● **PERSPECTIVE**

# Tuning of neocortical astrogenesis rates by *Emx2* in neural stem cells

Generation of astrocytes within the murine developing cerebral cortex mainly takes place during the first postnatal week, after neuronogenesis and prior to the bulk of oligogenesis. This process involves a great variety of highly complex regulatory mechanisms. Astrocytic outputs depend on two primary factors: progressive commitment of multipotent precursors to astroglial fates and proper tuning of proliferation of astrocyte-committed progenitors. To date, several regulatory mechanisms have been identified for the former process, while very little is known about modulation of astroblast proliferation (reviewed in Mallamaci, 2013). Intriguingly, astrogenic rates remain very low during the whole neuronogenic phase, although the mouse cortex is already able to generate astrocytes at E14.5–E15.5, thanks to specific chromatin reconfiguration (Fan et al., 2005). Poor proliferation of astroblasts may contribute to this effect (Seuntjens et al., 2009). Among different factors modulating astrocyte-committed proliferation, the Egf-receptor (*EgfR*) and the secreted ligand *Fgf9* both specifically promote it (Viti et al., 2003; Lum et al., 2009). *Emx2*, a pleiotropic hub (Gangemi et al., 2006) controlling a variety of neurodevelopmental processes, is highly expressed in the early neuronogenic pallium, while it fades out together with neuronogenesis ending. This temporal progression is possibly linked to the progressive decline of Wnt signals supporting *Emx2* expression (Theil et al, 2002) and late arousal of Fgf8 (http://developingmouse.brain-map.org/) antagonizing it (Garel et al., 2003). In a previous *in vitro* study (Brancaccio et al., 2010), we reported that *Emx2* overexpression in neural stem cells (NSCs) leads to a reduction of their astrocytic outputs, due to unknown mechanisms. In the paper highlighted here (Falcone et al., 2014), we showed that this phenomenon occurs also *in vivo* and dissected its cellular and molecular mechanisms.

At the beginning of our study, we verified that the decrease of the ultimate glial output of NSCs induced by *Emx2* overexpression takes place also *in vivo* and it is due to a shrinkage of the proliferating astrogenic pool. We injected a plasmid expressing *Emx2* into the lateral ventricular cavity of P0 pups and electroporated it into the cortex. The analysis of P4 mice electroporated cortices revealed a decrease of S100β$^+$ astrocytes and S100β$^+$Ki67$^+$ astroglial proliferating progenitors in *Emx2*-gain of function (GOF) samples, by about 30% and 50%, respectively (**Figure 1A**). [Frequencies of these cell types were conversely upregulated in the posterior cortex of E17.5 *Emx2*$^{+/-}$ embryos, suggesting that the inhibition of astrogenesis elicited by gain-of-function manipulations was not due to a dominant negative effect (**Figure 1B**)]. Then, these results were replicated in an *in vitro* model, set up to dissect molecular mechanisms involved in *Emx2* antiastrogenic function. E12.5 cortico-cerebral precursors were engineered for conditional *Emx2* overexpression, which was activated at the *in vitro* equivalent of P0. Following this manipulation, the final astroglial output was reduced approximately as much as *in vivo*. Moreover, it was associated to a prominent shrinkage of the astrogenic proliferating pool. Interestingly, *Emx2* impact on astrogenesis depended mainly on cell-autonomous mechanisms. This was verified by mixing a small amount of lentivirus-engineered precursors with an excess of isochronic wild type precursors, conditioning the medium. Even in this situation, *Emx2*-engineered cells expressing S100β were significantly reduced upon transgene activation.

