the mammalian cortex. Alternatively, this may be due to a poor sensitivity of our immunodetection technique in discerning subtle changes in antigen concentration.

Discussion

In this study, by integrating LNA-oligo *in situ* hybridization, electroporation of stage-specific reporters and immunofluorescence, we carefully reconstructed the miR-124 expression pattern in the developing mouse cerebral cortex. Moreover, by *in vitro* lentivirus-based gene transfer and *in utero* electroporation of gain-of-function plasmids, we investigated the activity of this molecule in the embryonic neuronogenic process.

We confirmed that miR-124 is progressively up-regulated during embryonic neuronogenesis, as previously reported [36,38,57,58]. In particular, with the appearance of the SVZ, we found that miR-124 displayed three distinct expression levels: low in the VZ [36,57], intermediate in the SVZ and high in more marginal layers (Figure 1; Additional file 1). The presence of isolated β-tubulin+ cells and Pax6- cells within the VZ, both of which expressed miR-124 highly, suggested that an abrupt upregulation of miR-124 might occur during direct neuronogenesis (Figures 2 and 7A). Conversely, the miR-124 expression profile appeared biphasic in indirect neuronogenesis; a transition from low to intermediate expression took place within Tbr2+ basal progenitors that had lost contact with the ventricular cavity, and a further upregulation was localized in basal progenitors or early post-mitotic β-tubulin+ neurons (Figures 2 and 7B). This scenario is reminiscent to what was found by Doetsch and colleagues in the adult SVZ [36]. In their study, a first upregulation of miR-124 occurred after the transition of neural stem cells to transit amplifying cells and a second upregulation was generally associated with the exit of neuroblasts from the cell cycle [36,56,59]. Finally, we specifically detected an accumulation of cells highly expressing miR-124 and with multipolar morphology at the border between the VZ and SVZ from E14.5 onward (Figures 1 and 2I, L). Such accumulation recalls the 'sojourn band' or 'multipolar accumulation zone', where newborn neurons settle before initiating their radial migration [1,60,61].

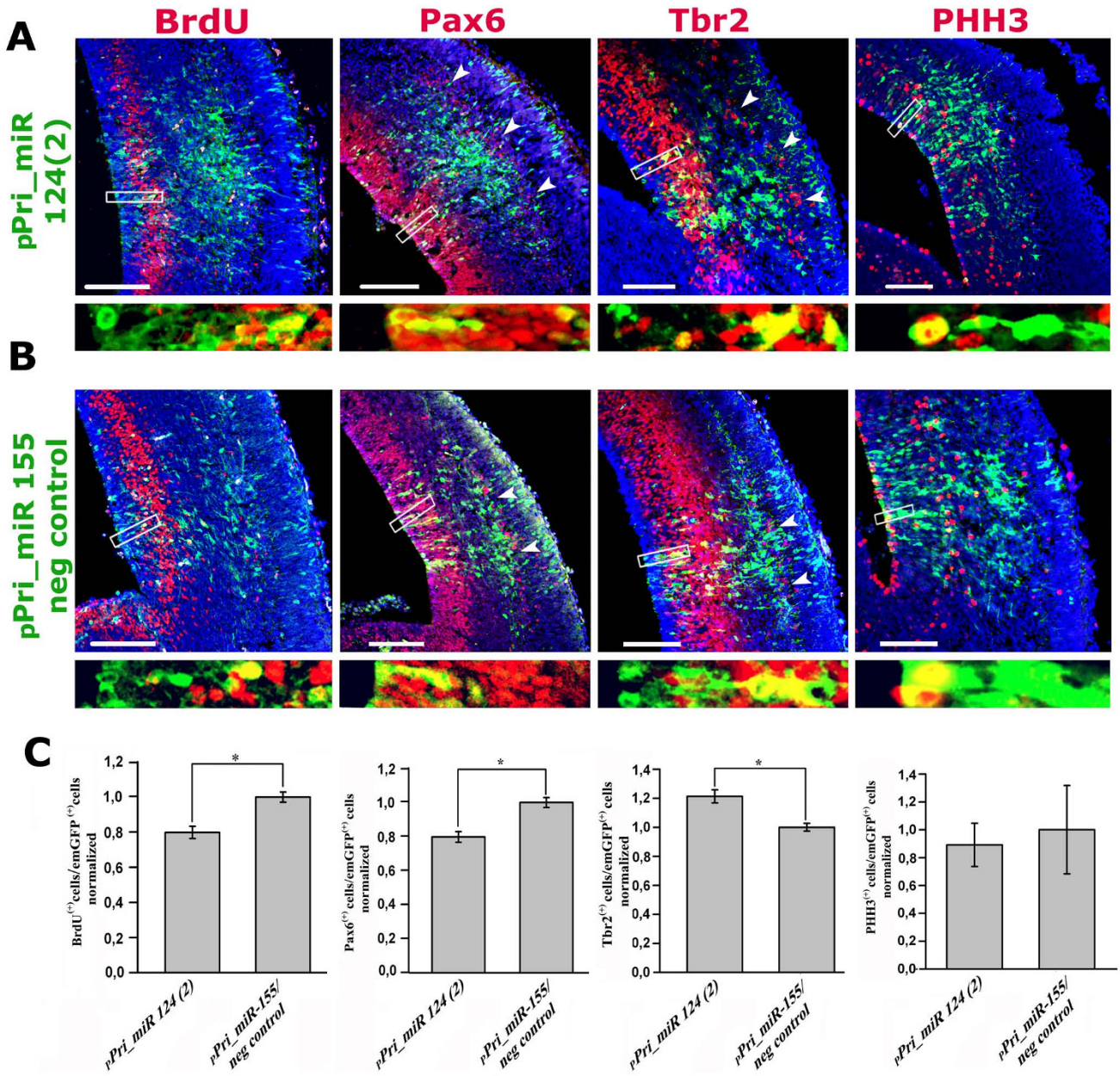

Figure 4

Immunoprofiling of cortical periventricular layers after *in utero* electroporation of plasmids pPri-miR-124(2) and pPri-miR-155/neg_control; part I. (A, B) Distribution of terminally administered BrdU, Pax6, Tbr2, phosphohistone3 (PHH3) and pCMV-driven EmGFP on E14.5 mid-frontal sections from brains electroporated *in utero* at E12.5 with plasmids pPri-miR-124(2) and pPri-miR-155/neg_control. Arrowheads point to abventricular displaced cells expressing Pax6 and Tbr2. Insets illustrate examples of EGFP/marker co-localizations, which were the subject of counting and statistical analyses. **(C)** Fractions of $EmGFP^+$ cells immunoreactive for BrdU, Pax6, Tbr2 and PHH3. *$P < 0.05$, as calculated by ANOVA test; N = 5+5 for BdU and Tbr2; N = 4+4 for Pax6; N = 3+3 for PHH3. Scale bars = 100 μm.

Electroporation of our gain-of-function constructs was followed by concerted upregulation of EmGFP and miR-124. Such upregulation was relatively weak for miR-124; however, levels of this miRNA in electroporated apical precursors were often above those of endogenously expressed miR-124 in the VZ, allowing functional perturbation of the system (Figures 3 and 6; Additional file 2). Limited production of miR-124 despite abundant levels of the EmGFP/Pri-miR-124(2) transcript available in apical progenitors might stem from suboptimal, regulated