CHAPTER IV

RESULTS

1. The effect of iron on Schwann cell proliferation

To examine the proliferation/cytotoxicity of iron on Schwann cells using MTT assay, Schwann D6P2T cells were exposed to various concentrations of iron citrate 50-1000 μg/ml) for 24 h. As shown in Figure 5, all concentrations of iron examined did not show cytotoxicity to cultured Schwann cells. On the other hand, at the concentration of 50 μg/ml iron appeared to induce the peak of Schwann cell proliferation, approximately 20% when compared with the untreated control. Furthermore, when concentration of iron was increased, percent proliferation of Schwann cells was decreased, but still higher than that of untreated control. Because the principle of MTT assay is based on the production of insoluble formazan, it is proportion to the activity of the dehydrogenase enzyme (a mitochondrial based enzyme). The activity of this enzyme is reasonably constant between cell types and therefore can give an indication of the number of cells present in each well. The number of viable Schwann cells in each condition was directly counted using trypan blue dye exclusion method. As shown in figure 6, the results showed that treatment of iron to Schwann cells increased number of viable cells. The highest cell number was observed at 50 μg/ml concentration which is approximately 20% higher than that of untreated control cells. The effect of iron on number of Schwann cell was similar to that obtained by the MTT method. Therefore, these results demonstrated that iron can induce Schwann cell proliferation. However its effect depends on the concentration used.

2. The effect of iron on TfR expression in cultured Schwann cells

To investigate the effect of iron on TfR expression on Schwann cells surface, D6P2T cells were stained with saturating concentration of FITC-labeled-mouse monoclonal antibody: anti-rat TfR (CD71) and subjected to FACs analysis. The cells were exposed to iron at various concentrations (50-1000 μg/ml) for 24 h. As shown in figure 7, the expression of TfR on the surface of Schwann D6P2T cells was detectable. Besides, exposure to iron at all concentrations examined equally shifted the histogram curves to the left of control indicating a reduction of TfR expression on the Schwann cell surface. This data also indicated that exposure of Schwann cells to iron caused iron to enter the cells where iron can regulate TfR-mediated iron uptake in these cells.

3. The effect of iron on ferritin expression in cultured Schwann cells

To examine the effect of iron on the expression of iron storage protein ferritin expression in Schwann cells, western blot analysis was performed. Schwann cells were exposed to iron at various concentrations for 24 h. Total cell lysates from untreated and treated cells were collected for SDS-PAGE. Equal amount of 10 μg/lane protein samples was separated on acrylamine gel and transferred to PVDF membrane. The presence of ferritin in the cell lysate was detected; using rabbit anti-ferritin antiserum. The results in figure 8 demonstrated that untreated Schwann cells expressed ferritin in an undetectable level (lane 1) induced ferritin expression. Exposure of Schwann cells to iron at all concentration examined (lane 2-6). It appeared that exposure to iron at 50 and 100 μg/ml induced a dose-dependent increase of ferritin (lane 2 and 3). However, with higher concentrations of iron (250-1000 μg/ml) the expression of ferritin was decreased (lane 4-6). The results demonstrated that iron added into the cell culture medium can enter the cells and is sufficient to induce cellular response of Schwann cells. After iron enters to the cells, it will be distributed for utilization prior to storage in ferritin. Thus, it raises a possibility that a reduction in ferritin expression as observed could reflect an increase in iron utilization within the cells.

4. The effect of iron on the mRNA expression of myelin marker associated with Schwann cell differentiation

From the previous experiments, it was thought that the high concentration of iron could affect Schwann cell differentiation. Then, these experiments were designed to determine the effect of iron on the mRNA expression of myelin marker associated with Schwann cell differentiation, P0 and MBP. The Schwann D6P2T cells were treated with the various concentrations of iron for 24 h and total RNA from each sample were isolated and examined by RT-PCR assay. The experiments were repeated at least two times and the representative gels were presented as shown in Figure 9, 10. The results showed that treatment of Schwann cells with iron increased the mRNA expression of P0 in a dose-dependent manner (Figure 9), which is similar to the mRNA expression of MBP (Figure 10). The expression of GAPDH was not affected by any treatment. These results demonstrated that iron exposure can increase transcription levels of P0 and MBP. Thus, iron could play a significant role in Schwann cell differentiation.

