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Assessment of the effects of virus-mediated limited Oct4 overexpression on the structure of the hippocampus and behavior in mice

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Recently, pluripotency induction or cellular reprogramming by introducing critical transcription factors has been extensively studied, but has been demonstrated only *in vitro*. Based on reports that Oct4 is critically involved in transforming neural stem cells into pluripotent cells, we used the lentiviral vector to introduce the Oct4 gene into the hippocampal dentate gyrus (DG) of adult mice. We examined whether this manipulation led to cellular or behavioral changes, possibly through processes involving the transformation of NS cells into pluripotent cells. The Oct4 lentivirus-infused group and the green fluorescent protein lentivirus-infused group showed a similar thickness of the DG and a comparable level of synaptophysin expression in the DG. Furthermore, our behavioral analyses did not show any differences between the groups concerning exploratory activity, anxiety, or memory abilities. This first trial for pluripotency induction *in vivo*, despite negative results, provides implications and information for future studies on *in vivo* cellular reprogramming. [BMB reports 2011; 44(12): 793-798]

INTRODUCTION

Octamer-binding transcription factor 4 (Oct4) is one of the members of the POU-domain transcription factors that play a role in the regulation of cell growth and differentiation in a variety of tissues (1). Oct4 is expressed in pluripotent cells, such as epiblast cells, primordial germ cells, and embryonic stem and germ cells of the developing embryo (2-5). Because Oct4 is critical for the self-renewal of embryonic stem cells (6), a loss-of-function mutation in Oct4 during the early embryonic stage of mice results in failure of the formation of inner cell mass and differentiation into the trophectoderm, with a loss of pluripotency (7).

Recent studies have shown that the introduction of specific transcription factors into somatic cells can induce them to acquire pluripotency (8, 9). Initially, pluripotent cells were generated via the ectopic expression of 4 factors (Oct4, Sox2, Klf4, and c-Myc) from mouse and human somatic cells (10-13). In previous studies, the combinations of specific factors that were able to stimulate the production of induced pluripotent stem (iPS) cells varied. However, Oct4 was apparently indispensable in all the combinations used. In neural stem (NS) cells, overexpression of Oct4 is sufficient to transform NS cells into pluripotent cells, which can differentiate into derivatives of all 3 germ layers and functional germ cells (14, 15).

The results of the above-mentioned studies showed that Oct4 expression is important for the regulation of pluripotency. However, all of these studies focused on the development of iPS cells, and to our knowledge, the effect of Oct4 overexpression *in vivo* has not been reported. In adult mammals, neural progenitor cells exist in only a few brain regions, one of which is the dentate gyrus (DG) subregion of the hippocampus. Therefore, we assessed the cellular and behavioral effects of *in vivo* Oct4 expression in the DG of mice by using lentiviral transduction.

RESULTS

Expression of Oct4 and EGFP in mouse DG

The DG is one of the niches in the adult brain where neurogenesis occurs; therefore, we decided to overexpress Oct4 in this region. We used a lentivirus system to overexpress Oct4 ectopically in the DG (Fig. 1A). All histological and behavioral experiments were performed 7 weeks after viral infusion (Fig. 1B). After performing all these experiments, we confirmed the pres-

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ence of overexpressed Oct4 by performing a reverse-transcriptase polymerase chain reaction (RT-PCR) with Oct4- specific primers. We used DG samples obtained from the Oct4 or enhanced green fluorescent protein (EGFP) from lentivirus-infused mice. As shown in Fig. 1C, only the Oct4 lentivirus-infused group showed significant expression of Oct4. Endogenous Oct4 was not detected in EGFP lentivirus-infused mice. This finding, which is consistent with those of previous studies, suggests that the expression of Oct4 is absent in adult mice (16). Brain slices of the EGFP lentivirus-infused mice showed green fluorescence signals exclusively in the DGs (Fig. 1D). These results indicate that the viruses specifically targeted the DG and that Oct4 was overexpressed by the lentivirus in the DG cells.

