# 最終試験の結果の要旨

| 報告番号 | 総研第 414 号 | | 学位申請者 | Sushil Panta |
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| 審査委員 | 主査 | 武田　泰生 | 学位 | 博士（医学・歯学・学術） |
| | 副査 | 金蔵　拓郎 | 副査 | 堀内　正久 |
| | 副査 | 原　博満 | 副査 | 久保田　龍二 |

主査および副査の５名は、平成 29 年 2 月 13 日、学位申請者 スシル パンタ君に面接し、学位申請論文の内容について説明を求めると共に、関連事項について試問を行った。具体的には、以下の様な質疑応答がなされ、いずれについても満足すべき回答を得ることができた。

質問１）In supplementary fig. 2 E, black bars (w/wo TNF$\alpha$) have significant change, but line 6 in page 10 of the manuscript says TNF$\alpha$ could not inhibit VEGF induced migration in p53 silenced cells. Explain it.
回答）The comparison is between scramble versus sip53 group under both TNF$\alpha$ and VEGF treatment condition. The extent of inhibition of VEGF induced migration by TNF$\alpha$ seen in scramble is not seen in p53 silenced group.
質問２）HUVEC migration only is not angiogenesis as whole. To see angiogenesis other experiments should be performed as well. How about sprouting and tube formation?
回答）TNF$\alpha$ has been reported to initiate sprouting initially but on later stages, it stops angiogenesis.
質問３）What is the concentration of TNF$\alpha$ during high grade inflammation in the body?
回答）High grade inflammation (e.g. Rheumatoid arthritis) has TNF$\alpha$ level as high as 100 pg/ml.
質問４）What happens with cell migration when you treat cells with high grade inflammation?
回答）There will be much stronger inhibition of cell migration along with the increased number of apoptotic cells.
質問５）What is the clinical setting of low grade inflammation?
回答）Low grade inflammation does not usually occur from infections but due to physiological mechanisms like obesity, diet or depression.
質問６）What is the serum level of TNF$\alpha$ in tumor?
回答）It is reported that there is about 2 pg of TNF$\alpha$ per mg of frozen tumor tissue.
質問７）In low grade inflammatory conditions, as high as 1 ng/ml of TNF$\alpha$ is seen in patients. How did you decide to have 0.1 ng/ml of TNF$\alpha$ as low grade inflammatory condition in experiments?
回答）1 ng/ml of TNF$\alpha$ resulted into significantly higher cell death. We wanted to use low dose of TNF$\alpha$ to mimic low grade inflammation, with nonlethal upregulation of p53 and get visible results without cell death.
質問８）What is the normal level of VEGF in healthy subjects and the concentration of TNF$\alpha$ in obesity patients?
回答）Normal serum VEGF in a healthy subject is around 30 pg/ml. In obesity patients, plasma TNF$\alpha$ level have been reported to be up to 40 pg/ml.
質問９）Explain the difference between physiological and pathological angiogenesis based on the molecules involved.
回答）Same molecules are involved in the two types of angiogenesis unless some external angiogenic factors are involved. The failure to keep the normal activities or mutation in certain proteins causes pathological angiogenesis.
質問１０）Is there any posttranscriptional regulation of Id1 by p53?
回答）Id1 has been reported to negatively regulate p53 translocation. We did not find any reports about posttranscriptional regulation of Id1 by p53.
質問１１）Is there any rules to prepare scramble siRNA? How did you make or check the function?
回答）We purchased commercially available scramble siRNA and siRNAs against p53 and Id1. The cells were transfected with siRNAs for around 5 hour using lipofectamine 2000.
質問１２）What is the concentration of 1000 U/ml of TNF$\alpha$?
回答）For TNF$\alpha$ 1000 U/ml is equal to 100 ng/ml.
質問１３）High dose of TNF$\alpha$ killed the cells. Is this the similar mechanism for low dose of TNF$\alpha$?
回答）High dose of TNF$\alpha$ kill the cells by upregulating a lethal level of p53 which takes cells into apoptosis. Low dose TNF$\alpha$ appears to function in similar manner but the level of p53 upregulated is not lethal. We also showed that