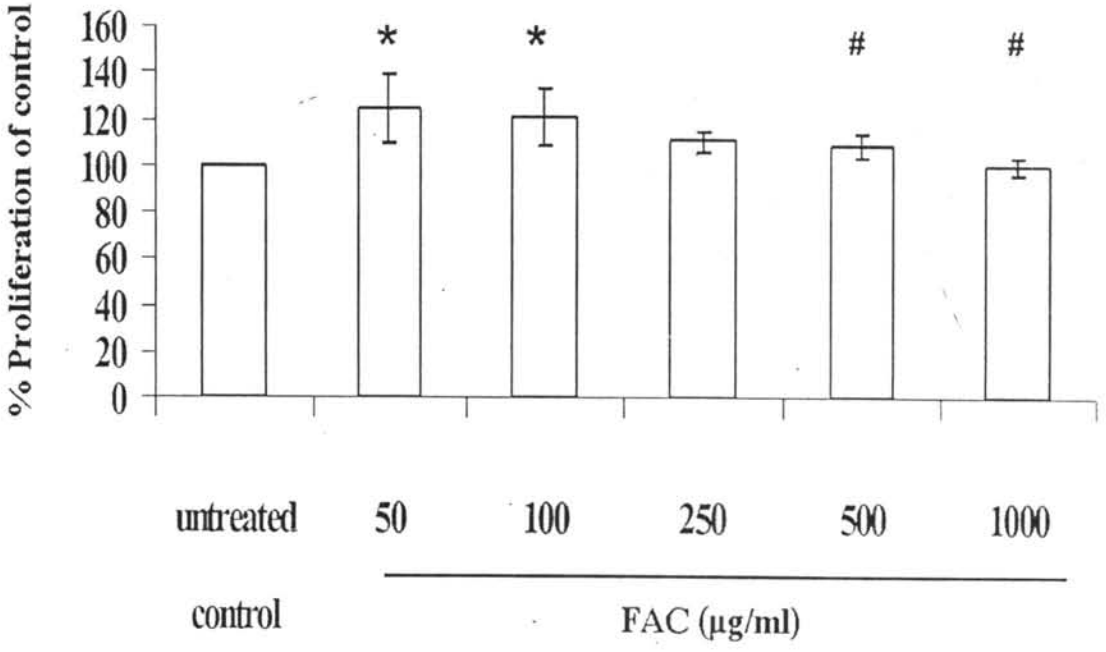

Figure 5 The effect of iron on Schwann cell proliferation determined by MTT assay

The Schwann D6P2T cells ($1x10^4$cells/well) in 96-well plates were exposed to various concentrations of iron citrate as indicated for 24 h. The MTT reagent was added to each well, incubated at 37° C for 2 h and completely removed. The purple precipitate inside the cells was dissolved by DMSO. Then the absorbance value at 570nm was measured by microplate reader. Values are the mean of three independent experiments, in which each condition was performed in triplicate, ±SEM.