Effect of Oct4 overexpression on the DG structure and synaptic density

We investigated the thickness of the DG subgranular zone because Oct4 expression might increase neurogenesis in the DG. The thickness did not vary between the Oct4 lentivirus-infused group and the control EGFP lentivirus-infused group (Fig. 2A). These results suggest that *in vivo* Oct4 expression cannot induce changes in the gross morphology of the DG.

Although overexpression of Oct4 did not cause a significant developmental change in the DG, as shown in Fig. 2A, it could alter synaptic development. Therefore, we examined whether Oct4 overexpression produced any changes in synaptic development which eventually affected the synaptic density. To this end, we measured the synaptophysin (SYP) protein level in the DG as a synaptic marker. We performed SYP immunostaining and analyzed signal intensities by using a fluorescence microscope. We found that the level of fluorescence signals for SYP was not different between the groups (Fig. 2B) ($P > 0.05$, unpaired *t* test). Taken together, all these data suggest that *in vivo* Oct4 expression cannot induce changes in the level of SYP expression and the gross morphology of the DG.

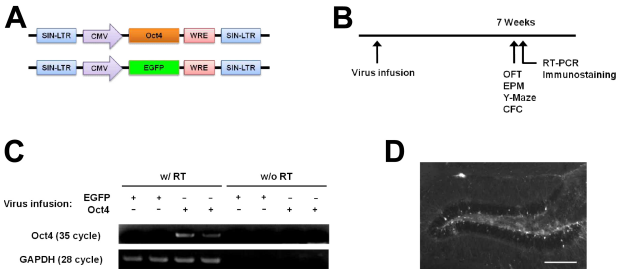

Fig. 1. The ectopic expression of Oct4 and EGFP in the DG of the mice. (A) A schematic map of the lentiviral construct. (B) Experimental scheme. (C) RT-PCR analysis of Oct4 by using cDNA extracted from the DG. The sample tissue was prepared 7 weeks after virus infusion. Only the Oct4 lentivirus-infused samples showed significant amplification signals. GAPDH was used as a control. (D) Green fluorescence expression in the DG of an EGFP lentivirus-infused mouse. Experiment was performed 7 weeks after virus infusion. Scale bar, 250 μm.

Behavioral analysis of the Oct4 and EGFP lentivirus-injected mice

The DG in the hippocampus is known to be critical in the regulation of cognitive functions and psychiatric disorders. We reasoned that if Oct4 overexpression elicits some functional changes in the DG, possibly via affecting NS cells, it might eventually lead to some behavioral changes. Therefore, we performed a variety of behavioral tests after Oct4 overexpression in the DG. However, the results of the behavioral tests on the Oct4 and EGFP lentivirus-infused mice were not different.

The anxiety levels and exploratory behavior of the mice were measured by the open field test (OFT) (17, 18). The percentages of time spent in the center divided by total times measured in the 10 cm^2 and 20 cm^2 sections were approximately equal (Fig. 3A) ($P > 0.05$, unpaired *t* test). Moreover, the overall movement of the Oct4 virus-infused mice did not differ from that of the EGFP controls (Fig. 3B) ($P > 0.05$, unpaired *t* test). The results of the OFT suggested that Oct4-overexpressing mice showed similar anxiety levels and locomotor activities to those in the EGFP control groups.

The elevated plus maze (EPM) has been adapted as a simple method for assessing the level of anxiety in rodents (19, 20). The rodent's anxiety is determined by measuring its tendency to stay in safe areas (closed arms) or to explore novel environments (open arms) (21). As shown in Fig. 3C, there were no significant differences between the anxiety level of the Oct4 lentivirus-infused group and the EGFP lentivirus-infused group, indicating that both groups presented a similar degree of anxiety-like behavior ($P > 0.05$, unpaired *t* test).