To cast light on molecular mechanisms mediating *Emx2* anti-astrogenic effect, we looked at a few well-known genes promoting the expansion of the astrogenic proliferating pool, including *EgfR* and *Fgf9*. We found that *Emx2* overexpression downregulates both *EgfR* and *Fgf9*. Consistently, the same genes were upregulated in *Emx2*$^{+/-}$ cultures. Functional relevance of *EgfR* and *Fgf9* to *Emx2* action was tested by delivering a lentivector driving *EgfR* expression and, alternatively, the *Fgf9* ligand to *Emx2*-GOF cultures at *in vitro* equivalent of P0. Both *EgfR* and *Fgf9* were able not only to rescue the normal astroglial output, but to restore wild type astrogenic proliferating rates too. The reconstruction of the pathways leading to *EgfR* and *Fgf9* downregulation represented a step forward in the understanding of *Emx2* anti-astrogenic activity. Both *EgfR* and *Fgf9* levels showed scarce sensitivity to exogenous *Fgf9* addition and *EgfR* overexpression, respectively, thus suggesting that *Emx2* regulation of astrogenesis may occur along two separated pathways. Regarding *EgfR* regulation, we suspected that it could be mediated by Bmp signaling. Indeed, *Emx2* promotes such signaling (Shimogori et al., 2004), which, in turn, inhibits *EgfR* expression (Lillien and Raphael, 2000). Interestingly, *Emx2* was able to upregulate two established endogenous reporters of Bmp signaling, Id3 and Msx1. Moreover Bmp inhibition by LDN193189 rescued *EgfR* expression levels in *Emx2*-GOF samples, while not perturbing them in controls. As for *Fgf9*, we hypothesized that its regulation might depend on *Sox2* repression. We found that *Emx2* overexpression almost abolishes *Sox2* expression in cortico-cerebral precursors at astrogenesis peak time. Moreover, *Sox2* overexpression rescued *Fgf9*-mRNA levels in *Emx2*-GOF cultures. Interestingly, a sort of upstream crosslink among these two regulatory branches exists. In fact, on one hand Bmp inhibition restored also *Fgf9* expression, on the other hand *Sox2* overexpression rescued *EgfR* levels. Besides, *Emx2*, while downregulating *Fgf9* in control conditions, increased *Fgf9* upon Bmp signaling inhibition. This suggests that Bmp signaling could inhibit *Fgf9* expression by counteracting an *Emx2*-dependent stimulatory pathway (**Figure 2**).

Finally, we evaluated the physiological relevance of *Emx2* to the confinement of the bulk of astrogenesis to postnatal life. First, we rigorously documented that both *Emx2* mRNA and protein levels progressively decrease in cortico-cerebral stem cells from embryonic towards perinatal stages. High *Emx2* levels are associated to the neuronogenic phase, whereas *Emx2* is barely detectable concomitantly with the arousal of astrogenesis. Then, we assessed consequences of short-term *Emx2* overexpression in embryonic NSCs on the size of the astrogenic lineage, unveiled by a Lif-supplemented, pro-differentiative medium. As expected, *Emx2* overexpression led to a reduction of the final output of both S100β$^+$ and GFAP$^+$ cells, a result mirrored by cultures loss-of-function for *Emx2*.

In summary: (1) *Emx2* overexpression in cortico-cerebral stem cells inhibits astrogenesis *in vivo* as well as *in vitro*, by shrinking the proliferating astrogenic pool and thus provoking a pronounced decrease of its ultimate astroglial output (**Figure 1**); (2) *Emx2* exerts its anti-astrogenic function by downregulating *EgfR* and *Fgf9*, *via* promotion of Bmp signaling and *Sox2* suppression (**Figure 2**). These phenomena may be instrumental to fine tuning of cortico-cerebral histogenesis, *i.e.*, the *in vivo* temporal progression of *Emx2* expression levels can help to hold back astrogliogenesis during the neuronogenic phase of development. On the other side, the sensitivity of astrogenic rates to *Emx2* expression levels points to *Emx2* as an appealing therapeutic tool, suitable for control of reactive gliosis as well as for selective channelling of neural precursors to neuronogenesis in processes of brain repair.
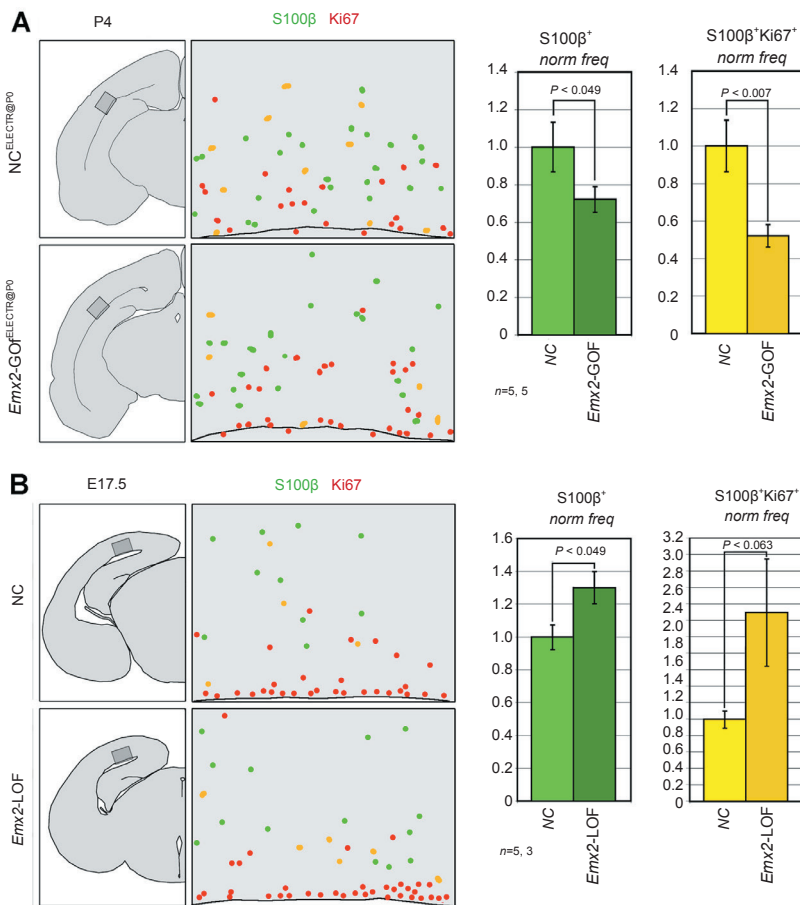
Figure 1 Altered astrocytogenesis upon *Emx2* manipulation *in vivo*.
(A) Distribution of S100β⁺ astrocytes and S100β⁺Ki67⁺ astroglial proliferating progenitors in the posterior parietal cortex of P4 pups electroporated at P0 with a control (NC) and, alternatively, a constitutive *Emx2* expressor plasmid (*Emx2*-GOF). (B) Distribution of S100β⁺ astrocytes and S100β⁺Ki67⁺ astroglial proliferating progenitors in the posterior parietal cortex of E17.5 embryos heterozygous for an *Emx2-null* allele and their littermate wild type controls.
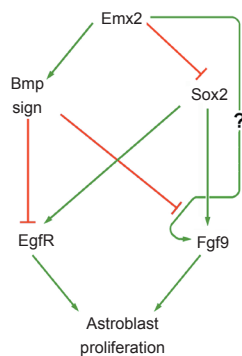


