PDGFRα controls formation and differentiation of neurospheres in the subventricular zone of the adult mouse brain

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Summary

Objective: To evaluate the role of PDGFRα for formation and differentiation of neurospheres in the subventricular zone of the adult mouse brain. *Subject and method*: We used *CAGG-CreER*, *Pdgfra*$^{flox/flox}$ mice (CAGG-Flox mice) as control group and *CAGG-CreER* mice as knockout mice to inactivate PDGFRα gene via tamoxifen treatment, then examine stem cell activities in adult mouse brain by expressing formation and differentiation of neurospheres. *Result*: Before tamoxifen treatment the number of neurosphere and cell number of differentiation are comparable between two groups, however after tamoxifen treatment those are severely suppressed in *CAGG-CreER* mice. *Conclusion*: Formation and differentiation of neurosphere were maintained by PDGFRα gene which expressed partly stem cell activities in adult mouse brain.

Keywords: PDGFRα, neurospheres, subventricular zone, mouse brain.

1. Background

The neural stem/precursor cells (NSCs) in the ventricular-subventricular zone (V-SVZ) contribute to adult neurogenesis and gliogenesis, however, the mechanisms regulating NSC phenotype and functions remain to be elucidated. In the present study, we uncovered roles for PDGFRα in adult NSCs isolated from the V-SVZ. This was achieved through *Pdgfra* inactivation using a ubiquitous promoter-driven, tamoxifen-inducible Cre recombinase. Cultured adult V-SVZ-derived NSCs largely depended on PDGFRα for their self-renewal, migration. Formation and differentiation of the neurosphere (FDN) by V-SVZ-derived NSCs were eliminated upon *Pdgfra* inactivation in adult mice, with its subsequent regeneration attributed to the repopulation of *Pdgfra*-expressing NSCs. The implication of PDGFRα in the control of NSCs via expression of FDN may be exploited in regenerative medicine.

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2. Subject and method

2.1. Subject

Conditional inactivation of *Pdgfra* in mice was achieved as described elsewhere (Horikawa et al, 2015). Briefly, the BAC genomic clone RP24-148N4 was used to genetically mutate the *Pdgfra* gene such that exons 4–5 were flanked by loxP sequences (creating *Pdgfra*$^{flox/flox}$). *Pdgfra*$^{flox/flox}$ mice were crossbred with *CAGG-CreER* mice (Hayashi and McMahon, 2002) to obtain *Pdgfra*-inactivated mice (termed KO mice) as previously described (Horikawa et al, 2015). Then we have CAGG-Flox mice as control group and *CAGG-CreER* mice as knockout mice when treated with tamoxifen.

2.2. Method

We analysed the stem cell activities in SVZ of adult mouse brain via formation and

differentiation of neurospheres in control and knockout groups.

Tamoxifen treatment

Tamoxifen (TM; Sigma-Aldrich) was administered orally to 8-week old mice once a day (225mg/kg body weight) for 5 consecutive days. For generation of TM-induced KO cells, 5mg of 4OH-TM (Sigma-Aldrich) were dissolved in 5ml of ethanol by continuously vortexing for 15 min to obtain a 10mM solution. From this, 1µL was added to 10mL of the culture medium, resulting in a final concentration of 1mM. Treatment lasted for 2 and 3 days for floating and differentiating cultures, respectively.

Cell culture

The neurosphere (NS) assay was conducted as previously described (Ishii et al, 2008). Mice were sacrificed by cervical dislocation, the periventricular explants were dissected and triturated with a fire-polished Pasteur pipette (IK-PAS-9P, Asahi Glass, Tokyo, Japan), and then single cells were collected by centrifugation. These cells were grown in floating culture on plates coated with methyl methacrylate using NeuroCult Neural Stem Cell Basal Medium with NeuroCult Proliferation Supplement (StemCell Technology, Vancouver, Canada), additionally supplemented with 20ng/mL EGF (Peprotech, Rocky Hill, NJ) and 10ng/mL FGF2 (Peprotech). Maintenance medium consisted of DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 1% N2 supplement (Vitrogen), 20ng/mL EGF, and 10ng/mL hFGF2. Fresh growth factors were added every 2 - 3 days in vitro and NSs (round clusters of free-floating cells with clear, smooth margins and diameters > 20µm (Yamamoto et al, 2005)) were fully grown after 14 days of incubation.

