REPORT
ISN-CAEN Award

CATEGORY 1A: Visit by the applicant to another laboratory

Applicant:	Dr. Sujira Mukda
Home Institution:	Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol University, Thailand
Host:	Professor Samuel H.H. Chan
Supervisor:	Associate Professor Steve Leu
Host Laboratory:	Institute for Translational Research in Biomedicine, Chang Gung Memorial Hospital, Kaohsiung, Taiwan
Duration of visit:	July 19, 2015 – October 28, 2015
Project Title:	The anti-apoptotic role of Pnn in oxidative stress in C6 glioma cells

Background of project

Pnn, a serine-arginine (SR) related protein, plays multiple roles in regulating cell proliferation, cell migration, cell-cell interaction as well as cell differentiation through its capacity in modulating alternative splicing and transcriptional regulation (1-5). Leu et al. (1) demonstrated previously that loss of Pnn alters the mRNA isoform expression of an apoptosis-associated gene, Bcl-x from anti-apoptotic Bcl-xL to apoptotic Bcl-Xs and triggers cellular apoptosis. Since Pnn has the capacity to modulate the alternative splicing of Bcl-x, it is conceivable that Pnn may play a role in regulating apoptosis under conditions such as oxidative stress.

Hypothesis

We hypothesize that Pnn plays a protective role against menadione-induced apoptosis in rat C6 glioma cells.

Specific aims

Does overexpression of Pnn reduce cellular apoptosis in rat C6 glioma cells induced by menadione?

Plan of work

1. To manipulate the Pnn levels, the lentivirus vector was used to deliver the Pnn cDNA to overexpress Pnn expression in the C6 glioma cells.

2. To determine the efficiency of overexpression constructs, the immunofluorescent staining was used to examine the expression levels of Pnn in the C6 glioma cells.
3. For increasing oxidative stress and promoting apoptosis in C6 glioma cells, H_2O_2 or menadione, a synthetic derivative of vitamin K, was used to generate reactive oxygen species.
4. To determine whether Pnn plays protective roles in neurons under oxidative stress, immunostaining against apoptotic proteins, i.e. cleaved caspase-3 and cleaved-PARP, were used to examine the apoptotic events.

Results

To deliver the pnn cDNA into the C6 glioma cells, the Lipofectamine® 3000 reagent (Invitrogen™) was used in this study. The efficiency of overexpression of Myc-tagged Pnn in C6 glioma cells was checked by immunostaining using antibodies against Pnn and Myc. Hydrogen peroxide (H_2O_2) or Menadione, a synthetic derivative of vitamin K, was used to generate reactive oxygen species and the expression of cleaved-PARP was determined to detect the apoptotic events. The results showed that the expression of cleaved-PARP in the C6 glioma cells was up-regulated by menadione which can be reversed by over-expression of Pnn.

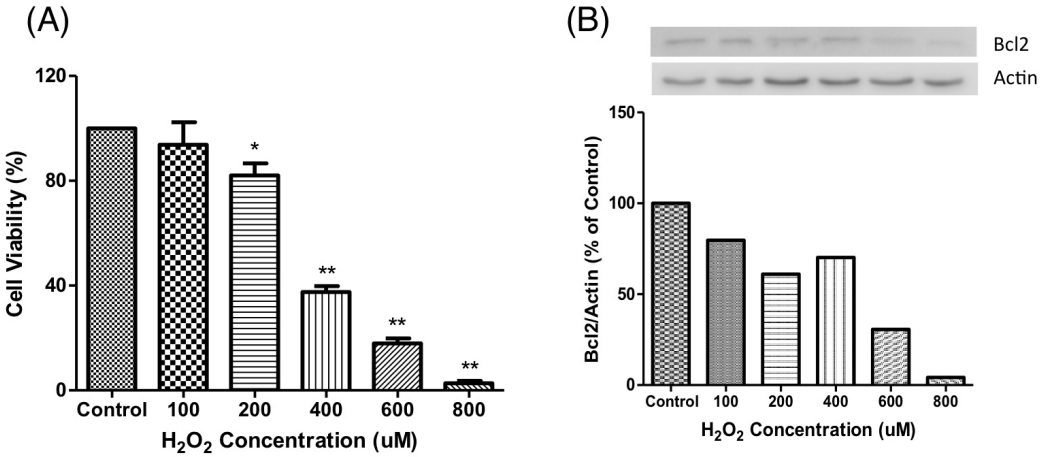

Figure 1. MTT assay (A) and Western blot analysis (B) to determine apoptosis-induced by oxidative stress. Cultured C6 cells were treated with H_2O_2 (0-800 µM) for 24 hours. After induction of oxidative stress, cells were either incubated with MTT or used for protein extraction followed by Western blots. Results showed that the cellular viability and expression levels of Bcl-2 were reduced by treatment of oxidative stress.

