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Reviewer evaluation on the diploma thesis by **Sára Kropáčková**

Subcellular insight into cholesterol-mediated proliferation of the tick cell line

In her thesis, Sára Kropáčková aims to address requirement and role of cholesterol primarily using a cell line derived from the tick *Ixodes ricinus*. The work stems from the established, yet incompletely understood, basis of dependence of arthropods (and other Ecdysozoa) on dietary cholesterol. The author performs experiments aimed to test a hypothesis that cholesterol is selectively required for tick cell proliferation and that the exogenous sterol is internalized by the cells. Next, the author attempts to link the cholesterol-dependent cell proliferation either with 20-hydroxyecdysone (20E) or with Hedgehog signaling. In the latter case preliminary experiments are conducted in live ticks.

The Introduction is logically composed, and the text reads smoothly. I must commend that the author chose to write her thesis in English. While the writing is far from perfect and is tainted with occasional mistakes in grammar and syntax, the language is relatively mature for a master student. The content of the Introduction is adequate and well gauged to inform the reader for the experimental part.

The downside of the Introduction is superficial knowledge of certain subjects, such as sterol metabolism and steroid hormone synthesis in insects. This is clear from citations of outdated literature instead of state-of-the-art references or use of obsolete terms (20-HE). Current research in this area is much advanced relative to what is captured on the top of page 8. An entire cascade of enzymes is known to carry out the individual steps in steroid hormone (ecdysone) synthesis, starting with the Rieske-type oxygenase Neverland, which is responsible for the initial step -- conversion of cholesterol to 7-dehydrocholesterol (the mutation of which makes certain flies dependent on cactuses). Introduction to Hh signaling is miniscule compared to the actual significance of this pathway and what is known about it.

Materials and methods. This section is written technically to contain sufficient information for each protocol. That is fine. However, the text would be much more accessible if appropriate background and rationale were provided as well. The reader should understand the purpose of BSA coupling, staining with Amido Black, Filipin, Nile red, Magic Red, and Trypan Blue or the principle of Vismodegib action the first time these tools are mentioned. This helps understanding adequacy of the approaches. I doubt that quantity of 20E was determined using HPLC alone, as mentioned in Section 4.8. Please specify.

Results. This experimental part is logically ordered and succinct. Description of individual experiments in some cases lacks rationale and/or is incomplete, making the reading less than smooth. Some critical controls are missing, making the data impossible to interpret (see below). As a general note, it is quite unusual for a diploma thesis to place data in a Supplement rather than to the main thesis body. I believe it is not the best choice when the relatively limited amount of data is to be compared side by side (such as Figures 4 and S2).

In Section 5.1, the author initially aimed to test if cholesterol was sufficient to restore proliferation in the tick cell line grown without cholesterol. The answer is yes. Closely related sterols such as ergosterol were unable to support growth, indicating selective requirement for cholesterol. However, this notion is rather questioned by the fact that linolenic acid was able to substitute for cholesterol.

Both cholesterol and linolenic acid were conjugated to BSA. While BSA itself was inactive, it is unclear whether the conjugation with BSA was required for cholesterol or linolenic acid activity, as critical controls - unconjugated sterols and linolenic acid - seem to be missing. Please comment.

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In Section 5.2, the author shows that the tick cells could uptake BSA-conjugated fluorescent version of cholesterol (BSA-CTL) but not of ergosterol (BSA-DHE), indicating selective requirement for cholesterol at the level of transmembrane transport. However, data for cell growth with BSA-CTL and BSA-DHE are not presented. Again, we are not told whether BSA conjugation is required for cholestatrienol and dehydroergosterol cellular uptake and proliferation. In addition, labeling in Figure 6 is inappropriate as cell were always cultured in LPDS (control means no sterol supplement, not a different medium). Please comment.

The "growth curves" in Figures 4, S2, 7 (and 12). It is surprising that growth curves in a cell culture appear linear, rather than exponential or sigmoidal. I suspect that, without cell counts provided for each day, these are really no curves but counts at two time points only. If so, the lines connecting the data are inappropriate and these data should be presented as scatter plots for the two discrete times with no line connecting them. Please comment.