Figure 2 Epistatic relationships among *Emx2* and mediators of its antiastrogenic activity.
The question mark highlights a hypothetical regulatory branch accounting for the divergent effects exerted by *Emx2* overexpression on *Fgf9*-mRNA levels in control conditions and upon Bmp signalling inhibition.

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## References

Brancaccio M, Pivetta C, Granzotto M, Filippis C, Mallamaci A (2010) Emx2 and Foxg1 inhibit gliogenesis and promote neuronogenesis. Stem Cells 28:1206-1218.

Falcone C, Filippis C, Granzotto M, Mallamaci A (2014) Emx2 expression levels in NSCs modulate astrogenesis rates by regulating EgfR and Fgf9. Glia doi: 10.1002/glia.22761.

Fan G, Martinowich K, Chin MH, He F, Fouse SD, Hutnick L, Hattori D, Ge W, Shen Y, Wu H, Ten Hoeve J, Shuai K, Sun YE (2005) DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development 132:3345-3356.

Gangemi RMR, Daga A, Muzio L, Marubbi D, Cocozza S, Perera M, Verardo S, Bordo D, Griffero F, Capra MC, Mallamaci A, Corte G (2006) Effects of Emx2 inactivation on the gene expression profile of neural precursors. Eur J Neurosci 23:325-334.

Garel S, Huffman KJ, Rubenstein JL (2003) Molecular regionalization of the neocortex is disrupted in Fgf8 hypomorphic mutants. Development 130:1903-1914.

Lillien L, Raphael H (2000) BMP and FGF regulate the development of EGF-responsive neural progenitor cells. Development 127:4993-5005.

Lum M, Turbic A, Mitrovic B, Turnley AM (2009) Fibroblast growth factor-9 inhibits astrocyte differentiation of adult mouse neural progenitor cells. J Neurosci Res 87:2201-2210.

Mallamaci A (2013) Developmental control of cortico-cerebral astrogenesis. Int J Dev Biol 57:689-706.

Seuntjens E, Nityanandam A, Miquelajauregui A, Debruyn J, Stryjewska A, Goebbels S, Nave KA, Huylebroeck D, Tarabykin V (2009) Sip1 regulates sequential fate decisions by feedback signaling from postmitotic neurons to progenitors. Nat Neurosci 12:1373-1380.

Shimogori T, Banuchi V, Ng HY, Strauss JB, Grove EA (2004) Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex. Development 131:5639-5647.

Theil T, Aydin S, Koch S, Grotewold L, Rüther U (2002) Wnt and Bmp signalling cooperatively regulate graded Emx2 expression in the dorsal telencephalon. Development 129:3045-3054.

Viti J, Feathers A, Phillips J, Lillien L (2003) Epidermal growth factor receptors control competence to interpret leukemia inhibitory factor as an astrocyte inducer in developing cortex. J Neurosci 23:3385-3393.