For NS differentiation, NSs were seeded onto 8-chamber culture slides coated with poly-D-lysine (for oligodendroglial differentiation) and incubated for 3 days in vitro with medium consisting of DMEM/F12, 1% N2 supplement, 20ng/mL hFGF2, and 20ng/mL PDGF-AA (Millipore, Temecula, CA).

3. Result

3.1. Neurosphere formation was PDGFRα-dependent, but regenerated after a transient depletion in Pdgfra-inactivated mice

In order to deplete PDGFRα in either cultured cells or adult mice, we generated *CAGG-CreER*; *Pdgfra* $^{flox/flox}$ mice (CAGG-Flox mice) by crossbreeding *Pdgfra* $^{flox/flox}$ mice (Flox mice) (Horikawa et al, 2015) and *CAGG-CreER* mice, which express CreER under the ubiquitous *CAGG* promoter (Hayashi and McMahon, 2002).

We, at first, examined the contribution of PDGFRα in V-SVZ-derived stem cells to neurosphere formation. Neurospheres were defined as floating round cell-clusters larger than 50µm in diameter that were induced after floating culture for 14 days (Figure 1A-i). The number of primary neurospheres obtained from each mouse were similar between Flox mice (primary-NS-Flox) and CAGG-Flox mice (primary-NS-CAGG-Flox) (Figure 1A-i, B, C, H, 206.3 ± 10.9 vs. 212.0 ± 15.6, respectively; difference not significant, n = 4 per group).

Table 1. Before tamoxifen treatment in vivo

	Primary-NS-Flox	Primary-NS-CAGG FLox
NS number	206.3 ± 10.9	212.0 ± 15.6

Then, to understand the in vivo role of PDGFRα, we prepared the mice with global *Pdgfra* inactivation (iKO mice) by treating CAGG-Flox mice with TM (Figure 1A-iii). Identically treated Flox mice were used as intact *Pdgfra*-expressing controls. Thereafter, we examined the neurosphere formation by V-SVZ cells isolated from these mice. The cells from control Flox mice with and without TM treatment (Figure 1B, C), excluding the direct effect of TM treatment. Neurosphere formation was examined in V-SVZ cells from iKO mice treated with TM at 1 day, 1 week, and 2 weeks before sacrifice (Figure 1A-iii,

E-G; NS-iKO^{vivoTM}-1d, NS-iKO^{vivoTM}-1w, and NS-iKO^{vivoTM}-2w, respectively). Neurosphere formation was severely depleted in NS-iKO^{vivoTM}-1d, where no neurospheres with a diameter larger than 50μm were formed (Figure 1E). Thereafter, neurosphere formation regenerated substantially in NS-iKO^{vivoTM}-1w (Figure 1F, 149.7 ± 11.6) and in NS-iKO^{vivoTM}-2w (Figure 1G, 222.3 ± 11.7). These data implicate PDGFRα in the neurosphere forming activity in the adult V-SVZ, in which, global *Pdgfra* inactivation suppressed the neurosphere forming activity in the adult V-SVZ, but the suppression was transient, with a subsequent recovery in iKO mice.

Table 2. At 1 day, 1 week, 2 weeks and control after tamoxifen treatment in vivo

	NS-iKO^{vivoTM}-1d	**NS-iKO^{vivoTM}-1w**	**NS-iKO^{vivoTM}-2w**	**NS-Flox^{vivoTM}**
NS number	0	149.7 ± 11.6	222.3 ± 11.7	201.0 ± 3.6