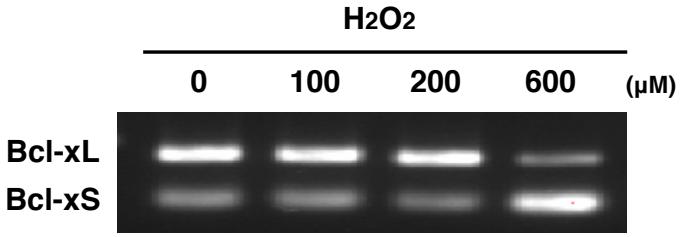

Figure 2. mRNA isoform expression of Bcl-x after H_2O_2 treatment. Cultured C6 cells were treated with H_2O_2 (0-600 μM) for 24 hours. After induction of oxidative stress, total RNA was extracted for RT-PCR to determine the mRNA isoform of Bcl-x. Results showed the isoform expression level of anti-apoptotic Bcl-xL was reduced by high dose (600 μM) of H_2O_2, while the level of apoptotic isoform Bcl-xS was increased by oxidative stress.

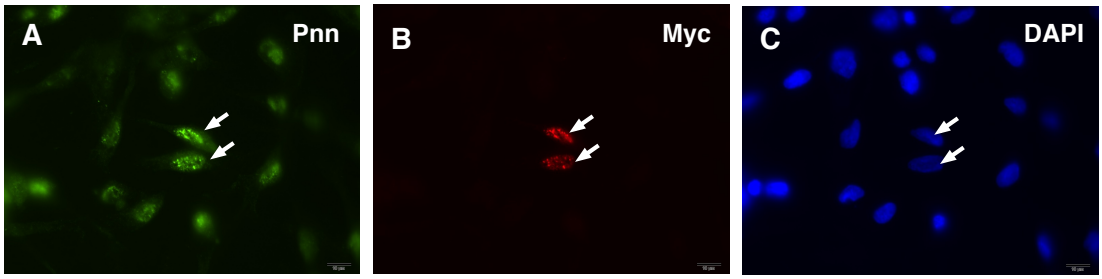

Figure 3. Over-expression of Pnn in cultured C6 cells. Twenty-four hours after transfection of myc-Pnn, C6 cells were fixed and incubated with specific antibodies against Pnn or myc-tag to detect the distribution of endogenous and exogenous Pnn. (A) The expression and distribution of Pnn within cell nucleus was detected by Pnn-specific antibody (green color). (B) The exogenous expression of Pnn was detected by antibodies against myc-tag (red color). (C) Cells were counterstained with DAPI for labeling the nuclei.

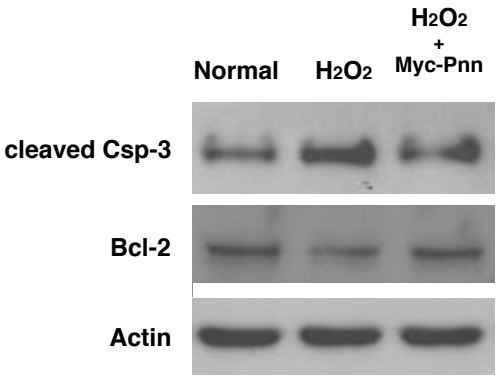

Figure 4. Over-expression of Pnn reduced H_2O_2-induced apoptosis in C6 cells. Twenty-four hours after transfection of myc-Pnn, C6 cells were treated with 600 μM H_2O_2 for 24 hours to induce oxidative stress. Total proteins were extracted for Western blots with antibodies against cleaved caspase-3 (Csp-3) or Bcl-2. Results showed the expression level of apoptotic index cleaved Csp-3 was increased by oxidative stress, while the level of anti-apoptotic Bcl-2 was decreased. The changes in expression of Csp-3 and Bcl-2 by H_2O_2 were reverted with over-expression of Pnn.

