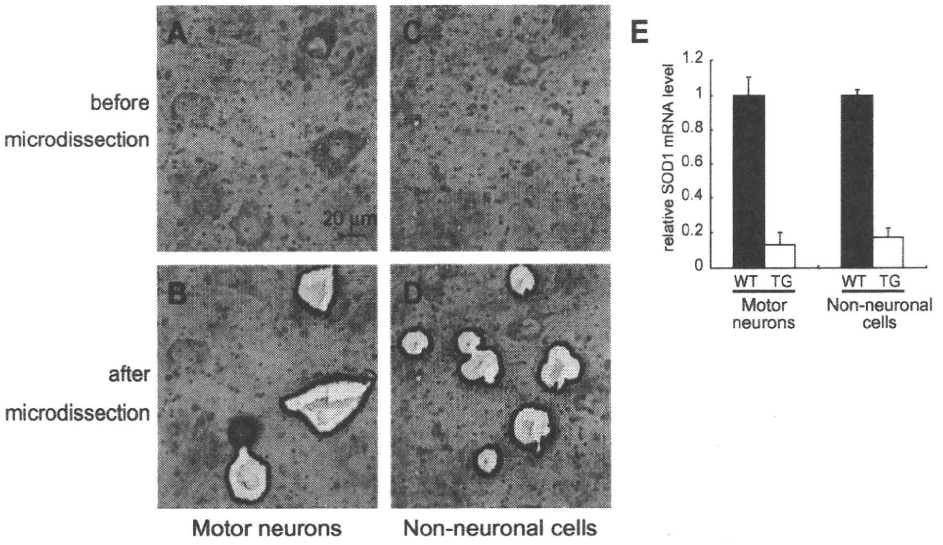

Fig. 3. Silencing efficiency in the neuronal and non-neuronal cells of the shRNA transgenic mice. (A–D) Microdissection of motor neurons and non-neuronal cells in the ventral horn of the lumbar spinal cord of the transgenic mice and age-matched wild-type littermates. Motor neurons (A and B) and non-neuronal cells (C and D) were dissected by laser microbeam. (E) Quantification of SOD1 mRNA in the motor neurons and non-neuronal cells by quantitative RT-PCR (mean and S.D., $n = 3$).

skeletal muscle (Fig. 2B). Since liver and skeletal muscle are mostly composed of hepatocytes and muscle fibers, respectively, these results indicate that the siRNA-silencing efficiency is different among cell populations in the shRNA transgenic mice.

3.4. The mechanism of tissue difference in siRNA-silencing efficiency

In order to study the mechanism of this tissue difference in siRNA-silencing efficiency, we first analyzed the expression levels of shRNA and siRNA with the probe to the guide strand of siRNA, and compared them to the expression level of the target mRNA in each tissue. The 54 mer shRNA was not detected in any tissue (data not shown), indicating that processing of shRNA by Dicer is excellent and not different among tissues. The processed guide strand of 21 mer siRNA was observed much more in the cerebrum than in the liver and skeletal muscle (Fig. 4A and B). As shown in Fig. 2C, in contrast, SOD1 mRNA level was relatively lower in the cerebrum in comparison with that in the liver. These clearly indicate that relative ratio of the processed siRNA to the target mRNA in tissues does not explain the difference in siRNA-silencing efficiency.

Next, to examine whether the guide strand of siRNA properly located in the cells, we performed Northern blot analysis after subcellular fractionation of the tissue homogenates. Most of the guide strand was detected in the cytoplasmic fraction in both of the cerebrum and liver (Fig. 4C). These results show that the shRNA is similarly exported from the nucleus to the cytoplasm and that the guide strand should be similarly processed in the cytoplasm in the cerebrum and liver. These suggest that the slicer/RISC function, siRNA-cleaving ability, was lower in the cerebrum than in the liver. Therefore, we finally analyzed expression of Ago2 protein which is considered to be the slicer in mammalian cells [17]. However, the expression of Ago2 protein was not lower in the cerebrum (Fig. 4D), which was previously reported [18,19]. These findings suggest that the lower silencing efficiency in the cerebrum could not be explained by Ago2 level. The exact molecular mechanism