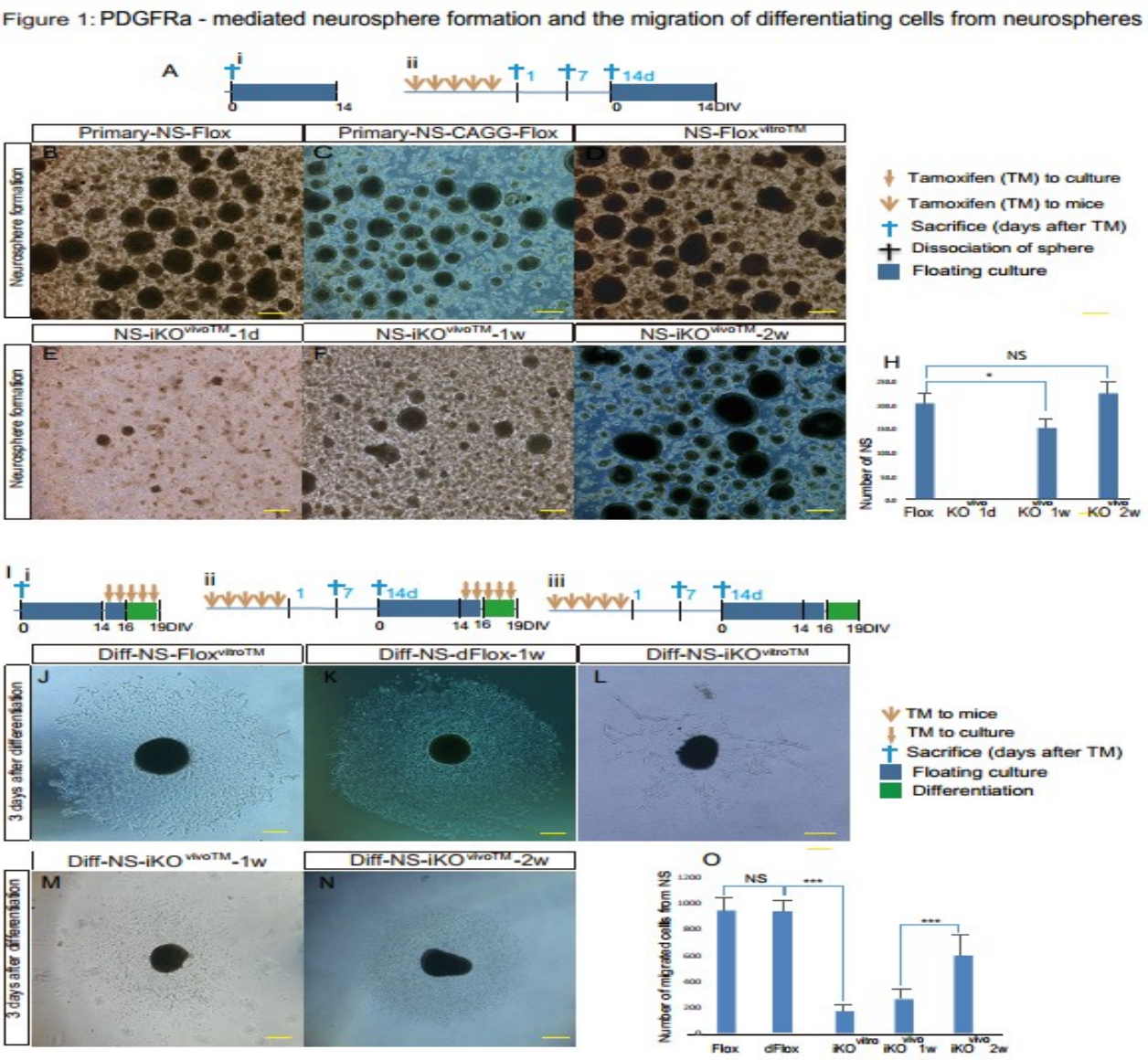

Figure legends

Figure 1. PDGFRα-mediated neurosphere formation and the migration of differentiating cells from neurospheres

(A) Schematic representation of the neurosphere formation assay. Primary neurosphere formation was examined 14 days after floating culture of cells from control *Pdgfra*$^{flox/flox}$ (Flox) mice and *CAGG-CreER*; *Pdgfra*$^{flox/flox}$ (CAGG-Flox) mice, termed primary-NS-Flox and primary-NS-CAGG-Flox, respectively (A-i). CAGG-Flox mice were administered TM orally (iKO mice) for 5 days and were sacrificed after 1 day, 1 week, and 2 weeks to obtain KO 1 day neurospheres (NS-iKO^{vivoTM}-1d), NS-iKO^{vivoTM}-1w, and NS-iKO^{vivoTM}-2w, respectively (A-ii). Control

neurospheres were obtained from Flox mice that were added 4OH intro (NS-Flox^{vitroTM}). (B–H) Micrographs of neurospheres. Primary neurospheres of primary-NS-Flox (B) and primary-NS-CAGG-Flox (C).

