Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

OPPONENT'S REVIEW ON BACHELOR THESIS

Name of the student: Fabian Moser

Thesis title: Cloning useful recombinant cDNA/ mRNA constructs to study cell cycle and Hippo-signalling in preimplantation mouse embryos

Supervisor: doc. Alexander W. Bruce, Ph.D.

Referee: Mgr. Martin Selinger

Referee`s affiliation: Institute of Chemistry, Faculty of Science, University of South Bohemia

	Point scale[1]	Points
(1) FORMAL REQUIREMENTS		
Extent of the thesis (for bachelor theses min. 18 pages, for masters theses min. 25 pages), **balanced length of the thesis parts** (recommended length of the theoretical part is max. 1/3 of the total length), **logical structure of the thesis**	0-3	3
Quality of the theoretical part (review) (number and relevancy of the references, recency of the references)	0-3	2.5
Accuracy in citing of the references (presence of uncited sources, uniform style of the references, use of correct journal titles and abbreviations)	0-3	2.5
Graphic layout of the text and of the figures/tables	0-3	2.5
Quality of the annotation	0-3	2
Language and stylistics, complying with the valid terminology	0-3	2.5
Accuracy and completeness of figures/tables legends (clarity without reading the rest of the text, explanation of the symbols and labelling, indication of the units)	0-3	2
Formal requirements – points in total		17
(2) PRACTICAL REQUIREMENTS		
Clarity and fulfilment of the aims	0-3	2
Ability to understand the results, their interpretation, and clarity of the results, discussion, and conclusions	0-3	2.5
Discussion quality – interpretation of the results and their discussion with the literature (absence of discussion with the literature is not acceptable)	0-3	1.5
Logic in the course of the experimental work	0-3	2.5

[1] Mark as: 0-unsatisfactory, 1-satisfactory, 2-average, 3-excellent.

Completeness of the description of the used techniques	0-3	2
Experimental difficulty of the thesis, independence in experimental work	0-3	2.5
Quality of experimental data presentation	0-3	2
The use of up-to-date techniques	0-3	3
Contribution of the thesis to the knowledge in the field and possibility to publish the results (after eventual supplementary experiments)	0-3	2
Practical requirements – points in total		20
POINTS IN TOTAL (MAX/AWARDED)	**48**	**37**

Comments of the reviewer on the student and the thesis:

The bachelor thesis of Fabian Moser aimed to clone and subsequently characterize the function and distribution of three proteins involved in either mitosis or early embryogenesis. In more detail, cloning and *in vivo* expression analysis was performed for EGFP-Tubulin-6 gene. Despite initial failure with cloning to pRN3P vector, TA-cloning approach into pGEM-T Easy vector proved to be successful. However, microinjection of the resulting *in vitro*-transcribed mRNA of EGFP-Tubulin-6 did not result in any observable signal in murine embryos. Concurrently, the same approach was used in case of YAP protein involved in the Hippo signalling pathway. The non-phosphorylatable variant HA-YAP1(S112A) was used for morphological *in vivo* studies in murine embryos. Due to the time limitations, *in vivo* experiments with generated Securin-GFP mRNA were not performed.

The thesis is very well written and I greatly appreciate its coherence. The length of 43 pages (including the list of references and appendix) is more than satisfactory. Despite a very good English and writing skills of the author, several typos are unfortunately present. These include mostly wrong spelling, missing spaces or dashes, incorrect forms of the verbs, *etc*. In addition, the form of restriction enzyme names was not uniform – sometimes written in italics, sometimes not. Also the abbreviation "*et al.*" was not written in italics. Nevertheless, these were just minor mistakes and generally, the formal side of the thesis is on a very good level.

The thesis is divided into seven chapters: Abstract, Concise Project Summary, Introduction, Aims, Materials and Methods, Results and Discussion, and Conclusions. The following text will comment each of the chapters with questions (in ***bold italics***) asked directly below the comments.

Abstract and Concise Project Summary

Both parts are fine. My only comment in this section goes to the "Concise Project Summary" part. I would have expected a mention about the results from the final experiment with EGFP-Tubulin-6 in murine embryos, even though the results were negative (page 29-30).

Introduction

This chapter covers 12 pages with 6 figures. It is generally very well written and coherent with 44 references being cited. Out of this number, 25 references are original research papers and 19 are review papers, which is in my opinion a very good ratio. However, some of the review papers are relatively old (Watson 1992; Amon 1999; Desai 1997; Straight 1997).

I am not sure about the statement on page 2, where author mentions Securin being an enzyme,

however further in the text, Securin is described as an inhibitor of the Separase enzyme, which needs to be degraded in order to activate the separation process of sister chromatids. Moreover, reference for the Figure 5 (page 8) is missing.

Aims

Aims are clearly stated, I have no comments here.

Materials and Methods

This chapter covers 10 pages and generally lacks a considerable amount of information. Therefore, I will go step-by-step through each method.

Cloning vectors

The vector maps for pcDNA3.1-HA-YAP(S112A) and pEGFP-Tubulin-6 are missing. I also lack the accession numbers of all three genes of interest. Moreover, the fact that YAP is cloned in the pcDNA3.1 vector leads to my first question:

Why is YAP cloned into the vector pcDNA3.1 and not in pRN3P as Securin? Does this mean that in vitro produced YAP mRNA does not include any harbouring UTRs? If so, what does it imply for your results?

Gel electrophoresis

The composition of TAE buffer is missing.