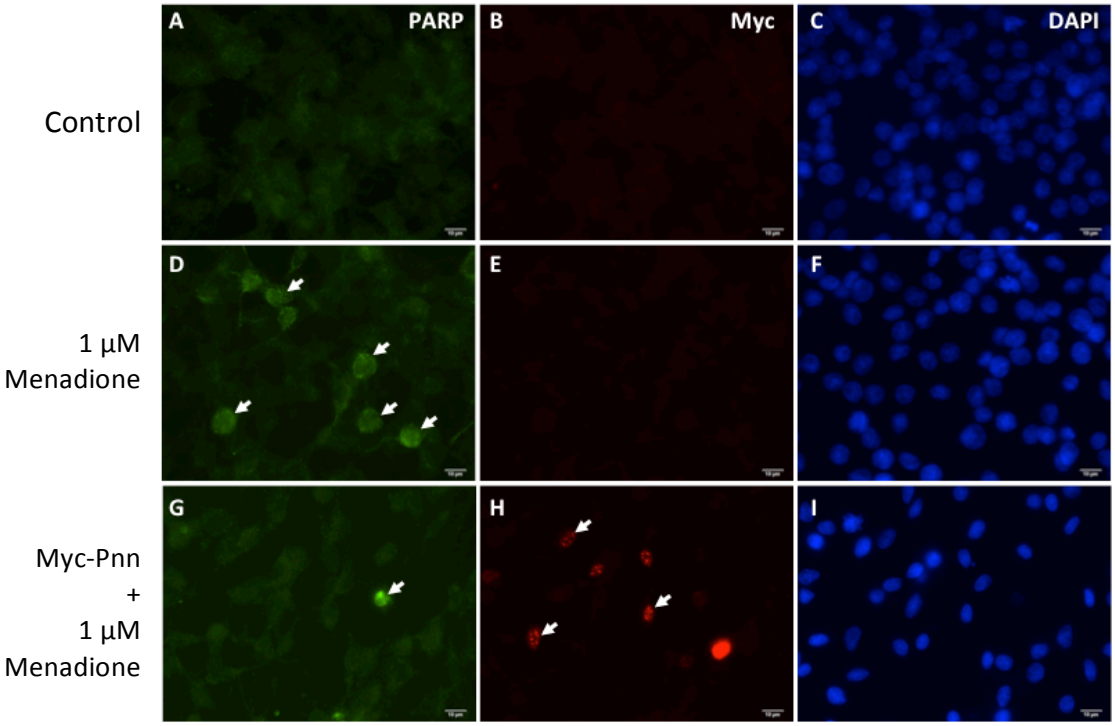

Figure 5. Over-expression of Pnn reduced menadione-induced increase of PARP in cultured C6 cells. Twenty-four hours after transfection of myc-Pnn, C6 cells were treated with 1 μM menadione for 2 hours to induce oxidative stress. The specific antibodies against PARP (green color) or myc-tag pnn (red color) were detected. Cells were counterstained with DAPI for labeling the nuclei.

References

1. Leu S, Lin YM, Wu CH, et al: Loss of Pnn expression results in mouse early embryonic lethality and cellular apoptosis through SRSF1-mediated alternative expression of Bcl-xS and ICAD. *Journal of cell science* 2012; 125:3164-3172
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Relevance to my research

Part of the finding of this research has been submitted as an abstract for poster presentation at The 13th Asia Pacific Federation of Pharmacologist (APFP) Meeting to be held in Bangkok, Thailand on February 1-3, 2016, under the title "The impact of Pnn on modulating apoptosis induced by menadione in rat C6 glioma cells".