(I) Schematic representation of the neurosphere differentiation assay. Primary-NS-Flox following induced differentiation were termed diff-NS-Flox. During the differentiation assay, primary-NS-CAGG-Flox were treated with 4OH-TM for 5 days to inactivate *Pdgfra* and were termed diff-NS-iKO^{vitroTM} (I-i). Primary-NS-Flox cells were used as a control, with the primary neurospheres of NS-Flox^{vivoTM} being treated again with 4OH-TM for 5 days and termed diff-NS-dFlox-1w (I-ii). Primary neurospheres from TM-treated mice (NS-iKO^{vivoTM}-1w and NS-iKO^{vivoTM}-2w) not treated with 4OH-TM were termed diff-NS-iKO^{vivoTM}-1w and diff-NS-iKO^{vivoTM}-2w (I-iii). (J–N) Micrographs of differentiated neurospheres. Differentiated primary neurospheres of diff-NS-Flox (J), diff-NS-dFlox-1w (K), diff-NS-iKO^{vitroTM} (L), diff-NS-iKO^{vivoTM}-1w (M), diff-NS-iKO^{vivoTM}-2w (N). (H) Quantification of neurosphere formation, similar numbers of neurospheres were identified in both primary-NS-Flox and primary-NS-CAGG-Flox cultures. *p<0.05, NS-Flox vs NS-iKO^{vivoTM}-1w; NS = non significance.

(O) Quantification of cells migrating from the neurosphere during the differentiation assay. Many migrating cells were counted in the diff-NS-Flox and diff-NS-dFlox-1w control samples, while diff-NS-iKO^{vitroTM}, diff-NS-iKO^{vivoTM}-1w contained very few and diff-NS-iKO^{vivoTM}-2w appeared relatively recovered (n = 3 – 4 mice per group). ***p<0.001, scale bars = 100µm.

3.2. Stem cell differentiation was PDGFRα-dependent, but regenerated after a transient decrease in iKO mice

Stem cell differentiation was examined by counting migrating cells following induced adherence of neurospheres to the bottom of culture-dishes (Figure 1I). Many migrating cells were found in the differentiation-induced primary-NS-Flox culture (diff-NS-Flox; Figure 1J) without TM treatment. A similar number of cells were found migrating from diff-NS-dFlox-1w neurospheres as from cultures prepared from Flox mice treated 1 week before sacrifice with TM and then treated again with 4OH-TM in vitro (Figure 1I-ii, K). Therefore, in vivo TM and in vitro 4OH-TM treatments did not directly affect the differentiation assay (Migrating cells, 945.8 ± 33.1 in diff-NS-Flox and 933.3 ± 30.0 in diff-NS-dFlox-1w, difference not significant, 127, 88 and 86 neurospheres from 4 and 3 mice per group, respectively). Migration from primary-NS-CAGG-Flox cultures treated with 4OH-TM in vitro (diff-NS-iKO^{vitroTM}) largely decreased (Figure 1I-i, L: migrating cells 169.6 ± 11.5, n = 129 neurospheres from 4 mice) in comparison with diff-NS-dFlox-1w (Figure K, p<0.001). These data suggest that the differentiation of NSCs is largely dependent on PDGFRα.