The Y-maze test is widely used to evaluate the spatial working memory and locomotor activities of mice (22). The hippocampus is known to be involved in the processing of spatial working memory. We evaluated spontaneous alteration, which is the tendency of mice to switch arm choices in successive trials. The mean spontaneous alteration rates (Fig. 4A) and the total number of arm entries (Fig. 4B) were not different be-

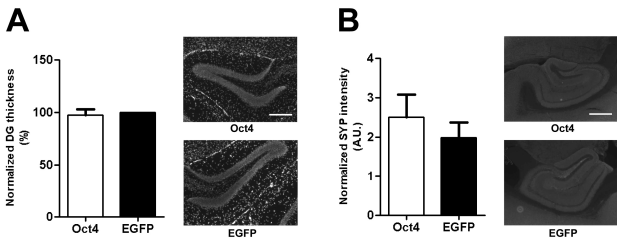

Fig. 2. The effects of Oct4 overexpression on synaptic density and DG structure. (A) Normalized DG thickness was similar in the 2 groups (n = 5). Scale bar, 250 μm. (B) Analysis of signal intensities after SYP immunostaining. No difference was observed between the SYP expression levels of the Oct4 lentivirus-infused mice and EGFP lentivirus-infused mice (Oct4: 2.50 ± 0.57, n = 4; EGFP: 1.99 ± 0.38, n = 5; $P > 0.4$, unpaired *t* test). Scale bar, 750 μm.

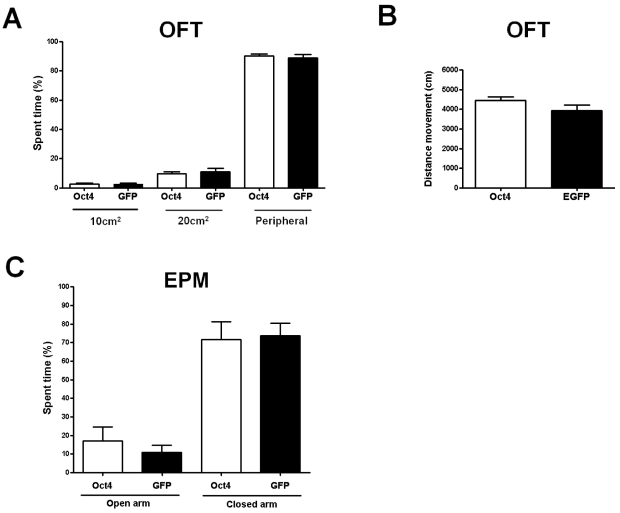

Fig. 3. The effects of Oct4 overexpression on locomotion and anxiety. (A) No difference was observed between the results of the OFT in the Oct4 lentivirus-infused mice (n = 12) and the EGFP lentivirus-infused mice (n = 9). The percentage of time spent in the 10 cm^2 (Oct4: 2.43 ± 0.70%; EGFP: 2.66 ± 0.57%), 20 cm^2 (Oct4: 9.69 ± 1.25%; EGFP: 11.1 ± 2.41%), and peripheral regions (Oct4: 90.27 ± 1.26%; EGFP: 88.82 ± 2.40%) was measured. (B) Total movement (cm) measured in the OFT was similar in the 2 groups (Oct4: 4,460 ± 158.6 cm, n = 12; EGFP: 3,938 ± 289.0 cm, n = 9; P > 0.4, unpaired *t* test). (C) The 2 groups showed similar anxiety-like behavior in the EPM test. Average time spent in the open arms was not different in the 2 groups (Oct4: 17.08 ± 7.41%, n = 6; EGFP: 10.85 ± 3.98%, n = 6; P > 0.4, unpaired *t* test). Both groups also showed similar durations of time spent in the closed arms (Oct4: 71.67 ± 9.42%, n = 6; EGFP: 73.66 ± 6.71%, n = 6; P > 0.8, unpaired *t* test).

