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Safrole oxide is a useful tool for investigating the effect of apoptosis in vascular endothelial cells on neural stem cell survival and differentiation in vitro

Le Su,[a,c] BaoXiang Zhao,[b,*] Xin Lv,[a,c] Jing Zhao,[a,c] ShangLi Zhang[a,c] and JunYing Miao[a,c,*]

[a]*Institute of Developmental Biology, School of Life Science, Shandong University, Jinan 250100, China*
[b]*Institute of Organic Chemistry, School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, China*
[c]*The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Health, Shandong University Qilu Hospital, Jinan 250100, China*

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Abstract—Previously, we found safrole oxide could promote VEC apoptosis, however, it is not known whether it can induce NSC apoptosis. It is reported that neural stem cells (NSCs) are localized in a vascular niche. But the effects of apoptosis in vascular endothelial cells (VEC) on NSC growth and differentiation are not clear. To answer these questions, in this study, we co-cultured NSCs with VECs in order to imitate the situation in vivo, in which NSCs are associated with the endothelium, and treated the single-cultured NSCs and the co-cultured NSCs with safrole oxide. The results showed that safrole oxide (10–100 μg/mL) had no effects on NSC growth. Based on these results, we treated the co-culture system with this small molecule. The results showed that the NSCs differentiation, into neurons and gliacytes was induced by VECs untreated with safrole oxide. But in the co-culture system treated with safrole oxide, the NSCs underwent apoptosis. The data suggested that when VEC apoptosis occurred in the co-culture system, the NSC survival and differentiation could not be maintained, and NSCs died by apoptosis. Our finding provided a useful tool for investigating the effect of apoptosis in vascular endothelial cells on neural stem cell survival and differentiation in vitro.

Recently, it is well established that NSCs are not randomly distributed throughout the brain. The expansion and differentiation of the stem cells are tightly regulated by the cells and proteins that constitute the extracellular environment that NSCs inhabit.[1] Usually we name the microenvironment 'niche'. In the adult, neural stem cells (NSCs) are concentrated around blood vessels in the hippocampus, the subventricular zone (SVZ), and the song bird higher vocal center.[2–4] Thus, NSCs are in close proximity to the vascular endothelial cells (VECs), which facilitates the coordinated interactions between these two cell types.[2] In the developing central nervous system (CNS), the communication between VECs and NSCs is observed. It is reported that endothelial cells could synthesize numerous basal lamina elements and growth factors involved in neurogenesis, such as brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF).[5,6] In the adult song bird, the stimulation of neurogenesis involves BDNF synthesis by endothelial cells.[4] Endothelial cells play an essential role in the proliferation of NSCs and their differentiation into neurons.[7] These findings identify that VECs are very important for NSC growth and differentiation, and form a niche for NSCs. But so far, there is no report about the effect of apoptosis in vascular endothelial cells on NSC growth and differentiation.

Safrole oxide, a newly synthesized compound, has been studied for many years.[8–10] Recently, we found that safrole oxide induced apoptosis in various cell types, including VECs, human lung cancer cells, and primary cultures of mouse neurons.[11–15] However, whether or not safrole oxide can induce NSC apoptosis is not

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* Corresponding authors. Tel.: +86 531 88364929; fax: +86 531 88565610; e-mail addresses: bxzhao@sdu.edu.cn; miaojy@sdu.edu.cn

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known. These findings trigger us to apply this small molecule to the co-culture system of NSCs and VECs to investigate the interactions between these two kinds of cells.[7]

NSCs that we isolated from the mouse embryo forebrains at the 14th day could form neurospheres by self-renewal. We examined the expressions of nestin, a useful marker of NSCs. Moreover, the cells derived from the NSCs exhibited intensive positive neuron-specific enolase (NSE), neurofilament-L (NF-L), and synapsin, which are specific markers for neurons, and glial fibrillary acidic protein (GFAP) that is a specific marker for astrocytes (Fig. 1). The positive results ensured that the cells obtained were multi-potential NSCs consistent with previous report.[16] Neurospheres were used before the third passage to avoid transformation observed after multiple passages.[17]

NSCs are the progenitors of neurons. However, the effect of safrole oxide on NSCs remains not to be defined. To address this question, mouse NSCs were exposed to 10–100 μg/mL safrole oxide (these concentrations are similar to that used to induce apoptosis in other cells in the presence of basic fibroblast growth factor (bFGF)).[11,15] We found that the safrole oxide had no effect on the growth of NSCs and the cell viability had no difference between the control group and the treatment group ($p > 0.05$) (Fig. 2A). The data suggested that 10–100 μg/mL safrole oxide could not affect the survival and proliferation of NSCs. The morphological changes of NSCs treated with safrole oxide were observed under a phase contrast microscope (Fig. 2B and C).

