Skoltech
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Thesis Changes Log

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PhD Program: Life Sciences

Title of Thesis: Development of Messenger RNA Delivery System via Virus-Like Particles

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Date of Thesis Defense: 17 May 2019

The thesis document includes the following changes in answer to the external review process:

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- Page 5: Abstract: "Applying messenger RNA (mRNA) has become a promising therapeutic modality in various spheres ranging from biotechnology to basic science" is changed to "Applying messenger RNA (mRNA) has become a promising therapeutic modality in various spheres ranging from basic science to biotechnology".
- Page 10: tRNA – transport RNA is corrected to transfer RNA
- Page 12:.. making it useful for gene therapy and vaccine development. => An appropriate reference is provided.
- Page 12:.. "Moreover, mRNA enables the defected protein" is changed to "Moreover, mRNA enables the defective protein"
- Page 16:.. figure 1.2 … A brief statement on the concept of dendritic cell vaccination after the figure legend is provided
- Pages 20-21:.. figure 1.4 … This is a table, so list it as table 1.1 or so and please reference it accordingly=> Table is deleted as author considered it as too redundant and duplicating the text. The schematic representation of Non-homologus end joining is added as a Figure 1.4.
- Page 39:.. Materials and methods Section: Missing source of chemicals/plasmids is included.
- Page 45:. Fig 2: Cells were plated at a density of 1.9-2.2 × 105 ; the units are missing. =.> Units are added.
- Page 49:. The membrane was washed 3 x 10 min=> Volume is added
- Page 53:. Fig 3.2: Change the Y-axis legend to relative mRNA incorporation=.> Changed
- Page 59: Fig 3.8. Time course experiment. How long does the EGFP positivity in cells increase and at what time point it starts to decay? If the information on the time course is available even with one of the constructs, please include them. This will establish that the production of target protein is transient.

-Unfortunately, the author does not have FACS analysis of cells inoculated with EGFP VLPs at late time points. According to the author's observations, the EGFP positivity started to decay after 24 hours and completely disappeared at a time point of about 36 hours. However, the author was always observing her plates under Keyence microscope and never measured EGFP

positivity after the cells were already dark under Keyence. Overall, the author does not have this experimental data that it is referred to.

- Page 69: Proteomics of VLP's and Table 3.21. What does the number indicates? Is it representing relative intensity or ion count? If yes, please provide a brief description on how the protein content was quantified. => added
- It is recommended that author provides a summary schematic figure similar to figure 1.8 to explain the different components assembled as part of the PhD thesis.

-The schematic representation of constructs used for VLPs preparation is depicted at Figure 3

- For figures throughout the document if the author is using the figure from the reference, state that the figure is adapted from the references quoted at the end of the figure legend. For e.g. Figure 4.3 [8], state figure adapted from reference 8.=> Corrected
- The delivery of spCas9 to the target cells has been demonstrated using qRT-PCR as well as western blot. However functional activity of the enzyme spCas9 still needs to be validated. If any data (however preliminary) regarding functional efficacy of the spCas9 enzyme expressed using VLPs is available, please include.

-The functional activity of SpCas9 , delivered by VSVG-L7Ae VLPs was demonstrated by applying these VLPs onto 293T reporter cell line that stably express sgRNA. In case functional SpCas9 is delivered to this cell line, EGFP positive signal appears. Figure 3.4B

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Chapter 3 "Results" starts from technical details. A general experimental strategy is not described, that greatly complicates understanding of logics of further experiments. The readers can see an overall description of the suggested experimental strategy only in Discussion (p. 76-77: "The schematic representation of the RNA delivery system we aimed to utilize is depicted at the Figure 4.1. We planned that fusing VSV-G with either MS2BP or L7Ae and cloning the cognate motifs in the mRNA structure would facilitate its incorporation into the VLPs and hence the delivery to the target cells. …"). I suggest to move this part from Discussion to Results.=> Moved

Fig.4.1: The depicted pathway of mRNA entrance from internalized vesicle into cytosol of the target cell is unclear. Specifically, it seems for me that the shown intermediate step of "mRNA in a circle" (the upper part of the cell on the right) is incorrect – mRNA should go directly into cytoplasm after fusion of VLP and cellular membranes.

-VSV-G is a coat protein that Vesicular Stomatitis Virus uses to enter the host cells. VSV-G interacts with Low density lipoprotein receptors (LDL-R) on the cell surface and enters the cells via clathrin-mediated endocytosis. For VSV-G to get free in the cytoplasm the endosome must get acidified and mature. This enables VSV-G to change its conformation, fuse with the endosomal membrane and get released to the cytoplasm (White and Whittacker, Fusion of enveloped viruses in endosomes, Traffic, 2016). That is why author tried to depict the similar way of cell entry with consequent endosomal maturation and escape pathways, assuming that VSV-G fusion with L7Ae does not affect this pathway.