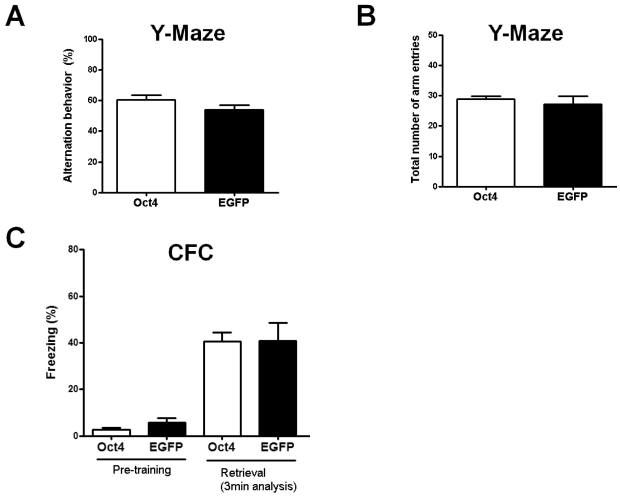

Fig. 4. The effects of Oct4 overexpression on cognitive memory function. (A) Spontaneous alteration values of Oct4 and EGFP lentivirus-infused mice. No significant difference between the 2 values (Oct4: 60.35 ± 3.17%, n = 12; EGFP: 54.01 ± 2.93%, n = 9; P > 0.1, unpaired *t* test). (B) Total number of arm entries was similar in the 2 groups (Oct4: 28.83 ± 1.09, n = 12; EGFP: 27.22 ± 2.58, n = 9; P > 0.53, unpaired *t* test). (C) The Oct4 and EGFP lentivirus-infused groups showed the same degree of freezing levels (Oct4: 40.66 ± 3.72%, n = 11; EGFP: 40.94 ± 7.55%, n = 6; P > 0.9, unpaired *t* test) in the CFC task. The mice received 1 unsignaled foot shock in the CFC test. Average freezing levels were not different in the 2 groups.

tween the 2 groups (P > 0.05, unpaired *t* test). These results suggested that both Oct4 and EGFP lentivirus-infused mice showed the same alternative and explorative behaviors, suggesting that the spatial working memory and locomotor activity of the mice were not affected by Oct4 overexpression.

We compared the associative learning abilities of the mice by using the contextual fear conditioning (CFC) paradigm, which is known to depend on the hippocampal function. In this experiment, a contextual environment as a neutral conditioned stimulus was paired with an aversive unconditioned stimulus such as an electric foot shock. A contextual environment that was previously paired with foot shock elicited freezing behavior in mice. Thus, associative memory could be assessed by the freezing level (23). As shown in Fig. 4C, freezing levels of the 2 groups were similar (P > 0.05, unpaired *t* test). These results indicate that Oct4 expression does not affect the associative memory, in which the hippocampus is critically involved.

DISCUSSION

In this study, we attempted to overexpress Oct4, which is a key regulator of stem cells, in the DG of the hippocampus of adult mice and assess its effect on the structure of the DG, emotions, and memory. Our results showed that, compared to the control mice, the mice showing Oct4 expression in the DG exhibited no differences in DG structure and behavior. The signal intensities measured after SYP immunostaining were similar in both groups. In addition, differences in the thickness of the DG in the 2 groups were not observed. In the behavioral experiments that measured exploratory activities, anxiety, and associative memory abilities, the Oct4 and EGFP lentivirus-infused mice showed similar results.

Accumulating reports have demonstrated that pluripotency induction from somatic cells or direct conversion of cell fate are possible in fibroblast culture, thereby shedding light on therapeutics for degenerative diseases. Although there is no report yet, if directed cellular reprogramming is possible *in vivo*, the technique might also be utilized for treating diseases. For example, non-neuronal cells in the brain could be converted to neurons to replace the lost neurons in neurodegenerative diseases or brain ischemia. It is worth trying to test if this type of *in vivo* approach could be useful.