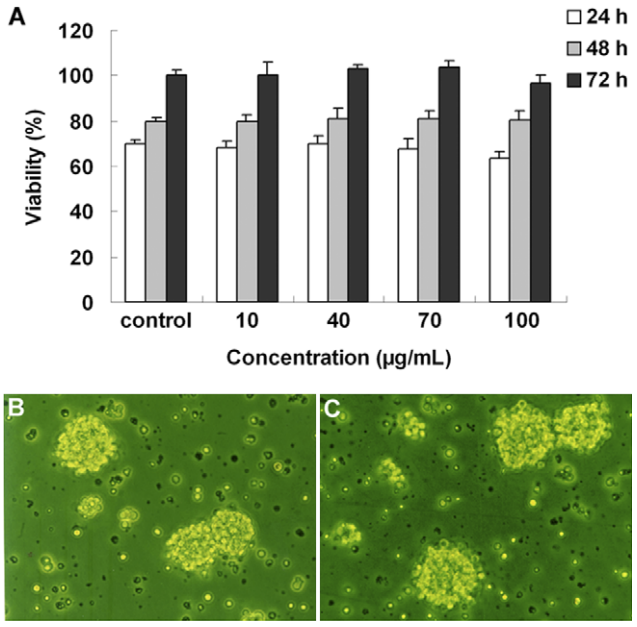

Figure 2. Effects of safrole oxide on the cell viability and morphology of NSCs. (A) The NSCs were treated with or without safrole oxide at various concentrations indicated for different periods. The viability of NSCs was determined by MTT assay as described in Materials and methods. There is no difference between the control group and the safrole oxide treatment group ($p > 0.05$). Mean values were derived from three independent experiments. (B) NSCs cultured in the supplemented medium. (C) NSCs treated with 70 μg/mL safrole oxide for 72 h. The morphological changes were observed under a phase contrast microscope (400×). The data presented are representatives of three independent experiments.

To elucidate the dynamic interactions between NSCs and vascular endothelial cells in the neurovascular niche, we used the co-culture system to imitate the situation in vivo. When co-cultured in the direct contact

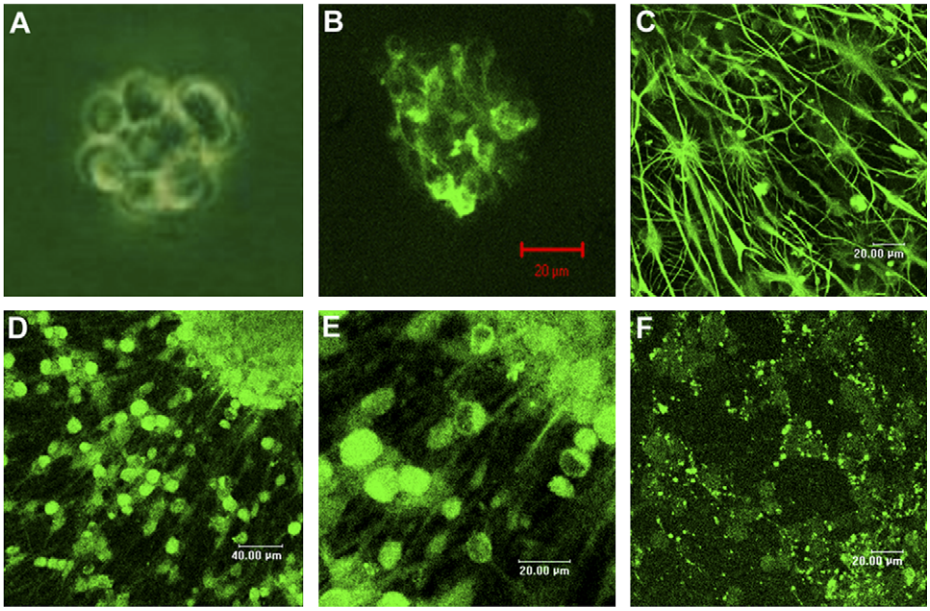

Figure 1. Identification of NSCs. (A) The NSCs formed neurosphere by self-renewal, which could be observed under a phase contrast microscope (Nikon, Japan) (400×). Positive immunocytochemical stainings for nestin in the NSCs (B), GFAP in the astrocytes derived from NSCs (C), NSE (D), NF-L (E), and synapsin (F) in the neurons derived from NSCs were observed, respectively. The photographs presented are representatives of three independent experiments.