Table 3. Control and knockout groups in vitro

	Diff-NS-Flox	Diff-NS-dFlox-1w	Diff-NS-iKO^{vitroTM}
Cell number	945.8 ± 33.1	933.3 ± 30.0	169.6 ± 11.5

Differentiation was examined in the NSCs isolated from V-SVZ of iKO mice with global *Pdgfra*-inactivation. This differentiation assay could not be carried out in NS-iKO^{vivoTM}-1d cultures, since neurosphere formation was depleted at 1 day after TM-treatment in iKO mice as described earlier (Figure 1E). The number of migrating cells was smaller in diff-NS-iKO^{vivoTM}-1w (Figure 1I-iii, M; 264.6 ± 15.8, n = 134 neurospheres from 4 mice) than in diff-NS-dFlox-1w (Figure 1K, 933.3 ± 30.0, p<0.001). The number of migrating cells then increased

significantly in diff-NS-iKO^{vivoTM}-2w (Figure 1I-iii, N; 600.7 ± 36.6 cells, n = 162 neurospheres from 4 mice) when compared to diff-NS-iKO^{vivoTM}-1w (Figure 1M, p<0.001), though this count was still lower than that seen in control diff-NS-dFlox-1w (Figure 1J, p<0.001). Therefore, the global *Pdgfra*-inactivation in vivo significantly suppressed the differentiation of NSCs, but the suppression was significantly recovered, in which recovery, the role of regenerated NSCs carrying an intact *Pdgfra* gene was indicated in the V-SVZ of iKO mice.

Table 4. Cell recovered after tamoxifen treatment in vivo at 1 day, 1 week and 2 weeks

	Diff-NS-iKO^{vivoTM}-1d	Diff-NS-iKO^{vivoTM}-1w	Diff-NS-iKO^{vivoTM}-2w
Cell number	N.D	264.6 ± 15.8	600.7 ± 36.6

4. Discussion

The V-SVZ attracts the interest of regenerative medicine as a niche for NSCs that contributes to the sustained neurogenesis and gliogenesis of adult mammalian brains; however, the regulatory mechanisms remain largely unknown. Here, we examined the role of PDGFRα in the regulation of neurosphere formation and differentiation (NFD) in the V-SVZ via the inactivation of *Pdgfra* both in vivo and in vitro through the use of TM-inducible Cre recombinase. *Pdgfra* inactivation in vitro severely suppressed NFD in cultured adult NSCs to indicate a role for PDGFRα in the self-renewal of NSCs. Furthermore, *Pdgfra* inactivation in vitro, severely suppressed radial cell migration from neurospheres.

The important role of PDGFRα in vivo was indicated through the neurosphere assay, a widely used read-out of in vivo stem cell characteristics (Pastrana et al, 2011), after global *Pdgfra* inactivation in adulthood. Intriguingly, neurosphere formation was totally depleted at one day but recovered at 1 and 2 weeks after *Pdgfra* inactivation. These recoveries were suggested due to repopulated NSCs with intact *Pdgfra* gene, because additional *Pdgfra* inactivation in vitro again severely suppressed the cell migration and differentiation of regenerated neurospheres. Altered stem cell activities intimately associating with depletion/repopulation of *Pdgfra*-expressing NSC population indicated the in vivo role of PDGFRα in adult neurogenesis and gliogenesis in V-SVZ (Decimo et al, 2012; Garcia et al, 2004; Lois and Alvarez-Buylla, 1993).

Our present study suggested in vivo and in vitro contributions of PDGFRα for the activation of V-SVZ-derived NSCs. These accumulated data may indicate that PDGFRα can also be involved in the homeostasis or regeneration of oligodendrocyte through the V-SVZ-derived oligodendrogenesis in adult brain.

We have shown that PDGFRα is important for the self-renew and multipotency of adult NSCs. Furthermore, the plasticity of NSC activities in adult V-SVZs was largely dependent on PDGFRα. This regulation of adult NSCs by PDGFRα may be a promising target of future regenerative medicine and rejuvenation strategies for the prevention of neurological diseases in the elderly.

5. Conclusion

We have shown that PDGFRα is important for the self-renew and multipotency of adult NSCs. Furthermore, the plasticity of NSC activities in adult V-SVZs was largely dependent on PDGFRα. Formation and differentiation of neurosphere were maintained by PDGFRa gene which expressed stem cell activities in adult mouse brain, this regulation of adult NSCs by

PDGFRα may be a promising target of future regenerative medicine and rejuvenation strategies for the prevention of neurological diseases in the elderly.

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