We provide the first experimental results attempting *in vivo* cellular reprogramming. However, despite the lack of an apparent effect of Oct4 overexpression in this study, we cannot simply conclude that Oct4 has no effect on cellular or behav-

ioral functions, and there remain some unresolved questions. First, a technical issue should be considered. The infection efficiency of our lentivirus was relatively low and uneven, as shown in the GFP expression pattern; therefore, the Oct4 lentivirus probably infected only a small population of NS cells in the DG. This technical limitation could be one of the possible explanations for the null effects of Oct4 overexpression in the DG. To assess this issue quantitatively, co-immunostaining of NS cell markers and Oct4 is required to show whether Oct4 was actually introduced into the NS cells, but the Oct4 protein was not detected by immunohistochemistry (data not shown).

This raises another question of whether Oct4-introduced cells could express a sufficient level of Oct4 protein. We could confirm that there were some infected cells, because we detected significant Oct4 mRNA levels compared to control EGFP lentivirus infused mice. Therefore, the level of Oct4 protein produced in the infected cell seemed to be below the limit of detection of our immunohistochemistry technique. The low level or lack of Oct4 protein might have been caused by suppression mechanisms. Oct4 is not normally expressed in differentiated cells. For example, the Oct4 promoter is methylated during the development of definitive NS cells from primitive NS cells, which is dependent on the expression of the germ-cell nuclear factor (GCNF). The primitive NS cells lose their pluripotency via restricted expression of Oct4 (16). Thus, *in vivo* ectopic expression of Oct4 may not be efficiently achieved because many other suppression mechanisms could affect the expression of Oct4, although our lentiviral delivery of Oct4 induces the expression of Oct4 mRNA.

However, it should be noted that we cannot rule out the possibility that the level of neurogenesis might be altered by Oct4 ectopic expression in spite of no alteration being found in either cell volume or synaptogenesis, because measurements of DG thickness and SYP immunoreactivity are indirect methods to determine the effect of Oct4 overexpression on neurogenesis. A further examination of neurogenesis using e.g. BrdU labeling after Oct4 overexpression would be required to clarify this point.

We only examined some factors affecting normal tissue morphology and basic behaviors, but there remains a possibility that Oct4 ectopic expression might have been effective in other conditions such as a neurodegenerative disease model or neurotoxic cell death.

In our behavioral tests, Oct4 overexpression did not seem to affect the locomotive and basal behaviors measured in the OFT and EPM test or the hippocampus-dependent cognitive behaviors such as spatial working memory and fear conditioning. However, a recent report demonstrated that increased hippocampal neurogenesis in the adult brain is not linked to enhancement of contextual fear conditioning associative-learning, but has a causal effect on the improvement of pattern separation ability in a similar environment (24). Therefore, further studies using other learning and behavioral paradigms in relation to Oct4 expression in the hippocampus are necessary.

The different environments between *in vivo* brain and *in vitro* culture systems raise some issues which should be considered in future studies. First, Nestin promoter/enhancer could be used in viral construction to specifically express Oct4 for NS cells in the DG. This study is the first trial for iPS cell induction *in vivo*, but its lentiviral construction was based on the previous *in vitro* studies (8, 9). Second, various combinations of transcription factors involved in reprogramming need to be tested. Oct4 expression alone might be insufficient to induce cellular reprogramming of NS cells *in vivo*. Nevertheless, our study provides implications and information for studies of *in vivo* cellular reprogramming.

MATERIALS AND METHODS

Animals

All animals were managed as previously described (25). C57BL/6 male mice (8-weeks old) were used for the virus infusion.