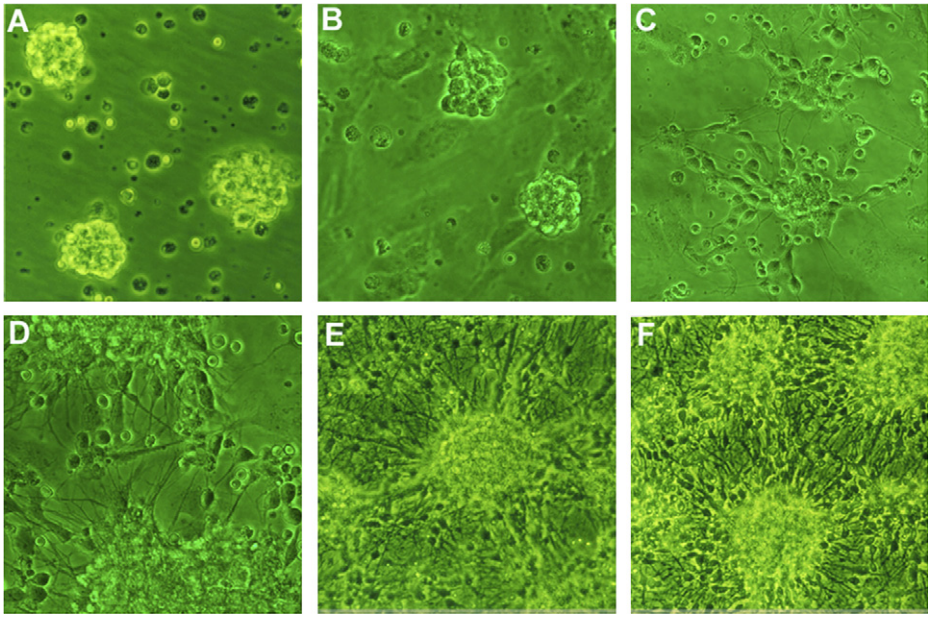

Figure 3. Neurospheres co-cultured with VEC monolayer. (A) NSCs cultured alone in the supplemented medium. Neurospheres co-cultured with VEC monolayer for 0 h (B), 10 h (C), 24 h (D), 48 h (E), and 72 h (F), respectively. The photographs presented are representatives of three independent experiments (400×).

with VEC monolayer, the NSCs underwent differentiation. Neurite outgrowth of NSCs was observed after 10 h (Fig. 3C). The neurites became more and longer after 24 h (Fig. 3D). After 3 days, the adjacent neurospheres formed network by neurites (Fig. 3F). Meanwhile, NSCs still suspended as neurospheres in the control group (Fig. 3A). To further determine whether the NSCs differentiated into neurons or gliacytes after co-cultured with VECs, we examined the expressions of NSE, NF-L, synapsin, and GFAP by immunofluorescence assay after 3 days (Fig. 4). The positive results ensured that the NSCs differentiated into neurons and gliacytes after co-cultured with VECs.

In our previous studies, we found that 70 μg/mL safrole oxide could induce VEC apoptosis after 10 h.[18] Then we exposed the co-culture system to 70 μg/mL safrole oxide to know the effect of VEC apoptosis on NSC survival and differentiation. As shown in Figure 5, the NSCs presented neurite outgrowth after co-cultured with VECs for 5 and 10 h. Meanwhile, some NSCs also presented neurite outgrowth in the safrole oxide treatment group after co-cultured for 5 h (Fig. 5E). But after the co-culture system was treated with 70 μg/mL safrole oxide for 10 h, at this time safrole oxide induced VEC apoptosis obviously,[18] the neurites of NSCs degenerated and the NSCs underwent apoptosis (Fig. 5F). The results showed that the apoptotic VECs could significantly inhibit the survival and differentiation of NSCs after the cells were treated with safrole oxide for 10 h.

