

The First Viral Expression Model in Human Induced Pluripotent Stem Cells to Observe Enteroviral Infections *In vitro*

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Introduction

Viruses recently caused global outbreaks of potentially severe diseases. The newly recognized corona virus, which is causing SARS-CoV-2 is the most recent example for this scenario. Potentially severe but not as prominent as the corona virus is the Coxsackievirus B3 (CVB3), which can cause fatal outcomes of myocarditis, meningitis, encephalitis and diabetes type 1 due to chronic infections of the heart, the brain, and the pancreas [1,2]. Although, CVB3 is long-time known to cause these pathologic phenotypes, less is known about the molecular interactions of CVB3 and its host-cell. Recently, a new concept for viral model systems was developed, which allows the detailed observation of viruses, here CVB3, in human iPSC-derived cells [3]. The genome of a non-infectious variant of CVB3, CVB3ΔVPO, was inserted into the hiPSC-genome via PiggyBac transposase together with a doxycycline-inducible tet-on system. This dose-dependently and time-dependently controllable system showed typical infection phenotypes for CVB3-infected cells like mitochondrial alterations, re-structuring of cellular membranes and changes in cardiac action potential generation [4-6]. Hard to observe virus-host-cell interactions could potentially be resolved in this system, leading to a new understanding of viral pathology. With this new tool in hand, virologic research could make a huge step forward towards a wide range of potential vaccines, therapies and prevention strategies to be able to counter newly appearing viruses more efficiently as possible nowadays.

The Expression Model

The investigation of viral diseases and their

pathophysiology has become a huge issue during the past years due to several epidemic and pandemic events caused by viruses worldwide. Only in the 2020's, society experienced the outbreak of SARS, MERS, Ebola and recently Sars-CoV-2 with tremendous financial and social consequences for local and global populations [7-10]. To resolve the mechanisms of infection and pathophysiology to counter appearing viruses should be a main focus of today's society to prohibit another global pandemic. The newly invented viral expression system by Peischard et al. paves the way for novel findings and a new understanding of viral infections on the example of CVB3.

For the new viral expression system, a stable transfected cell-line was generated, which inherits the genome of CVB3. This CVB3 genome contains two-point mutations in the so called VPO precursor protein, which prevent the generation of functional VP2- and VP4-capsid proteins and so disable the virus to form functional capsids [11]. So, this cell model is rendered non-infectious and can be handled under reduced safety level. The expression of the non-infectious CVB3 virus variant, called CVB3ΔVPO, is controlled via a co-transfected, doxycycline-activated tet-on system (Figure 1a). In normal culture media, CVB3ΔVPO is not expressed and leaves the cells unchanged towards viral effects. The addition of doxycycline in low concentrations of 2 µg/ml and less leads to the detectable expression of CVB3ΔVPO within a few hours. The applied doxycycline binds to a co-expressed transactivator, which then binds to the TRE-element of a desired gene. This binding enables gene expression as in this case of CVB3ΔVPO. Thereby, the experimenter can adjust the desired virus load by titrating the doxycycline concentration applied to the cells [3]. This dose-dependence of this system enables for the simulation