*** Significantly different from untreated control by one way ANOVA followed by LSD analysis at $p < 0.05$**

 # Significantly different from 50 µg/ml concentration by one way ANOVA followed by LSD analysis at $p < 0.05$

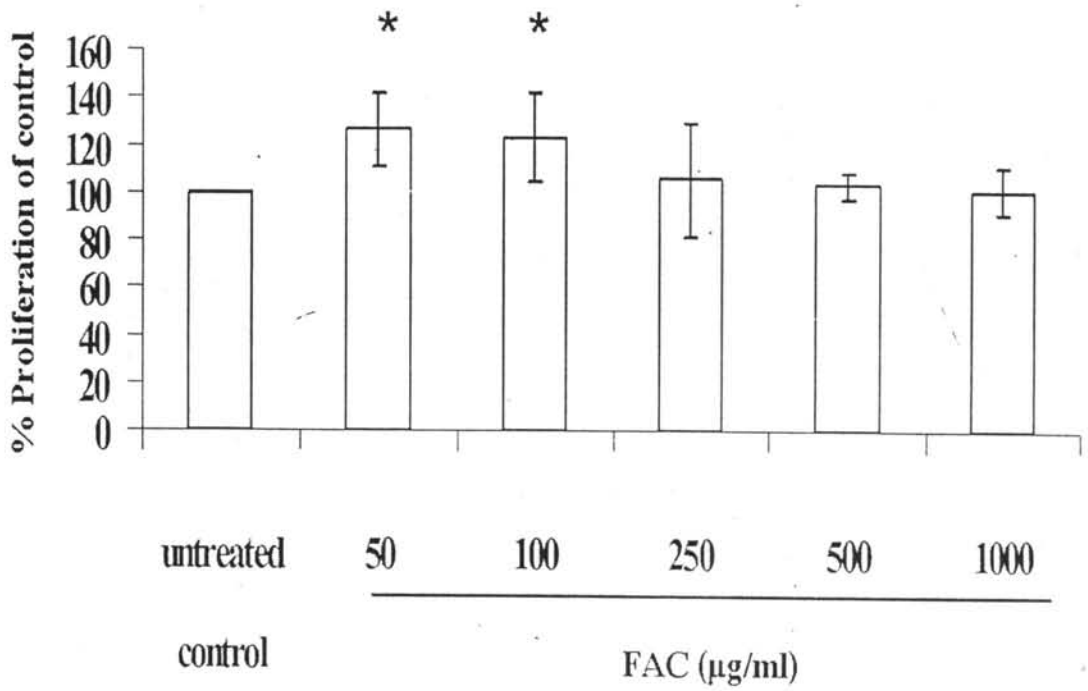

Figure 6 The effect of iron on Schwann cell proliferation determined by trypan blue dye exclusion method

The Schwann D6P2T cells ($1x10^4$cells/well) in 96-well plates were exposed to various concentrations of iron citrate as indicated for 24 h. Cells were trypsinized, pelleted and resuspented in 50 µl of serum free medium. 0.4% trypan blue dye was added into the cell suspension and incubated at 37° C for 5 min. The number of viable (unstained) cells was counted on a hemocytometer. The percentage of viable cells in each condition was calculated from that of untreated control [% proliferation of control = (viable cells in each condition/viable cells from untreated control) x 100]. Values are the mean of three independent experiments, in which each condition was performed in triplicate, ±SEM.