We next examined whether safrole oxide could induce the co-cultured NSC, necrosis, an extreme end of neurotoxicity. Results from LDH assays showed that there was no significant difference in LDH release between the test group and control group at 10 h (Fig. 6A) ($^{\&}p > 0.05$ vs. $^{\#}$, $n = 3$), suggesting that safrole oxide did not induce necrosis in the co-cultured cells. Moreover, nuclear condensation, DNA fragmentation, and apoptotic body formation, which are characteristics of apoptosis, were observed clearly (Fig. 6B). The apoptosis rate of NSCs equaled to the number of cells that had the apoptotic characteristics divided by the total number of NSCs. Compared with the co-culture group, the

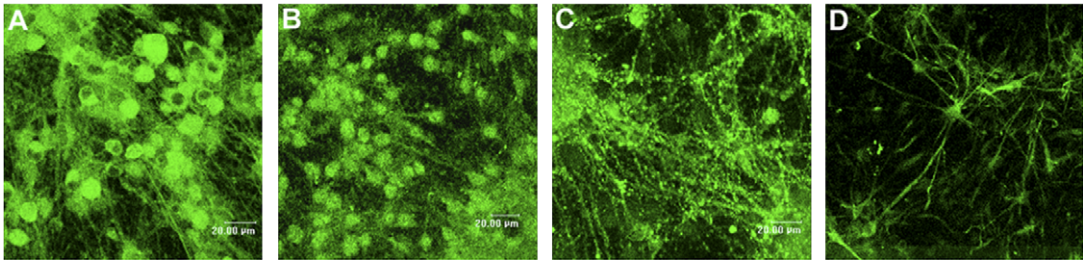

Figure 4. Identification of differentiated NSCs in the co-cultured system. NSCs were co-cultured with VEC in the supplemented medium for 3 days and the expressions of NSE (A), NF-L (B), synapsin (C), and GFAP (D) were examined by immunofluorescence staining as described in Materials and methods. The photographs presented are representatives of three independent experiments.

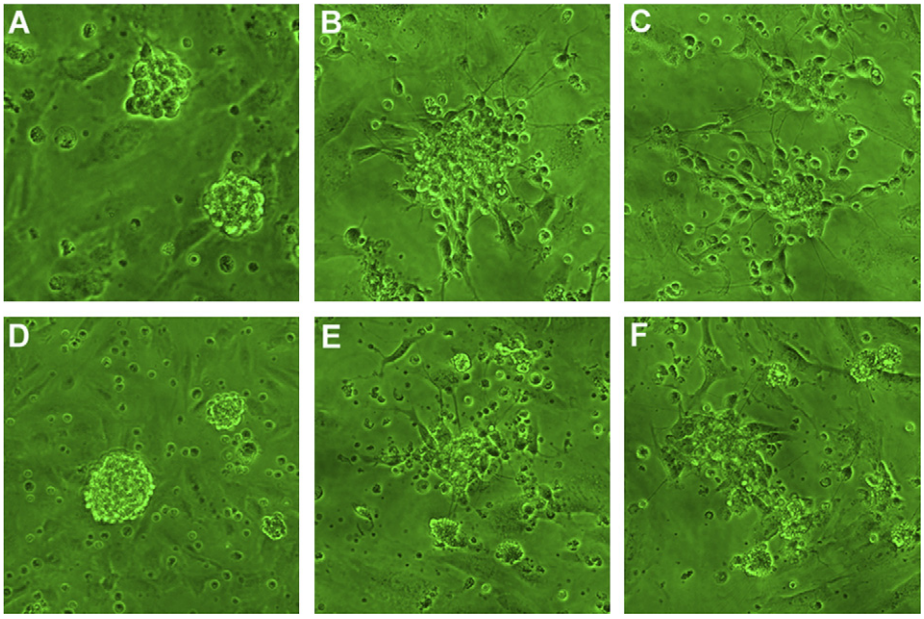

Figure 5. Effects of safrole oxide on the morphology of neurospheres co-cultured with VEC monolayer. Neurospheres co-cultured with VEC monolayer in the supplemented medium for 0 h (A), 5 h (B), and 10 h (C), respectively. The co-culture system was treated with 70 μg/mL safrole oxide for 0 h (D), 5 h (E), and 10 h (F), respectively. The pictures presented are representatives of three independent experiments.