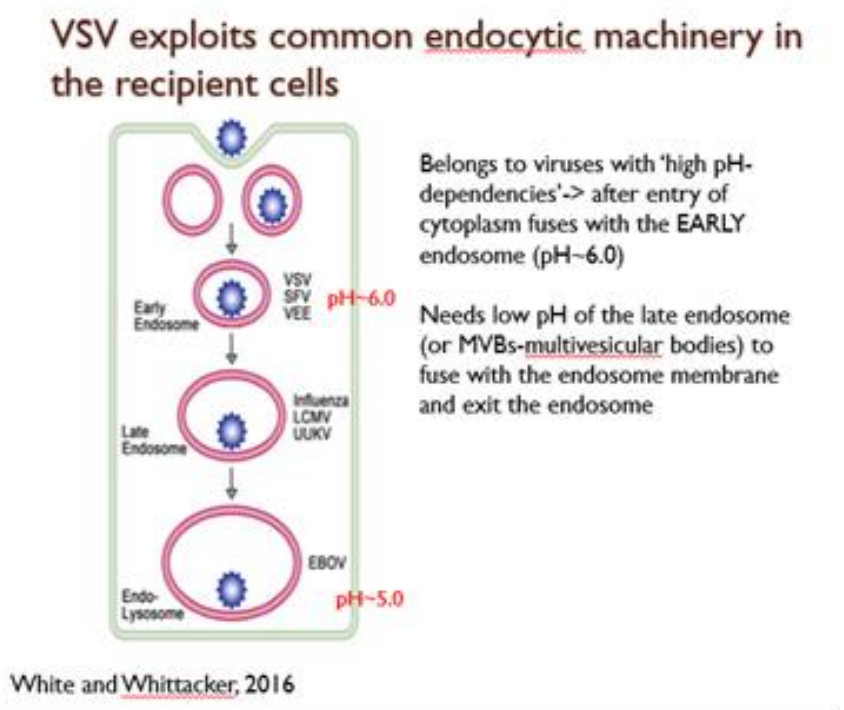

Fig. 3.1 legend: "n = 3, ± S.D", but there is no SD in the plots.
-Figure 3.1 includes histograms, however, author added the same results in a triplets as a graph bar to include error bars there (Figure 3.1 B)

Incorrect figure number (p. 62): "we proceeded with the qRT-PCR. EGFP mRNA was bound with VSVG-L7Ae and enriched more than 600-fold compared with the control VSVG-WT (Figure 2D). However, enrichment of the bound RNA was also observed with the endogenous mRNAs, such as GAPDH (~160-fold) (Figure 2E), β-actin (ACTB, ~110-fold) (Figure 2F), and 7SL lncRNA (RN7SL1, ~90-fold) (Figure 2G)" – in fact, it is Figure 3.13 and it contains a single histogram. => Corrected

Page 15, section «1.2 Applications of mRNA delivery»: «The applications may be as following: to devise viral vaccines, to generate autologous dendritic cell vaccines for cancer

immunotherapy, to replace disease-related defective protein in gene therapy, and to introduce genome-editing platforms to cells». An important direction was missed – delivery of mRNA for reprogramming of cells (induced pluripotent stem cells, iPSC) (e.g., see Bernal JA. RNA-based tools for nuclear reprogramming and lineage-conversion: towards clinical applications. J Cardiovasc Transl Res. 2013, 6:956-68). It was briefly mentioned in the Discussion (p. 75 "mRNA delivery has several exciting applications, such as mRNA vaccines, cellular reprogramming, and genome editing [86]" but without any details.
-Application of mRNA delivery for the reprograming is added as the section 1.2.4

1.3 and 1.4 are in fact should be sub-sections within 1.2. => Corrected

Figure 1.2 –This big scheme taken from a review looks too complex here to illustrate just single sentence. I would like to see some simplified scheme that focuses on points important for the present work (preferably, depicted by the author).=> A simplified scheme depicted by author is added instead (Figure 1.2)

Pages 39, 42, Tables on recombination reaction: "Destination vector – 150 ng/μl". How many microliters? => The volume is added.

Page 48, Table on qRT-PCR does not show concentration of the primers, just "1 μl".=> Concentration is added.

Many references in the list have incorrect formatting. Mostly, it is absence of the journal title, but some other misprints can be found (e.g., "K. Institutet" (i.e. Karolinska Institutet) instead of the author name in Ref. 57). I found mistakes in the following references: 16, 17, 41, 54, 57, 58, 61, 68, 70-72, 77, 85, 86, 90, 91, 99, 106, 122, 123, 125, 127, 135-149. => All references are corrected

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Major concerns:

- There is no discussion/explanation on the unexpected loss of VSVG-L7Ae activity in comparison to WT for EGFP delivery by retroviruses (p. 68);

  -The author included one of the possible explanations of this phenomenon in the Discussion section, p 92

- RNA-seq data was not confirmed by qPCR or alternative methods=> It is very unfortunate but the author does not possess this kind of data.

- Most of Western Blot data is presented as photos of a membrane, without calculations of the protein changes=> It is very unfortunate but the author does not possess this kind of data.

- Minor points:
    - Experimental procedure for protein transfer to a membrane (p. 49) should be added=> added
    - “Gene therapy” (p.17) – described examples of mRNA applications do not deal with gene therapy – all genes remain intact. This is a substitutional therapy; => changed
- -Sleng like “annealed oligonucleotides were run on the gel for 30 min” (p. 41) should be changed => corrected
- -I recommend intensive text proofreading to remove typos and improve the text – for example, “5’-methylation of cytosine” (p. 14); also figure legends (Fig. 1.6 (p. 23), Fig. 2 (p.45)) should be corrected.=.> corrected