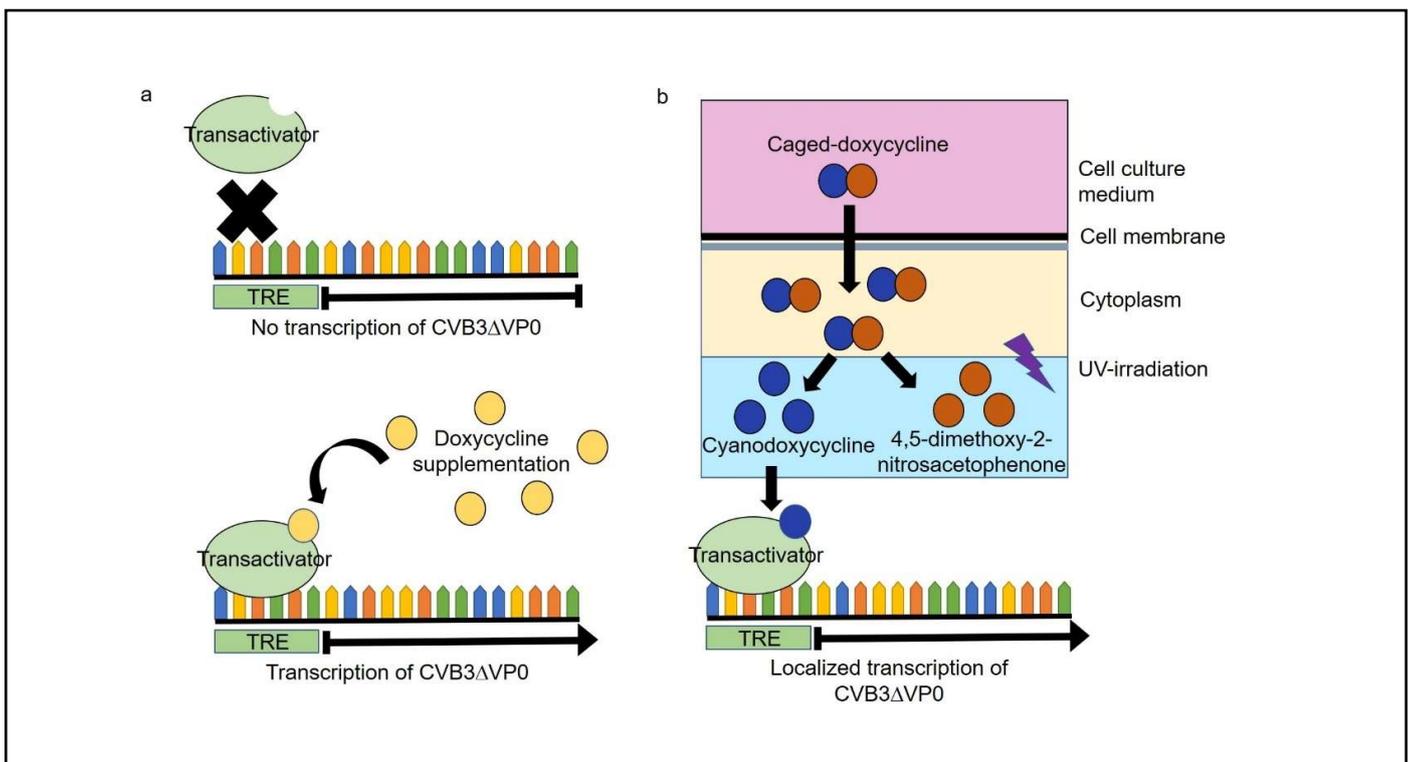


Figure 1: Principle of general and localized CVB3ΔVP0 expression via tet-on system. a) General principle of a tetracycline-dependent tet-on system used for CVB3ΔVP0 expression. Doxycycline supplementation to the cell culture medium leads to the binding of a transcription transactivator to the TRE-element ahead of the gene of interest. Transactivator binding to TRE enables gene expression. **b)** Principle of localized gene expression via caged-doxycycline (c-dox) application and localized UV-irradiation. Membrane permissive c-dox enters the cell through the cell membrane. Selective UV-irradiation of cell layer parts leads to the cleavage of c-dox to cyanodoxycycline and non-toxic 4,5-dimethoxy-2-nitrosacetophenone. Cyanodoxycycline is able to activate the transcription transactivator in the same way as doxycycline, but allows for highly localized virus protein expression replicating infection patterns typical for CVB3 infection.

of various infection scenario from strong, acute infections to chronic infections with long-lasting, low viral loads. The viral expression system by Peischard et al. is hiPSC-based which makes it very flexible for various research areas from classical virology, cardiology, neurology etc. The stable transfected hiPSC which can express CVB3ΔVP0 are pluripotent, which means that they can be transdifferentiated into any cell type of the human body. While CVB3 is also infecting different cell types in human patients including cardiomyocytes, neurons and pancreatic cells, it is possible to model viral infections in all of these tissues in a culture [1]. First promising experiments with this expression system were performed on hiPSC-derived ventricular-like cardiomyocytes. Thereby, typical alterations for CVB3 infections were found in CVB3ΔVP0-expressing cells proving the functionality of the generated system. An infection of host-cells with CVB3 wildtypes was found to be associated with the elevation of mitochondrial reactive oxygen species (ROS) together with pathologic mitochondrial phenotypes [12]. The elevation of mitochondrial ROS-levels in hiPSC-derived cardiomyocytes expressing CVB3ΔVP0 verified the functionality of the system [3]. The direct effects

of these ROS are still unclear and hard to study. Due to rapid apoptosis of cells infected with CVB3 wildtype strains makes a detailed view on mitochondrial changes almost impossible and long-term, chronic infections are non-observable. In the hiPSC-based expression system of Peischard et al. no significant elevation of apoptosis was detected after CVB3ΔVP0 expression, because no competent viruses are produced [3]. This enables for long-term observation of mitochondrial effects induced by CVB3ΔVP0. Further ROS effects, for example DNA and organelle damage may be observed and studied in more detail as well. Possible effects of mitochondrial ROS production may be mitochondrial apoptosis, DNA damage and potential cellular ageing towards a senescent phenotype [13,14]. The transfer of such findings towards a clinical phenotype may resolve many questions on how viruses lead to lethal cardiac outcomes and how potential therapies may revert viral interplay with cardiac cells.