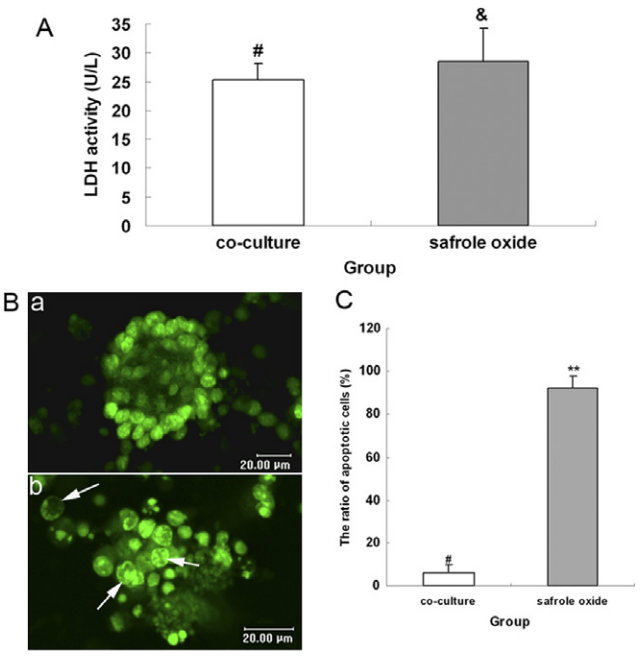

Figure 6. Safrole oxide triggered the co-cultured NSCs to undergo apoptosis. (A) NSCs co-cultured with VECs in the supplemented medium were treated with or without safrole oxide (70 μg/mL). After 10-h treatment, LDH assay was performed by using a LDH kit as described in Materials and methods ($^{\&}p > 0.05$ vs. $^{\#}$). Mean values were derived from three independent experiments. (B) The co-culture system was treated with (b) or without (a) 70 μg/mL safrole oxide. Ten hours later, the cells were stained with acridine orange for 5 min and then the nuclear condensation and fragmentation were observed under the laser scanning confocal microscope. The arrowheads show the typical nuclear fragmentations. The data presented are representatives of three independent experiments. (C) The quantity of apoptotic cells. ($^{**}P < 0.01$ vs. $^{\#}$). Mean values were derived from three independent experiments.

safrole oxide treatment group showed an overwhelming majority of apoptotic NSCs that increased to 92% (Fig. 6C) ($^{**}p < 0.01$ vs. #, $n = 3$). These results showed that the death of NSCs belonged to a typical kind of apoptosis. Therefore, our results showed that the apoptotic VECs triggered by safrole oxide induced NSC apoptosis.

Based on these results, we reported that endothelial cells played a key role in regulating neurogenesis by stimulating NSC differentiation into neurons. This result is in accordance with results reported recently.[7,19] In this study, it was also showed that the stimulation of astrocytic differentiation by endothelial cells is consistent with a previous report and evokes the close relationship between astrocytes and endothelial cells in the functioning of the blood–brain barrier.[20,21]

Apoptosis in response to lack of adhesion or inappropriate adhesion has been termed anoikis, a Greek word meaning 'homelessness'.[22] Signals propagated from cell–ECM adhesion complexes activate a number of well-characterized pathways, many of which have been suggested to play a role in the suppression of apoptosis.[23] Most cell–ECM interactions depend on integrins, transmembrane heterodimeric receptors for ECM proteins that associate with a frightening number of proteins on the cytoplasmic face of the plasma membrane, forming cell–ECM adhesion complexes.[24,25] The destruction of the cell–ECM adhesion complexes would induce cell anoikis.[26,27] In the previous studies, we found that safrole oxide could induce VEC and neuron apoptosis.[11,15] Both of them are adherent cells. VEC apoptosis induced by safrole oxide was mediated by integrin β4 and the attachment was destructed during the apoptosis process.[12] In this study, we found that safrole oxide had

no effect on NSC growth and could not induce apoptosis of NSCs which are suspended cells in the single culture. The data suggested that safrole oxide might induce apoptosis in adherent cells through integrins but not in suspended cells.

Based on the different effects of safrole oxide on VECs and NSCs, we treated the co-culture system with 70 μg/mL safrole oxide to investigate the effect of apoptotic VECs on the growth and differentiation of NSCs. We found that the apoptotic VECs could inhibit the differentiation and induce apoptosis in NSCs. Several recent publications have described the roles of endothelial cells in modulating the self-renewal and neurogenesis of neural stem cells and dorsal root ganglion.[7,19,28] These studies are consistent with a complex, dynamic coupling of neurogenesis and vasculogenesis.[29] Our data suggested that, when the VEC apoptosis occurred, the stem cell niche would be destroyed and the NSCs underwent apoptosis.

In summary, our system is a powerful tool to identify the factors dependent on endothelial cells and influencing neural stem cells and may help in the knowledge of the neural stem cell niche. Investigation of the factors involved in these functions is essential to increase the understandings of neurogenesis mechanism and pathological processes such as neurodegenerative diseases, in which endothelial cells may play a key role.[30] In light of the small molecule safrole oxide, we will take an important step forward in our understanding of the neural stem cell niche.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.03.032.