*** Significantly different from untreated control by one way ANOVA followed by LSD analysis at $p < 0.05$**

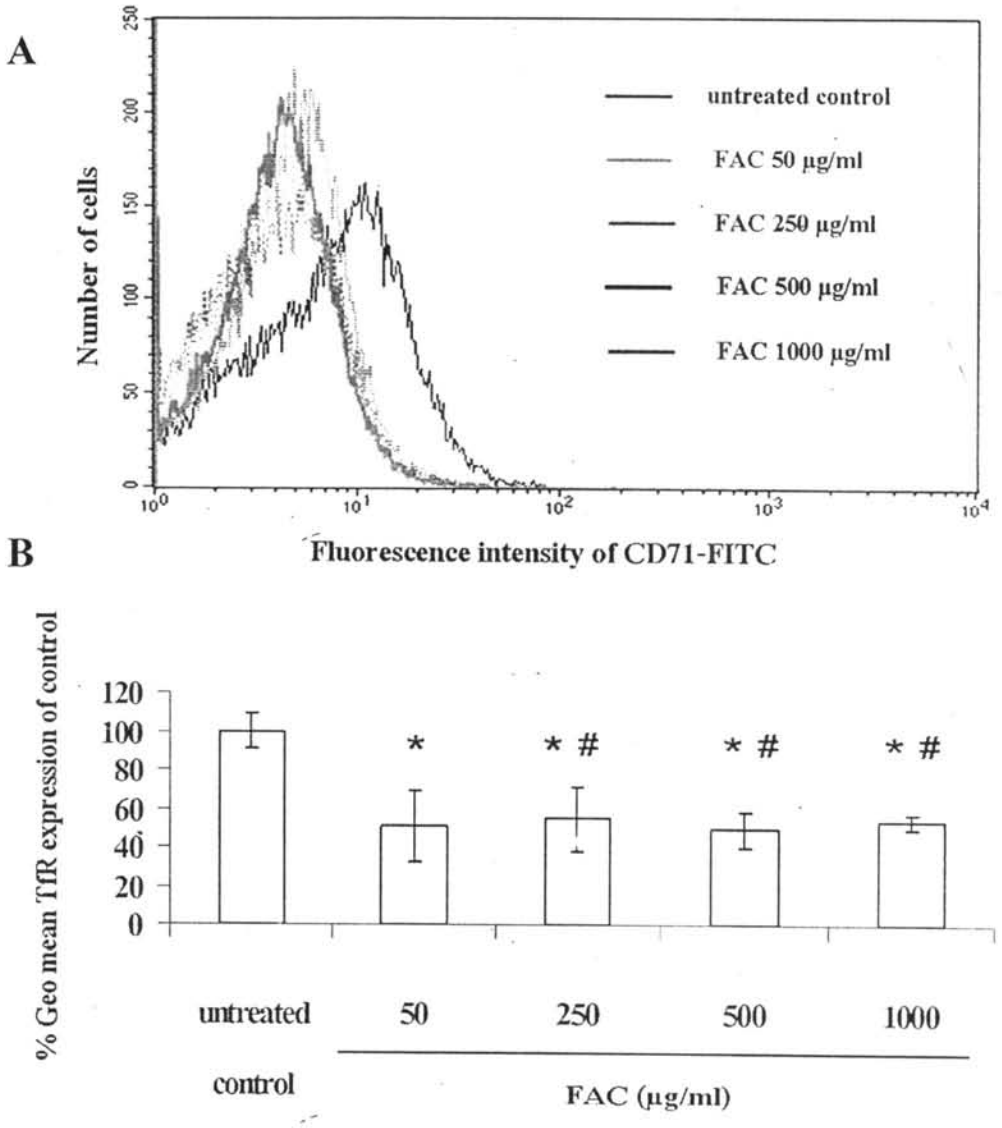

Figure 7 The effect of iron on TfR (CD71) expression in cultured Schwann cells determined by FACs analysis

(A) Histogram showing TfR expression of Schwann cells following 24 h exposure to iron citrate at various concentrations as indicated. Cells in each condition were washed, trypsinized, resuspended and fixed in 4% paraformaldehyde containing PBS/BSA solution. Then, the fixed cells were stained with FITC-conjugated anti TfR (CD71) for 1 h at room temperature and expression of TfR on the Schwann cell surface of 30,000 cells was analyzed by flow cytometer. (B) The percentage of TfR (CD71)-positive cells were calculated using the following formula [% Geo mean TfR expression of control = (Geo mean TfR expression in each conditions/ Geo mean TfR expression

from untreated control) x 100]. Values are the mean of two independent experiments, ±SD.

