最終試験の結果の要旨

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On February 12, 2019, the degree applicant named Muhammad Subhan Amir made a presentation on his thesis paper and was interviewed and questioned about the paper and related matters by Chief and Vice examiners of total 5 peoples. Specifically, the following questions and answers were made and the examiners were able to obtain satisfactory answers.

Question 1: What is the reason why you did not show the control protein expression for JNK, p38, and SMAD1/5?

Answer: The purpose of this study was to explore the functional role of Hif-1α. We included the data for MAP kinase and SMAD1/5 phosphorylation to prove that our stimulation with BMP9 worked properly.

Question 2: Phosphorylation levels of ERK isoforms seem to be different. What caused the difference?

Answer: The different phosphorylation levels of ERK 1 and 2 isoforms in BMP9-stimulated cells represent their different sensitivities to BMP9 stimulation. It is known that ERK1 and 2 isoforms have different biological functions in various cell types.

Question 3: Did you examine the direct effect of Chrysin on PHD protein?

Answer: We have no data of PHD protein expression after Chrysin treatment. However, our data showed that PHD mRNA was not affected by Chrysin treatment. Chrysin is a well-established specific activator of PHD.

Question 4: Figure 5 showed that ALP mRNA was decreased by Hif-1α and PDK1 knockdown. But ALP mRNA still increased in Hif-1α and PDK1 knockdown compared to day 0. What is your speculation about another regulator? What is the candidate for that?

Answer: We have concluded that the essential role of Hif-1α is the induction of PDK1 in BMP9-induced osteoblast. We recognized that ALP expression was not completely blocked by either Hif-1α or PDK1 knockdown. As the expression of Runx2 and Osterix was not affected, and these two transcription factors are important regulators of ALP expression, I believe they are good candidates for the promoter of ALP expression under Hif-1α or PDK1 knockdown condition. Other possible candidates are Lrp5, Dlx5, Msx2 and ZBTB16, which are also important regulators of osteoblast differentiation.

Question 5: In figure 6, you summarized this study. Please explain how BMP9 inhibits PHD activation.

Answer: We showed that Chrysin, a PHD activator, significantly reduced protein expression of Hif-1α in BMP9-stimulated osteoblasts. We thus believe that BMP9 decreases the catalytic activity of PHD rather than decreases its protein expression.

Question 6: What do you think about the possibility of BMP9 as an in vivo drug? And what is the source of BMP9?

Answer: BMP9 is a promising drug because BMP9 possesses the highest osteoinductive profile among BMP members. In-vivo experiment of BMP9 will add the basic knowledge of its possibility as a clinical drug. BMP9 resides inside the bone matrix and its main producer is in the liver.

Question 7: BMP2 are already available in clinical use but there are some side effects. Do you think it is possible to use BMP9 instead of BMP2 clinically? What is your recommendation regarding the use of BMP9 in clinical use?

Answer: Our study is a basic science. There will be many phases before BMP9 becomes ready for the clinical use. Up until the recent data, BMP9 is still one of the promising drugs in bone regeneration.

Question 8: What do you think about the combination of Hif-1α stabilizer and BMP9 in clinical use?

Answer: Hif-1α stabilization is observed in some diseases including cancer. I therefore suggest we should be careful about the use of Hif-1α stabilizer in line with BMP9 in clinical use. Nowadays cancer treatments targeting Hif-1α are under development. In connection to that, our data may recommend that BMP9 should not be simultaneously prescribed with anti-Hif-1α drugs.

Question 9: In figure 1, you showed the data of BMP2 compared to BMP9. Why did you use BMP2 in this experiment? Do you think BMP2 has similar results in inducing Hif-1α and PDK1? How about the effects of other BMPs, like BMP 4 or BMP 7?

Answer: We used BMP2 in comparison to BMP9 to examine if other BMPs would have the similar pattern to BMP9 in inducing Hif-1α protein expression. We expect the similar results in other BMPs. But since our data only showed the Hif-1α protein expression after BMP2 stimulation, further experiments should be performed for others BMPs.

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Question 10: Could you explain the BMA technique?

Answer: BMA is an abbreviation for Bone Marrow Aspiration. This technique provides autologous source of osteoprogenitor cells.