Was the RNA electrophoresis performed under the same conditions using the same buffers as the DNA electrophoresis? Including the marker and loading dye? If so, why did you not use the denaturing gel for RNA separation?

Plasmid transformation

The procedure itself is very strange. Following the data in Table 13 (ligation reaction), the resulting amount of DNA added to the bacteria was ca. 390 ng! From my experience, the amount of input DNA should be in tens of nanograms (depending on the bacterial strain).

What strain of E. coli was used? Was it a home-made stock or a commercially available one?

After the transformation and subsequent incubation of bacteria in SOC medium for 1 hour at 37°C, author describes that the bacteria were centrifuged and the resulting pellet was resuspended in water.

Why water? Do you think this step may have somehow affected the viability of bacterial cells?

PCR + Colony PCR

First of all, these two chapters should be merged just into one. Information is sometimes duplicated and sometimes even different, therefore, it is quite confusing. In addition, concentrations of primers are missing. Table 3 is incomplete – the final volume of the reaction is missing, the amount of AmpiGen Taq Mix is remarkably low (only 2.5 ul), which would point to only 5 ul reaction.

What were the primer concentrations and what was the final volume of the colony PCR reaction? Why was the annealing time as well as the elongation time that long?

Organic extraction

What was the concentration of sodium acetate?

In vitro transcription

Was the purity of the DNA/RNA sample measured after the extraction? If so, why is it not mentioned either in the methods or the results?

The step of the plasmid linearization is necessary in order to get the RNA with a desired length. Is there any other possible way how to get to the IVT RNA of precise length without the linearization reaction?

DNA digestion

I am not sure about the usage of NEBuffer 2.1 described in Table 11 in the case of pRN3P-e and

Tubulin-GFP. The same set of restriction enzymes (BamHI and NotI) was used in other cases with either CutSmart buffer of NEBuffer 3.1. Since the activity of NotI is only 50% in the NEBuffer 2.1, please explain this.

Ligation reaction

The molar ratio of insert:vector is missing.

TA-cloning

Ligation reaction described in Table 15 is different in comparison to the ligation reaction in Table 13. Did you use a different T4 ligase kit? If not, please explain why there are remarkably different amounts of input DNA and ligation reaction buffer.

mRNA microinjection

H2A-RFP abbreviation is mentioned for the first time and is not explained. Furthermore, probably by a mistake, author refers to YAP construct as "EGFP-YAP1(S112A)", however the rest of the thesis operates with HA-YAP(S112A).

Author provides the information about the final concentration of mRNA samples, however what was the amount of mRNA microinjected into the cells?

Confocal microscopy

The same problem with YAP construct as above. Even the settings of the microscope are dealing with the GFP signal counting!

Results and Discussion

According to its name, this chapter combines the description of the results directly followed by their discussion. The length of 9 pages is satisfactory, however, the number of references is very low. Only 2 out of 4 citations are relevant to the discussion part (van der Ent *et al.*, 2006 and Nishioka *et al.*, 2009). I know it is hard to discuss the results of a rather methodological work, but even though, I would have expected at least one or two more papers related to the issue of cell fate decision process in an early embryogenesis. Especially comparing the methods and constructs which were used in these papers.

4.1 Molecular cloning of EGFP-Tub-6

The description of data in Figure 9 is unclear.

What restriction enzymes were used for pEGFP-Tubulin-6 digestion? Why there is no plasmid visible on the gel?

Figure 11 is a mess. First, the quality of images is low (***Why do you use photographed prints and not the original images?***). Second, restriction reactions for both clones should have been analysed on the same gel and uncut plasmids should have been shown also for both clones. Moreover, figures in theses should meet certain standards, so next time, please avoid gels with unrelated samples.

Even though the microinjection of EGFP-Tubulin-6 mRNA did not result in any detectable signal, I would have shown the images anyway. The negative results are still results. In connection to this, I have few questions concerning this particular experiment.

What was the amount of EGFP-Tubulin-6 mRNA microinjected into embryo blastomeres? Did you also use H2A-RFP mRNA as a positive control? Does the H2A-RFP mRNA include the 5' and 3' UTRs? From which construct is this mRNA derived?

4.2 Embryo blastomere microinjection of HA-YAP(S112A) mRNA

As is stated in the thesis, the recombinant YAP protein carries also the HA-tag. I don't understand the reason, why the HA-tag antibodies were not used directly. Even though if you would have

seen significant changes towards to TE fate, it would be necessary to verify the presence of YAP anyway. Why did you not use the anti-HA antibodies during your experiments?

Have you considered the presence of an endogenous YAP protein? Do you think it will not functionally interfere with your YAP(S112A) variant?

4.3 Production (IVT) of Securin-GFP RNA

I would be really careful about the statement that the generation of Sec-GFP mRNA was successful. The only conclusion I can get from Figure 14 is that you know nothing about the integrity and size of analysed RNA.

Please, explain, why do you think the in vitro transcription of Sec-GFP mRNA was successful.

Conclusion:

Despite all the comments, it is clear that there is a lot of work behind the thesis. It is also without any doubts that author proved to be capable of an independent scientific work and thinking. Therefore, I **recommend** the thesis for the defense and I suggest the grade **2.**[2] However, the final grade will depend on the performance of the candidate during the defense and his answers to my questions.

In **České Budějovice** date **20.1. 2020**

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signature

[2] You can suggest a grade, which can be modified during the defense based on the presentation. However, if the reviewer is not present at the defense, the grade will not be counted. Grades: excellent (1). Very good (2), Good (3), Unsatisfactory/failed (4).