*** Significantly different from untreated control by one way ANOVA followed by LSD analysis at $p < 0.05$**

 # Significantly different from 50 µg/ml concentration by one way ANOVA followed by LSD analysis at $p < 0.05$

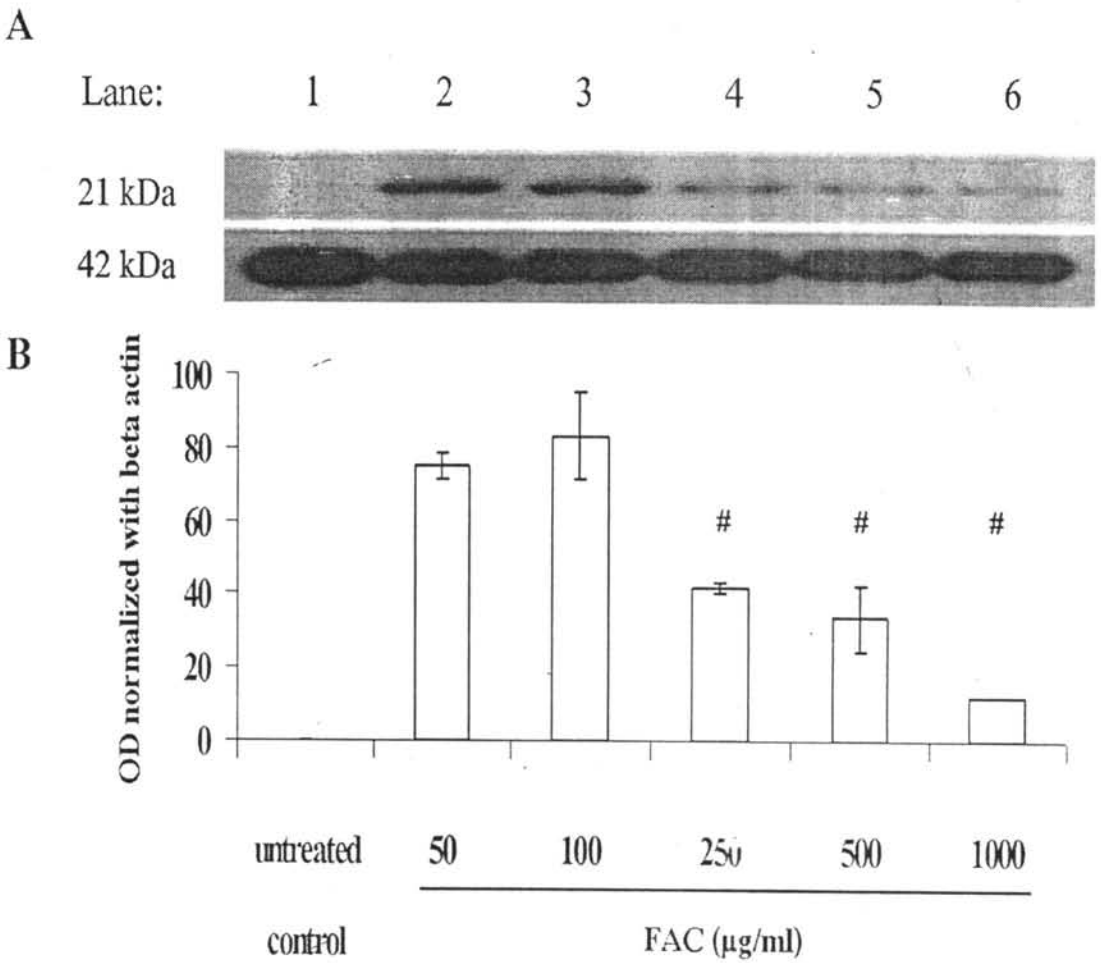

Figure 8 The effect of iron on ferritin expression in cultured Schwanns cells determined by western blot analysis

(A) Immunoblot analysis of ferritin protein in untreated and iron-treated Schwann D6P2T cells. Equal amounts (10 µg) of total cell lysates were loaded in each lane, electrophoresed and immunobloted with rabbit polyclonol antisera specific for ferritin protein (21 kDa). Beta actin (42 kDa) bands served as internal control of protein loading. (B) The densitometric quantification of ferritin normalized with that of beta actin in each lane. The results are expressed as mean ±SD of two independent experiments.