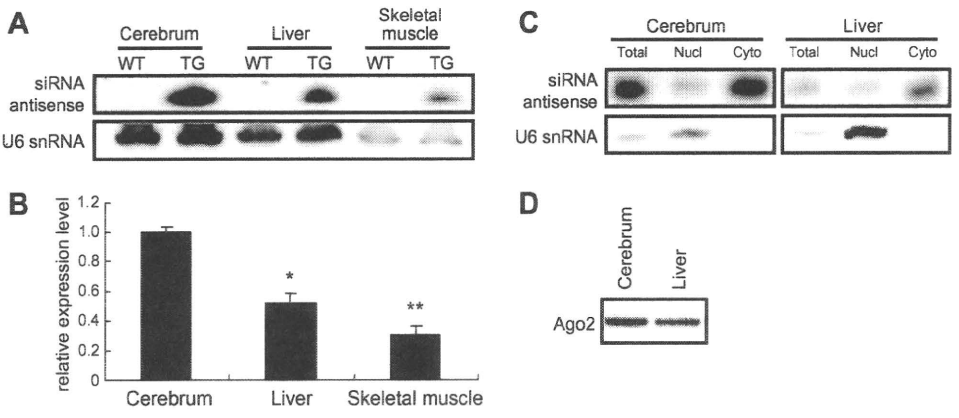

Fig. 4. The processing of shRNA/siRNA in the tissues of the transgenic mice. (A) Detection of siRNA guide strand in the cerebrum, liver and skeletal muscle on Northern blot analysis. (B) Quantification of their band intensity on Northern blot analysis. Values are the ratio to cerebrum (mean and S.D., $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$; significance compared to cerebrum). (C) Subcellular localization of the siRNA guide strand in the cerebrum and liver. U6 snRNA is used as a marker of nuclear fraction. (D) Ago2 protein in the cerebrum and liver on Western blot analysis.

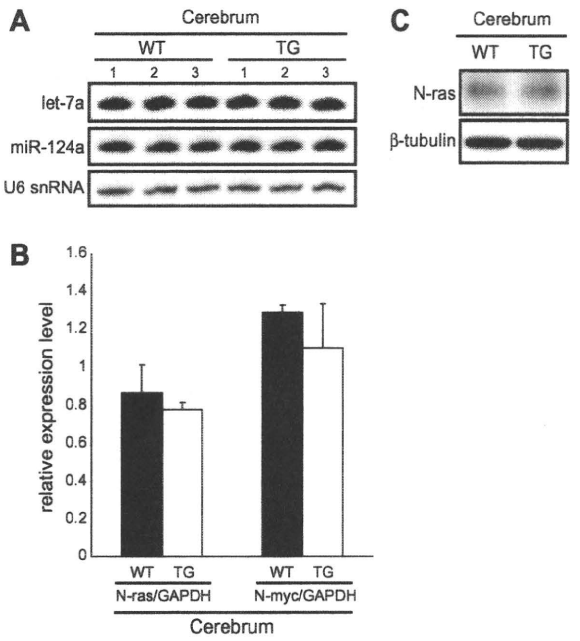

Fig. 5. Endogenous microRNA pathway in the shRNA transgenic mice. (A) Endogenous levels of miRNAs, let-7a (upper) and miR-124a (middle), in the cerebrum on Northern blot analysis. (B) Quantification of N-ras and N-myc levels, which are predicted as target genes of let-7a, in the cerebrum by quantitative RT-PCR (mean and S.D., $n = 3$). (C) N-ras protein in the cerebrum on Western blot analysis.

for the tissue difference in siRNA-silencing efficiency remains to be elucidated.

3.5. Endogenous miRNA pathway is not affected in the cerebrum of shRNA transgenic mice

To analyze whether competition between shRNA and miRNA occurred in the shRNA transgenic mice, we evaluated the expression levels of miRNAs and their target genes in the cerebrum of shRNA transgenic mice. There was no remarkable change in levels of let-7a and miR-124a on Northern blot analysis (Fig. 5A). Expression levels of N-ras and N-myc mRNAs, which were the predicted target genes of let-7a [20,21], were not altered on quantitative RT-PCR (Fig. 5B). Expression level of N-ras protein was not altered on Western blot analysis (Fig. 5C). These results clearly indicate that endogenous miRNA pathway is preserved in the shRNA transgenic mice.

The reproducibility of all results was confirmed by at least two experiments.

4. Discussion

We demonstrated the tissue difference in siRNA-silencing efficiency in the anti-SOD1 shRNA transgenic mice, but could not make clear the exact mechanism for the difference. However, the silencing effects in the tissues were generally good (>80%), and the anti-SOD1 shRNA transgenic mice could recapitulate the phenotype of fatty liver and female infertility as seen in SOD1-null mice [15,16].

Overexpression of shRNA from transgene did not induce apparent adverse effect including inhibition of endogenous miRNA pathway in our transgenic mice. It is of note that abundant shRNA/siRNA exogenously delivered by adeno-associated virus (AAV) vectors can cause drastic toxicity in the liver or brain possibly due to oversaturation of endogenous miRNA pathway [9,22]. The absence of the toxicity in the shRNA transgenic mice is probably due to its lower expression, because such a tissue toxicity is dependent on expression level of shRNA/siRNA [9,22]. Alternatively, there might be a difference in the processing pathways between shRNA expressed from transgene and that exogenously expressed by viral vector.

In conclusion, even with tissue difference in siRNA-silencing efficiency, endogenous miRNA pathway being well preserved, the transgenic RNAi approach is considered to be a useful method for analysis of gene function in vivo.

Acknowledgements

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