Virus production

HEK293T cells were placed on T175 dishes (1.2×10^7 cells/plate) containing OptiMEM (GIBCO, 31985) with 5% fetal bovine serum (FBS) 16 h prior to transfection. The culture medium was changed 2 h prior to transfection, and 67.5 μg of plasmid DNA was used for the calcium phosphate-mediated transfection of each T175 dish. VSV-G envelope construct and a Δ8.9 packaging construct were used. Lentiviral supernatants were prepared by cotransfection of HEK293T cells with 30 μg of the lentiviral vector (pLL3.7-Oct4 and pLL3.7-GFP), 22.5 μg of the packaging vector, and 15 μg of the envelope vector.

Virus infusion

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (0.16 mg/kg) and xylazine (0.01 mg/kg) in saline. For microinjection, a guide cannula was positioned such that the final coordinates would be −2 mm anterior to the bregma, ±1.5 mm lateral to the middle line, and −2.3 mm ventral to the surface of the skull. After placing the cannula, virus infusion was conducted using a Hamilton syringe (Hamilton, Reno, NY, USA). Lentivirus dissolved in saline was delivered into the DG (4.5 μl/h).

RT-PCR

Total RNA was extracted from the DG by using TRIzol Reagent II (Invitrogen, Carlsbad, California, USA, 15596). cDNA was synthesized by using Superscript III reverse transcriptase (Invitrogen, 56575) with random hexamers as the primers. These cDNAs were used as templates for the RT-PCR reactions of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (sense: 5′-TGCACCACCAACTGCTTA-3′, antisense: 5′-GGATGCAGGGATGATGTTC-3′) and the Oct4 gene (sense: 5′-CCCCAGGGCCCCATTTTGGTACC-3′ (Takahashi *et al.*, 2007), anti-sense: 5′-GCTATACGAAGTTATTAGGTCC-3′). RT-PCR amplification was performed

using Ex Taq (Takara, Otsu, Shiga, Japan, RR001) for 28 to 35 cycles (94°C, 15 s; 60°C, 30 s; 72°C, 30 s). The PCR products were visualized on a 2% agarose gel.

Histochemistry

The brains were fixed with 4% paraformaldehyde (PFA) in PBS. The following day, the PFA was replaced with 30% sucrose in PBS, and the extracted brains were maintained overnight at 4°C for cryoprotection. Brain sections of 30-μm thickness were cut in a cryostat (LEICA, Wetzlar, Germany, CM1900). For immunohistochemical analysis, we used rabbit anti-SYP antibodies (1 : 250, Santa Cruz Biotechnology, Santa Cruz, California, USA, SC9116) and secondary antibodies conjugated with Alexa 488 fluorescent dye (1 : 300). For normalization, each signal intensity was divided by the signal intensity of the negative control (without secondary antibodies). Sections were mounted with Vectashield solution containing 4'-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, California, USA, H1200). The signals were visualized under a fluorescence microscope (OLYMPUS, Tokyo, Japan, IX51).

Behavioral procedure

OFT, Y-maze, and CFC tests were performed as previously described (25). The anxiety behavior of the mice was assessed by using an EPM made of acrylic with white-paper attachments. The maze consisted of 4 arms: 2 open arms without walls and 2 arms enclosed by walls, which were 18 cm high, 65 cm long, and 8 cm wide. The mice were placed facing an open arm at the junction of the 4 arms in the maze, and the time spent by the mice in each arm was recorded for 5 min using video-track software. Mice that fell were excluded from the data analysis.