The impact of Pnn on modulating apoptosis induced by menadione in rat C6 glioma cells

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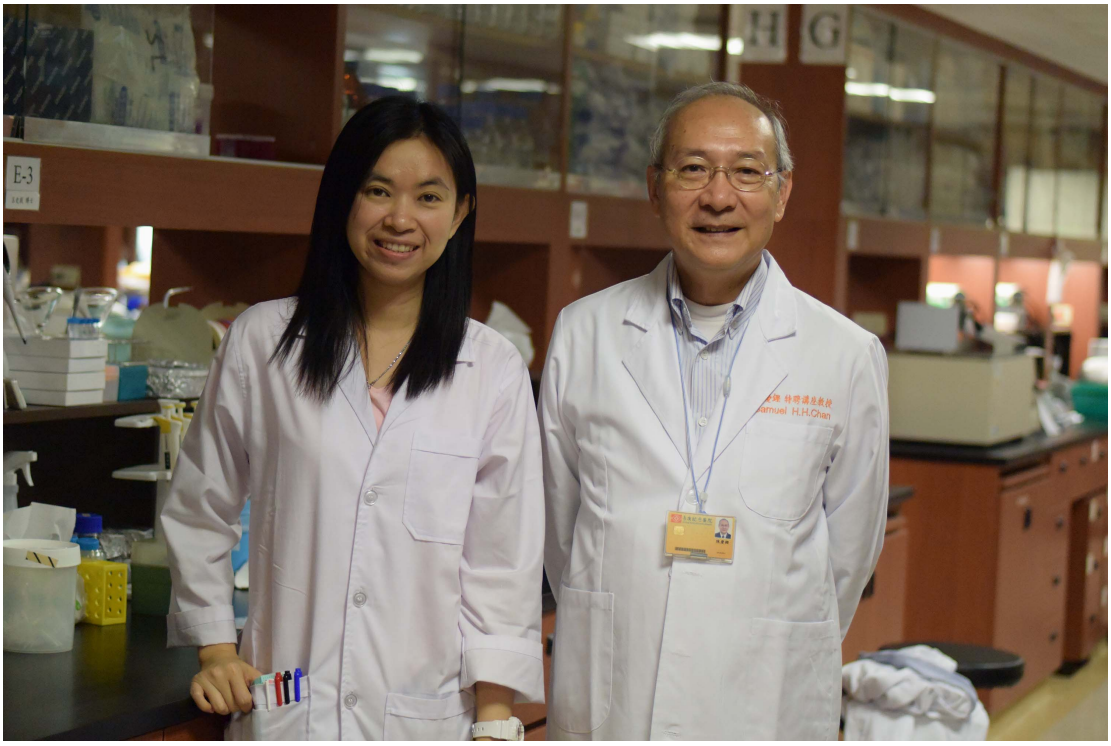
Pnn, a multi-functional protein, participates in regulating gene transcription and mRNA alternative splicing. Recent studies have demonstrated that Pnn plays essential roles in murine embryonic development as well as in modulating apoptosis in tumor cells. However, the role of Pnn in stress response remains unclear. In present study, we applied cultured glioma cells to evaluate the effects of Pnn expression levels on modulating apoptosis induced by oxidative stress. Results from immunofluorescent staining and Western blots showed that expression levels of apoptotic indices, including mitochondrial Bax, cleaved PARP, and cleaved caspase-3, were upregulated by hydrogen peroxide (H_2O_2)-induced oxidative stress, while the Bcl-2 expression level and cellular metabolic activity detected by MTT assay were reduced by menadione. The alteration in expression levels of apoptosis-associated proteins was then revered by over-expression of Pnn. Of importance, the loss of Pnn by siRNA oligos further enhanced the apoptosis in C6 cells with oxidative stress. According to the capacity of Pnn in regulating mRNA alternative splicing, the mRNA isoform of several apoptosis-associated genes were also determined. RT-PCR results showed that both oxidative stress and Pnn expression levels could modulate mRNA isoform expression of Bcl-X, an apoptosis-associated protein whose splice variants have been reported with distinctly opposite functions. However, The over-expression of anti-apoptotic Bcl-x isoform (Bcl-xL) in C6 cells could not rescue the apoptosis induced by oxidative stress or loss of Pnn. In conclusion, Pnn could modulate the mRNA alternative splicing on apoptosis-associated gene in C6 cells. Besides, the expression level of Pnn was associated with cellular apoptosis and stress responses to oxidative stress in glioma cells.