The disintegration of cellular membranes is a major symptom observed in CVB3 infected cells [6,15]. The destabilization of the membranes belonging to the golgi-apparatus is often referred to the generation of viral

replication complexes, vesicular structures in which the viral assembly takes place [16]. Transport of these viral replication complexes to the cell membrane and additional membrane destabilization finally leads to the release of viral progeny to infect neighbouring cells. Obvious membrane destabilization of the cellular membrane and of the membrane of cytoplasmic structures was observed by Peischard et al. in hiPSC-derived cardiomyocytes as well [3]. While no viral progeny is produced during CVB3ΔVPO expression, the effects of the expressed viral proteins potentially lead to the formation of viral replication complexes and to the destabilization of the cell membrane. Further influence of the CVB3ΔVPO on the membrane of other cellular organelles like the endoplasmic-reticulum (ER) or the sarcoplasmic-reticulum (SR) in hiPSC-derived cardiomyocytes has still to be verified. From wildtype studies it is known that CVB3 viroporin 2B commonly inserts into the membranes of cellular organelles, especially into those of the golgi-apparatus, the ER and SR [17-20]. The ER and especially the SR are known to be significant calcium storages and are key players in the contraction of muscle cells. Viroporin insertion into the ER and SR leads to a calcium outflow from the ER/SR into the cytoplasm, where it can potentially change the ability of muscular cells to perform correct contraction [21]. This may be one possible cause for arrhythmic events in viral myocarditis patients. Other effects and interplay of CVB3 and cellular organelles may be resolved in follow-up studies.

As CVB3 patients who develop myocarditis can die because of stress-triggered arrhythmic events, potential influence of CVB3ΔVPO on cardiac action potential generation was investigated as well. Little is known about viral influences on electrical signal generation, while just a few studies address this question. A study by Seebohm et al. could show that the expression of CVB3 in *Xenopus laevis* oocytes changes the distribution of ion channels and so potentially leads to changes in action potential generation and propagation [22]. This could be verified in the viral expression system by Peischard et al. with multielectrode array measurements. The expression of CVB3ΔVPO in hiPSC-derived cardiomyocytes lead to significantly elongated cardiac field potentials measured by the eQT-interval length. In addition, CVB3ΔVPO-expressing, hiPSC-derived cardiomyocytes were not affected by β-adrenergic stimulation, while non-CVB3ΔVPO-expressing cells reacted with an expected QT-Interval shortening.

A very interesting application of the controllable, CVB3ΔVPO-expressing hiPSC line by Peischard et al. is the possibility to locally express CVB3ΔVPO in a generated tissue. While the expression of CVB3ΔVPO is generally controlled via doxycycline supplementation in the media to initiate the viral expression in all cells simultaneously, the treatment with caged doxycycline enables for

localized expression of CVB3ΔVPO. Therefore, cells were incubated with caged doxycycline, which is basically not initiating the expression of CVB3ΔVPO via tet-on system. Caged-doxycycline enters the cells through the plasma membrane due to its hydrophobicity. By applying short UV-irradiation, caged doxycycline is decomposed into cyanodoxycycline and non-toxic 4,5-dimethoxy-2-nitrosacetophenone [23]. Thereby, cyanodoxycycline can initiate the localized expression of CVB3ΔVPO via the tet-on system. This only happens in cells, which experienced UV-irradiation (Figure 1b). So, by applying different UV-irradiation patterns onto a cell layer, various viral infection patterns can be simulated. This application resembles the nature of wildtype CVB3 infections, because these infections do not affect a whole organ at once, but start in regionally distinct infection foci from where they spread towards other regions of an organ [24]. By modelling known infection patterns of human patients, one could get more insight into the pathologic interaction of CVB3-infected tissue with surrounding healthy tissue. Concerning cardiac cells, it may also be possible to study the electrical signal conduction in CVB3ΔVPO-expressing cardiac tissues or the propagation of electrochemical signals from non-infected tissue parts into infection foci.

Conclusion

The number of new applications, which can be performed with the new CVB3 expression model by Peischard et al. is extensive. The differentiation of many cell types, the simulation of many different infection scenario including long-term infection and healing events opens up new applications for the scientific community. The localized expression of CVB3ΔVPO, the coupling of several cell types and the monitoring of cell signalling through CVB3-infected tissue, the simulation of infection foci may thereby lead to a more detailed understanding of virus-host interactions on the physiological and molecular level potentially leading to novel innovative therapeutic approaches. Additionally, it is possible to adapt the CVB3ΔVPO-based expression model to other viruses. Potentially, this model system will be used for a better understanding of viruses and their host-cell interaction and for the development of therapies and vaccines to counter a newly rising pandemic.

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