this nonlethal level of TNF$\alpha$ is involved in inhibition of cell migration.
質問１４）How does TNF$\alpha$ upregulate p53?
回答）TNF upregulates p53 by stabilizing Mdm2 and by upregulating p38.
質問１５）Did you confirm Mdm2 regulation of p53?
回答）We found that TNF$\alpha$ stabilized Mdm2.
質問１６）How did Id1 control migration through integrins?
回答）Cells migrate with the help of filopodia for which integrins are necessary. Id1 upregulates integrins.
質問１７）Could you explain the relationship between Id1 and p53?
回答）Depending on the stimulus, p53 and Id1 act to inhibit each other's synthesis and nuclear shuttling. p53 inhibit Id1 through DEC1 in DNA damage response pathway while Id1 inhibit shuttling of p53 from cytoplasm to the nucleus.
質問１８）Did the downregulation of $\beta_3$-integrin seen in Fig. 4 D occurred in Fig. 4 A setting also?
回答）Yes, we observed downregulation of $\beta_3$-integrin in Id1 downregulated condition also.
質問１９）What are the ligands for $\beta_1$-integrin and $\beta_3$-integrin?
回答）Ligands for $\beta_1$-integrin are collagens, laminins, fibronectin and VCAM-1. Ligands for $\beta_3$-integrin are fibronectin and vitronectin.
質問２０）Does p53 directly bind to Id1?
回答）There are no reports on direct binding of p53 and Id1 till date.
質問２１）What is the benefit of low grade inflammation in cancer?
回答）In cancer cells carrying wild type p53, low grade inflammation upregulate p53 leading to the inhibition of cancer cell migration and regression of tumor, e.g. BCG treatment of bladder cancer.
質問２２）What is the relationship between in vivo and in vitro angiogenesis?
回答）In vitro assays are very useful to study the different steps of angiogenesis and to screen anticancer drugs. Multiple in vitro assays have been designed to mimic the in vivo environment where the microenvironment might be different. Efforts are continuously being made to closely mimic the biological environment by adding supporting proteins or cells to the culture and make in vitro data more useful to extrapolate.
質問２３）In Fig. 1, how did you scratch the cells? The scratch size differs in different groups.
回答）The scratch was made by using 200 $\mu$l pipette tip. The scratches appear to be different but the quantification was done by measuring the ratio of area after 18 hour to area at 0 hour. We collected triplicate of such ratio of area covered by cells and the difference in scratch between two groups does not interfere with the data.
質問２４）In Fig. 1 A, does TNF$\alpha$ only treatment increase the space? It seems to be bigger than control.
回答）Cell migration takes place in multiple direction. Especially in case of TNF$\alpha$ treatment we found that the cells change their morphology to the more elongated one and had aligned arrangement. This movement and positioning of cells might be the reason why the TNF$\alpha$ treated group had increased space compared to the control. However, there was no difference in cell viability between the two groups.
質問２５）In Fig. 2 A and B, TNF$\alpha$ increased p53 but not the p53 mRNA. Explain this phenomenon.
回答）Normally p53 is in latent state in the cell. Induction or activation of p53 due to minor stress occur largely due to the alteration in the protein. The rate of transcription of p53 play a minor role in its upregulation.
質問２６）What is the mechanism of the synergistic effect of TNF$\alpha$ and VEGF?
回答）The synergistic effect of TNF$\alpha$ and VEGF is reported for the first time. We think PTEN is the protein which is activated by TNF$\alpha$, can modulate VEGF signal, binds to p53 and has the capacity to increase the half-life of p53.
質問２７）The characters of HCT116 cells are different from the endothelial cells. Explain about their migration.
回答）In spite of the two cells namely HUVECs and HCT116 cells being totally different, our experimental results in the two types of cells were similar. We wanted to check the TNF$\alpha$ and VEGF activity in a cancer cell line which is adherent (to perform scratch assay) and which carried wild type p53.
質問２８）What is the difference between the rate of migration of HUVECs and HCT116 cells?
回答）The migration rate of the two cell types were not checked for the purpose of comparison but as per our observation, HCT116 cells had lower migration rate compared to the HUVECs. However, many researchers have carried out scratch assay with HCT116 cells previously.
質問２９）Id1 is a factor of proliferation. Did the increase of migration occur due to cell proliferation?
回答）We did not perform proliferation assay but the increase of cell migration results from both proliferation and migration of cells due to the VEGF treatment. VEGF strongly upregulates Id1 for the process of proliferation and migration.

以上の結果から、５名の審査委員は　申請者が大学院博士課程修了者としての学力・識見を有しているものと認め、博士（医学）の学位を与えるに足る資格を有するものと認定した。