 # Significantly different from 50 µg/ml concentration by one way ANOVA followed by LSD analysis at $p < 0.05$

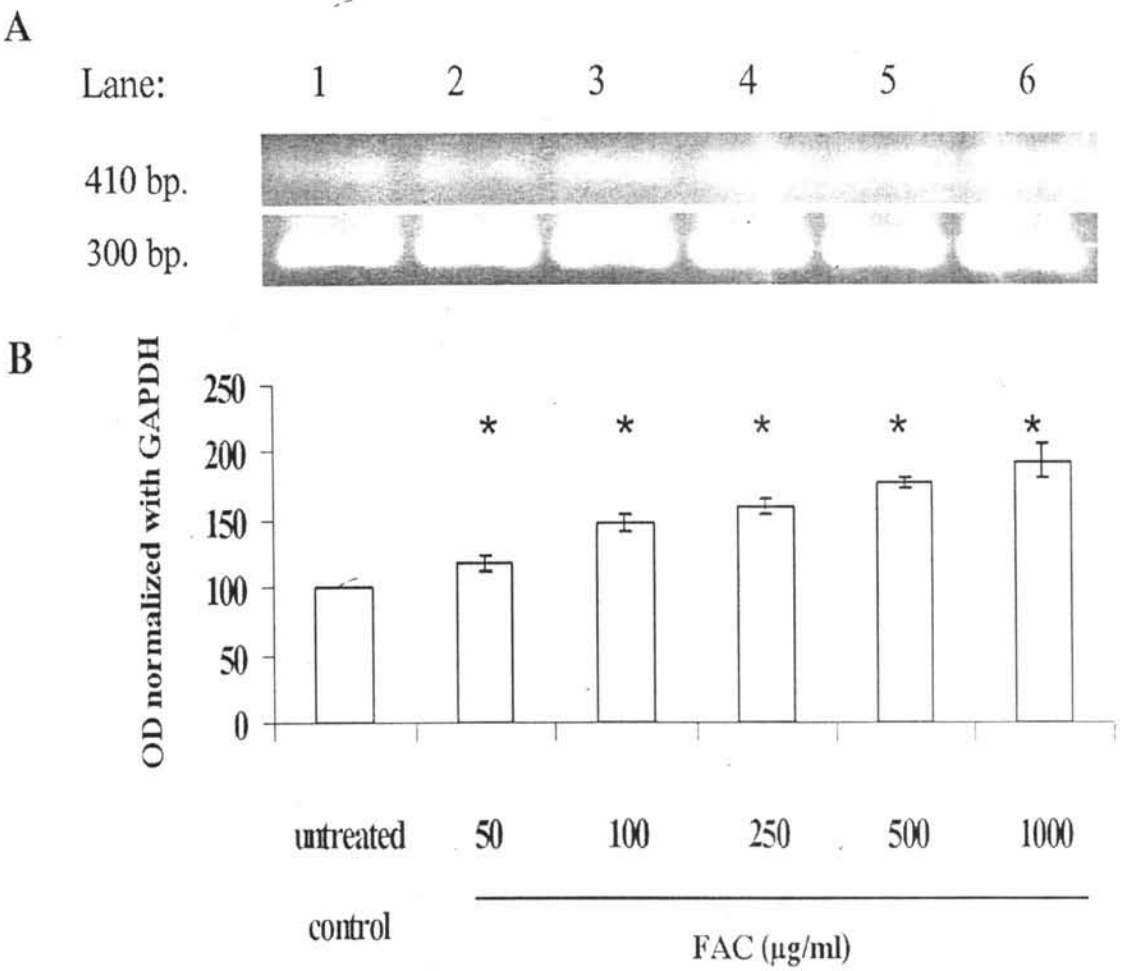

Figure 9 The effect of iron on the mRNA expression of myelin protein zero (P0) in cultured Schwann cells determined by RT-PCR

The Schwann D6P2T cells were exposed to iron citrate at the various concentrations (50-1000 μg/ml) for 24 h. Untreated cells were served as control. (A) Representative gel demonstrated that exposure of iron induced the mRNA expression of P0 in a dose-dependent manner. The expression of GAPDH was served as an internal control. (B) Densitometric analysis of P0 bands. Values are the means, ±SD from two-independent experiments.