The aim of Section 5.3 is conceptually flawed. It makes no sense to test whether cholesterol is "essential as a substrate for ecdysteroid biosynthesis" in the tick cell line. In insects, only special endocrine gland cells have the capacity to synthesize ecdysone (the immediate 20E precursor) from dietary sterols. It is extremely unlikely for any isolated arthropod cell line to synthesize ecdysone. Do these tick cells express all orthologs of the insect steroidogenic enzymes? Figure 8B shows that 20E is (as expected) undetectable in these cells unless it had been added. However, what is the point in quantifying the artificially added hormone? How does this experiment address ecdysteroid synthesis? I see no logic in this setup. Please comment.

On the other hand, 20E is known to stimulate cell proliferation in some contexts, so testing 20E for cell growth is an interesting idea. Indeed, supplying 20E to tick cells grown without cholesterol did increase cell numbers relative to medium alone (Fig. 8A), although the effect was minor relative to that of BSA-conjugated cholesterol. However, the data cannot be interpreted because - again- critical controls are missing. How can BSA-cholesterol conjugate be compared to unconjugated 20E? Both BSA-20E conjugate and cholesterol without BSA should have been included. Please comment.

Section 5.4. The observation that intracellular stores and distribution of cholesterol appear to be unaffected when cholesterol is absent in the culture medium is remarkable and surprising. The fact that the cells fail to proliferate normally even when cholesterol is internally available seems counter-intuitive. This finding prompted the author to test for a signaling role of extracellular cholesterol leading to cell proliferation in Section 5.5. Since Hh signaling involves cholesterol that can activate the Smo protein, the author decided to inhibit Smo by using a chemical antagonist of Smo (Vismodegib) and compare effects to those of cholesterol depletion. Indeed, Vismodegib was able to block cell proliferation in tick cell line.

While this result is interesting, it is insufficient to conclude that availability of (extracellular) cholesterol "drives Hh signaling mediated cell proliferation" (page 25). Claiming that "By disrupting the Hh signaling pathway in cells, their growth is thus disrupted in the same way as when cholesterol is removed from the media" cannot be sustained, much the conclusion "we confirmed that cells without external cholesterol in the medium do not have an activated Hh pathway which causes them not to multiply" (Discussion, page 31). These are pure speculations.

The fact that cells stopped dividing by no means proves that the defect caused by cholesterol depletion and Smo inhibition have common molecular grounds. There are many ways to block cells cycle independent of cholesterol of Hh, and the outcome would be no different. Arrested proliferation is too non-specific a phenotype to reflect failure in one particular pathway. As the author correctly

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points out at the end of Discussion, it is necessary to examine specific Hh pathway readouts such as expression of the downstream gene Gli.

Moreover, yet another missing control makes the data difficult to interpret, particularly in Figure 12 (but also in other experiments monitoring cell growth). Cell numbers may decline not only because of reduced cell division but also due to reduced cell viability or increase in cell death. In the Discussion (page 28), the author claims that "Even though the LPDS cells do not reproduce, they do not die." This statement is invalid without testing cell viability.

Sections 5.6 and 5.7 are dedicated to experimenting in live ticks, first by showing expression of smo and ptc mRNAs in selected organs and its relative decrease in the gut during feeding. Not surprisingly, feeding ticks with the Smo antagonist Vismodegib was detrimental to tick growth. These are nice data, however, too preliminary to make a causal link between Hh signaling and cholesterol by any means.

Discussion. Although typically the most difficult section for students, the Discussion here is probably the best part of the thesis. I like the systematic logic and clarity in the presentation, smooth flow of the text. The writing shows good command of English (for the most part) and good thinking.

Admittedly, my evaluation of this thesis was quite harsh, in a style of an editor or reviewer judging a real manuscript submission. I believe that the purpose of having diploma thesis is to learn something, rather than being pampered. I also believe the comments may help the early author to improve prospective manuscripts for publication.

Notwithstanding my critical comments above, I would like to conclude that despite some experimental shortcomings, I consider Sara's thesis very good and acceptable as a basis for awarding master's degree. Through this project, Sara became acquainted with many molecular and biochemical methods, microscopy techniques, and experimentation on live ticks. She tackled an important scientific problem, a difficult project indeed, in which she has demonstrated knowledge and skills as a starting scientist and author. In summary, I do not hesitate to recommend that, upon successfully defending his thesis, Sára Kropáčková will have met the requirements to earn her degree.

With kind regards,

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