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REFERENCES

1. Ryan, A. K. and Rosenfeld, M. G. (1997) POU domain family values: flexibility, partnerships, and developmental codes. *Genes Dev.* **11**, 1207.
2. Okamoto, K., Okazawa, H., Okuda, A., Sakai, M., Muramatsu, M. and Hamada, H. (1990) A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell* **60**, 461-472.
3. Rosner, M. H., Vigano, M. A., Ozato, K., Timmons, P. M., Poirie, F., Rigby, P. W. J. and Staudt, L. M. (1990) A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345**, 686-692.
4. Pesce, M. and Scholer, H. R. (2001) Oct 4: gatekeeper in the beginnings of mammalian development. *Stem Cells* **19**, 271-278.
5. Hochedlinger, K., Yamada, Y., Beard, C. and Jaenisch, R. (2005) Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. *Cell* **121**, 465-477.
6. Niwa, H., Miyazaki, J. and Smith, A. G. (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24**, 372-376.
7. Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H. and Smith, A. (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379-391.
8. Takahashi, K. and Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676.
9. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-872.
10. Meissner, A., Wernig, M. and Jaenisch, R. (2007) Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nat. Biotechnol.* **25**, 1177-1181.
11. Okita, K., Ichisaka, T. and Yamanaka, S. (2007) Generation of germline-competent induced pluripotent stem cells. *Nature* **448**, 313-317.
12. Lowry, W., Richter, L., Yachechko, R., Pyle, A., Tchieu, J., Sridharan, R., Clark, A. and Plath, K. (2008) Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc. Natl. Acad. Sci.* **105**, 2883.
13. Park, I. H., Zhao, R., West, J. A., Yabuuchi, A., Huo, H., Ince, T. A., Lerou, P. H., Lensch, M. W. and Daley, G. Q. (2007) Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451**, 141-146.
14. Kim, J. B., Greber, B., Arauzo-Bravo, M. J., Meyer, J., Park, K. I., Zaehres, H. and Scholer, H. R. (2009) Direct reprogramming of human neural stem cells by OCT4. *Nature* **461**, 649-643.
15. Kim, J. B., Sebastiano, V., Wu, G., Arauzo-Bravo, M. J., Sasse, P., Gentile, L., Ko, K., Ruau, D., Ehrich, M. and van den Boom, D. (2009) Oct4-induced pluripotency in adult neural stem cells. *Cell* **136**, 411-419.
16. Akamatsu, W., DeVeale, B., Okano, H., Cooney, A. J. and van der Kooy, D. (2009) Suppression of Oct4 by germ cell nuclear factor restricts pluripotency and promotes neural stem cell development in the early neural lineage. *J. Neurosci.* **29**, 2113.
17. Belzung, C. and Griebel, G. (2001) Measuring normal and pathological anxiety-like behaviour in mice: a review. *Behav. Brain Res.* **125**, 141-149.
18. Ko, H. G., Jang, D. J., Son, J., Kwak, C., Choi, J. H., Ji, Y. H., Lee, Y. S., Son, H. and Kaang, B. K. (2009) Effect of ablated hippocampal neurogenesis on the formation and extinction of contextual fear memory. *Mol Brain* **2**, 1.

19. Pellow, S., Chopin, P., File, S. E. and Briley, M. (1985) Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Meth.* **14**, 149-167.
20. Kanai, M., Funakoshi, H., Takahashi, H., Hayakawa, T., Mizuno, S., Matsumoto, K. and Nakamura, T. (2009) Tryptophan 2,3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behavior in mice. *Mol. Brain* **2**, 8.
21. Walf, A. A. and Frye, C. A. (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat. Protoc.* **2**, 322-328.
22. Bertholet, J. Y. and Crusio, W. E. (1991) Spatial and non-spatial spontaneous alternation and hippocampal mossy fibre distribution in nine inbred mouse strains. *Behav. Brain Res.* **43**, 197-202.
23. Phillips, R. and LeDoux, J. (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav. Neurosci.* **106**, 274.
24. Sahay, A., Scobie, K. N., Hill, A. S., O'Carroll, C. M., Kheirbek, M. A., Burghardt, N. S., Fenton, A. A., Dranovsky, A. and Hen, R. (2011) Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature* **472**, 466-470.
25. Park, S. W., Ko, H. G., Lee, N., Lee, H. R., Rim, Y. S., Kim, H., Lee, K. M. and Kaang, B. K. (2010) Aged wild-type littermates and APPswe+ PS1/¥ÄE9 mice present similar deficits in associative learning and spatial memory independent of amyloid load. *Genes & Genomics* **32**, 63-70.