*** Significantly different from untreated control by one way ANOVA followed by LSD analysis at $p < 0.05$**

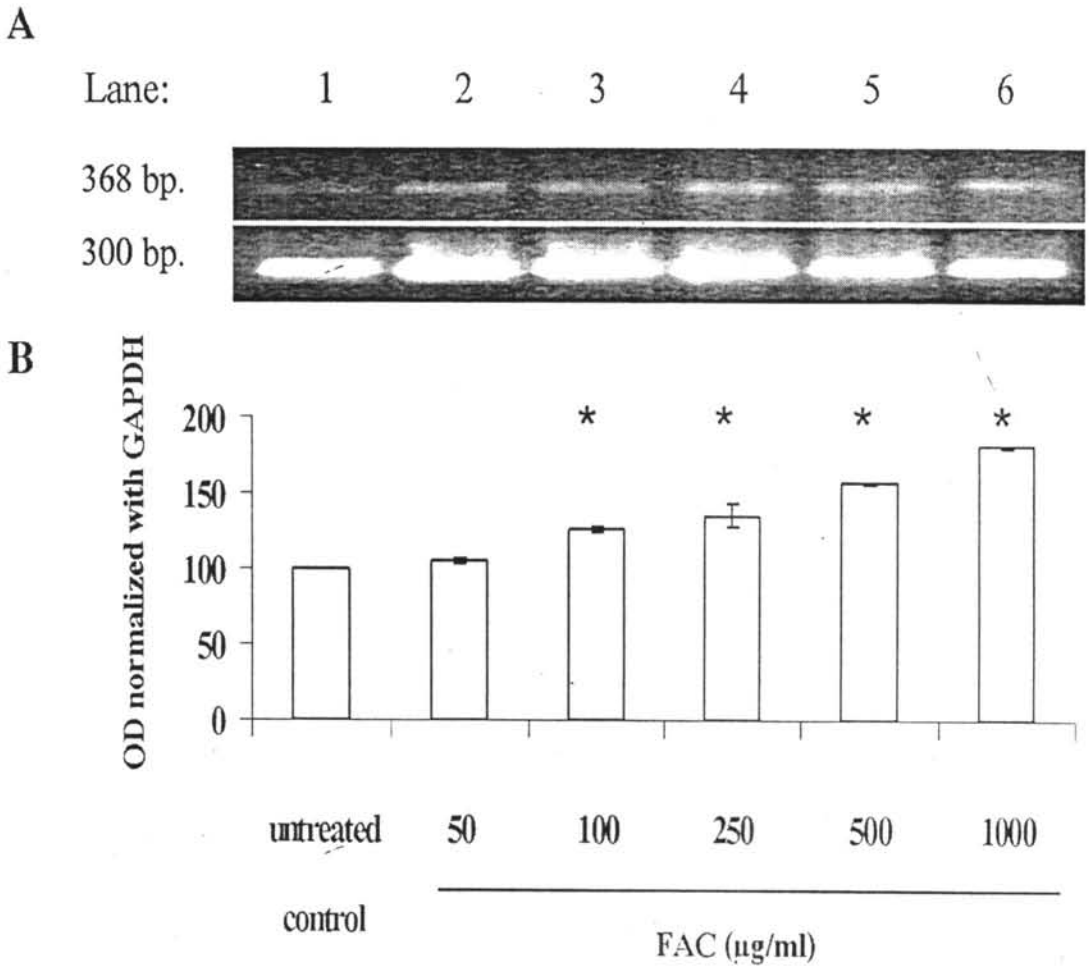

Figure 10 The effect of iron on the mRNA expression of myelin basic protein (MBP) in cultured Schwann cells determined by RT-PCR

The Schwann D6P2T cells were exposed to iron citrate at the various concentrations (50-1000 μg/ml) for 24 h. Untreated cells were served as control. (A) Representative gel demonstrated that exposure of iron induced the mRNA expression of MBP in a dose-dependent manner. The expression of GAPDH was served as an internal control. (B) Densitometric analysis of MBP bands. Values are the means, ±SD from two-independent experiments.

*** Significantly different from untreated control by one way ANOVA followed by LSD analysis at $p < 0.05$**