Acknowledgements

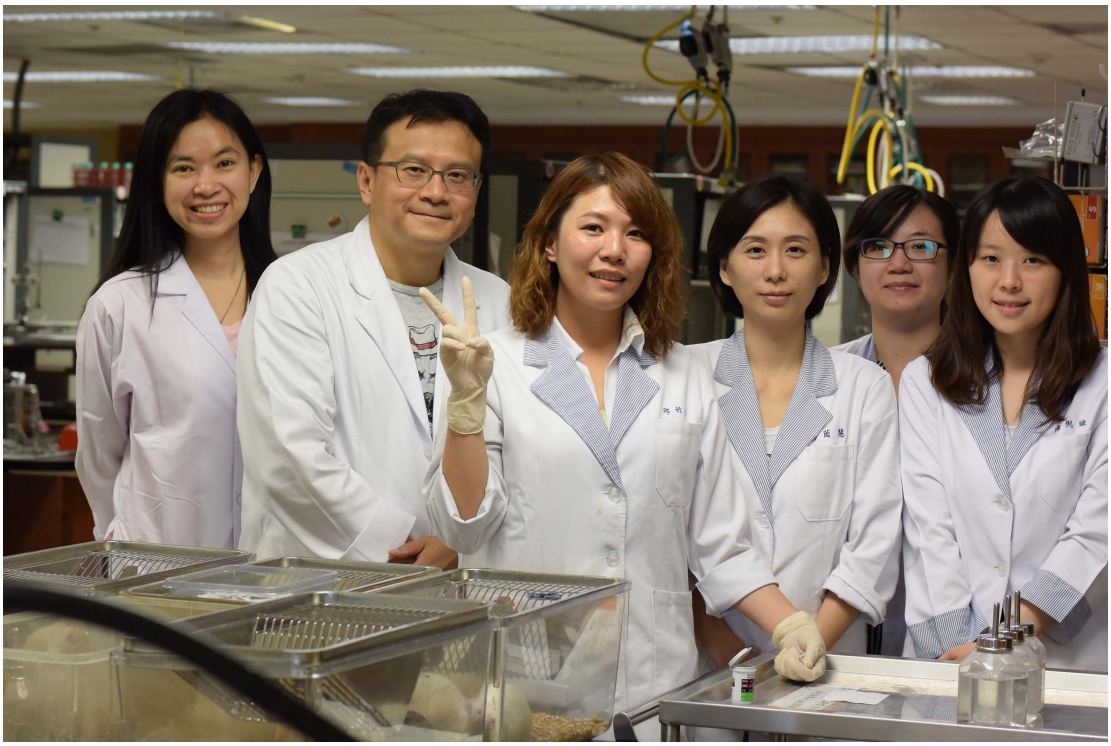
I would like to thank CAEN and ISN for providing me a financial support under the Category 1A to visit the Institute of Translational Research in Biomedicine, Chang Gung Memorial Hospital, Kaohsiung, Taiwan. My special appreciation and thanks are due to Prof. Samuel Chan for giving me a great opportunity to work in his Institute. Special thanks also for his support, generosity and encouragement. My thanks are also due to Associate Professor Steve Leu for allowing me to participate in his research project, which gave me the opportunity to manipulate the lentivirus vector into the cells in order to overexpress or silence the genes of interest. With this work, I received the hands-on training on techniques in cell culture, immunocytochemistry, western blotting, and RT-PCR. I would also like to express my thanks to Dr. Jenq-Lin Yang for giving me the chance for hands-on experience in primary cultures of hippocampal and cortical neurons from E17 rats. My special thanks also go to Dr. Ching-Yi Tsai for giving me the experience in stereotaxic surgery and microinjection in mouse model. Thanks also for all her kind support and assistance during my stay in Kaohsiung. I would also like to extend my appreciation to all the staff members at the Institute of Translational Research in Biomedicine for their warm welcome and hospitality, and for all their assistance in the laboratory experiment. My special thanks are also due to my home institute for allowing me to leave. Not only the scientific knowledge I have gained for my career development, I also have chance to explore Taiwan and learn more about their cultures and language which was a great experience for me.

Photographs

From left: Prof. Samuel H.H. Chan, Me, Associate Professor Steve Leu, Dr. Jenq-Lin Yang



With Prof. Samuel H.H. Chan



With Associate Professor Steve Leu and his lab members

Farewell dinner with Associate Professor Steve Leu, Professor Samuel H.H. Chan (left), Dr. Jenq-Lin Yang, Dr. Ching-Yi Tsai, Professor Julie Y.H. Chan (right), and members of Professor Chan's group.