Question 11: Why did you focus only on PDK1? Did you also examine the other family of PDKs like PDK 2, PDK 3 or PDK 4?

Answer: Based on the previous study, PDK1 is the direct target of Hif-1α. And we also found that BMP9 stimulation induced the expression of PDK1 but not the other PDKs including PDK2, PDK3 and PDK4.

Question 12: Explain XIAP.

Answer: XIAP is an abbreviation of X-linked inhibitor of apoptosis. XIAP is a protein involved in a parallel pathway responsible for controlling the level of the Hif-1α protein expression.

Question 13: What is the reason you did not measure the ATP production? Did you check if the function of mitochondria is decreased in your experiment?

Answer: We did not measure the ATP production or mitochondrial activity in our study because our main focus was the signaling pathway of BMP9-induced Hif-1α protein expression. Future studies in metabolism of the cells with BMP9 stimulation are needed to explore the ATP production and to explore the mitochondria function.

Question 14: Why can NSC697923 (Ubc13 inhibitor) decrease XIAP protein expression?

Answer: In an alternative pathway of Hif-1α stabilization and translocation, XIAP binding to Ubc13 is needed. After Ubc13 inhibitor treatment, we found that XIAP expression was decreased. XIAP protein stability was presumably decreased in the presence of NSC697923.

Question 15: What is the definition of hypoxia? What is the percentage of oxygen in hypoxia? How did you construct the hypoxia condition in your experiment?

Answer: Hypoxia is the condition where oxygen level is lower than the physiological oxygen tension. Our experiments used 2% oxygen because the induction of Hif-1α was better than that at 5% oxygen. We have an incubator with an adjustable oxygen level.

Question 16: Your study showed the Hif-1α pathway was activated in BMP9 treatment. What are other pathways activated in BMP9 stimulation?

Answer: The canonical and non-canonical pathways of BMP were activated after stimulation with BMP9. Our data in figure 1 showed that the phosphorylation levels of Smad 1/5 and MAPK family were increased after BMP9 stimulation.

Question 17: When Hif-1α or PDK1 was knocked down, ALP mRNA expression was only partially decreased. Does this mean the other pathways are still activated?

Answer: Yes, it is likely that the other pathways are still activated after BMP9 treatment with Hif-1α or PDK1 knockdown.

Question 18: Explain the genes coding Hif-α isoforms. Are they coded by different genes? Could you explain in detail the difference between Hif-1α, Hif-2α and Hif-3α?

Answer: There are three Hif-α isoforms and are encoded by 3 different genes respectively. Hif-1α is most active during short periods (2-24hours) of intense hypoxia (<0.1% O_2), and preferentially induces genes that encoded glycolytic enzyme, that are involved in pH regulation and that promote apoptosis. Hif-2α is active under mild or physiological hypoxia (<5% O_2), and continues to be active after 48-72 hours of hypoxia. Hif-2α induces genes that are involved in invasion and the stem cell maintenance. Hif-3α is not functional, as it lacks activation domain.

Question 19: You have the data of Hif-2α. Did you check the expression of Hif-3α and Hif-1β?

Answer: We did not check Hif-3α and Hif-1β because Hif-3α is not functional and Hif-1β is stably expressed.

Question 20: What is the mechanism of Hif-1α in regulation of PDK and LDHa genes? Are they direct or indirect targets of Hif-1α?

Answer: Upon translocation to the nucleus, the Hif heterodimer binds to the hypoxia-response element (HRE). Functional HREs tend to be localized in the promoters of target genes including PDK and LDH.

Question 21: Could you explain the difference of mineralization degrees between addition of Hif-1α and PDK1 inhibitors? Is it influenced by the concentration of each inhibitor?

Answer: The influence of the concentration is possible in this experiment because we only used one concentration for each inhibitor.

Question 22: What receptors of BMP9 are involved in this study?

Answer: BMP9 is supposed to signal through the receptor complex comprising BMPR-I, BMPR-II, and a co-receptor, Endoglin.

Based on the above results, the five examiners recognized that the applicant had academic ability and insight as a graduate school doctoral course and was qualified to have a degree of Doctor Philosophy in Dental Science.