References and notes

1. Fuchs, E.; Tumbar, T.; Guasch, G. *Cell* **2004**, *116*, 769.
2. Palmer, T. D.; Willhoite, A. R.; Gage, F. H. *J. Comp. Neurol.* **2000**, *425*, 479.
3. Capela, A.; Temple, S. *Neuron* **2002**, *35*, 865.
4. Louissaint, A., Jr.; Rao, S.; Leventhal, C.; Goldman, S. A. *Neuron* **2002**, *34*, 945.
5. Mabie, P. C.; Mehler, M. F.; Kessler, J. A. *J. Neurosci.* **1999**, *19*, 7077.
6. Jin, K.; Zhu, Y.; Sun, Y.; Mao, X. O.; Xie, L.; Greenberg, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11946.
7. Shen, Q.; Goderie, S. K.; Jin, L.; Karanth, N.; Sun, Y.; Abramova, N.; Vincent, P.; Pumiglia, K.; Temple, S. *Science* **2004**, *304*, 1338.
8. Noller, R. C.; Kneeland, P. D. *J. Am. Chem. Soc.* **1964**, *68*, 201.
9. Qato, M. K.; Guenthner, T. M. *Toxicol. Lett.* **1995**, *75*, 201.
10. Luo, G.; Guenthner, T. M. *Drug Metab. Dispos.* **1996**, *24*, 1020.
11. Miao, J. Y.; Zhao, B. X.; Li, H. H.; Zhang, S. L.; Du, C. Q. *Acta Pharmacol. Sin.* **2002**, *23*, 323.
12. Zhao, J.; Miao, J. Y.; Zhao, B. X.; Zhang, S. L. *FEBS Lett.* **2005**, *579*, 5809.
13. Du, A. Y.; Zhao, B. X.; Yin, D. L.; Zhang, S. L.; Miao, J. Y. *Bioorg. Med. Chem.* **2005**, *13*, 4176.
14. Du, A. Y.; Zhao, B. X.; Yin, D. L.; Zhang, S. L.; Miao, J. Y. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 81.
15. Su, L.; Zhao, B. X.; Lv, X.; Wang, N.; Zhao, J.; Zhang, S. L.; Miao, J. Y. *Life Sci.* **2006**. doi:10.1016/j.lfs.2006.11.041.
16. Lv, X.; Wang, N.; Su, L.; Zhang, S.; Miao, J. *Dev. Neurosci.* **2006**, *28*, 499.
17. Morshead, C. M.; Benveniste, P.; Iscove, N. N.; van der Kooy, D. *Nat. Med.* **2002**, *8*, 268.
18. Zhao, J.; Miao, J.; Zhao, B.; Zhang, S.; Yin, D. *Int. J. Biochem. Cell Biol.* **2006**, *38*, 1603.
19. Mathieu, C.; Fouchet, P.; Gauthier, L. R.; Lassalle, B.; Boussin, F. D.; Mouthon, M. A. *Exp. Cell Res.* **2006**, *312*, 707.
20. Mi, H.; Haeberle, H.; Barres, B. A. *J. Neurosci.* **2001**, *21*, 1538.
21. Hawkins, B. T.; Davis, T. P. *Pharmacol. Rev.* **2005**, *57*, 173.
22. Frisch, S. M.; Francis, H. *J. Cell Biol.* **1994**, *124*, 619.
23. Frisch, S. M.; Screaton, R. A. *Curr. Opin. Cell Biol.* **2001**, *13*, 555.
24. Geiger, B.; Bershadsky, A.; Pankov, R.; Yamada, K. M. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 793.
25. Critchley, D. R. *Curr. Opin. Cell Biol.* **2000**, *12*, 133.
26. Valentijn, A. J.; Zouq, N.; Gilmore, A. P. *Biochem. Soc. Trans.* **2004**, *32*, 421.
27. Gilmore, A. P. *Cell Death Differ.* **2005**, *12*, 1473.
28. Mompeo, B.; Engele, J.; Spanel-Borowski, K. *J. Neurocytol.* **2003**, *32*, 123.
29. Wurmser, A. E.; Palmer, T. D.; Gage, F. H. *Science* **2004**, *304*, 1253.
30. Barcia, C.; Emborg, M. E.; Hirsch, E. C.; Herrero, M. T. *Front. Biosci.* **2004**, *9*, 277.