REPorter system for RNA-based therapy detecting apoptosis and cellular stress in ORGanoid models - REP-ORG systems.

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BACKGROUND

In recent years, the increasingly in-depth understanding of the many RNA functions has led to the development of new RNA-based drugs that directly regulate aberrant gene expression in diseases. They include messenger RNA (mRNA), short-interference RNA (siRNA), and RNA aptamers. **Figure 1**. Despite their potential, using RNA molecules presents several limitations related to their multiple functions and structural malleability. This has driven the establishment of new methodologies to improve these drugs' correct administration and functioning. This study is part of a research project aimed at testing the off-target effects of RNA-based therapeutics. **Figure 2.** Therefore, we designed new reporter systems to standardize tests for analyzing the long-term effects of RNA-based drugs.

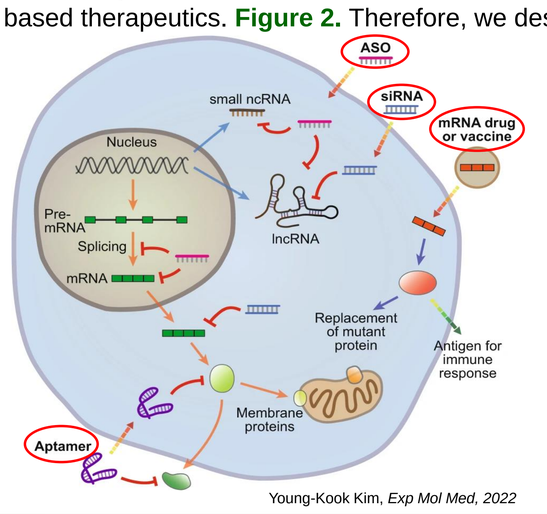

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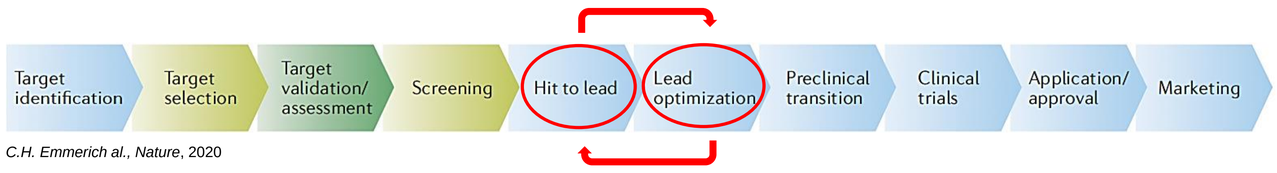
Figure 1. RNA therapeutics are expected to expand the range of druggable targets from proteins to RNAs and DNAs. Cell surface, extracellular, and intracellular proteins remain favorable targets for the development of small-molecule and protein (e.g., antibody) therapeutics, as well as RNA aptamer drugs. Actually, the majority of human genome sequences transcribed as functional ncRNAs largely outnumbered mRNAs to be translated into proteins. Both mRNAs and ncRNAs can be directly targeted by RNA drugs such as ASO/asRNAs, miRNAs, and siRNAs. Once introduced into cells, mRNA therapeutics may be developed for protein replacement therapy or vaccination.



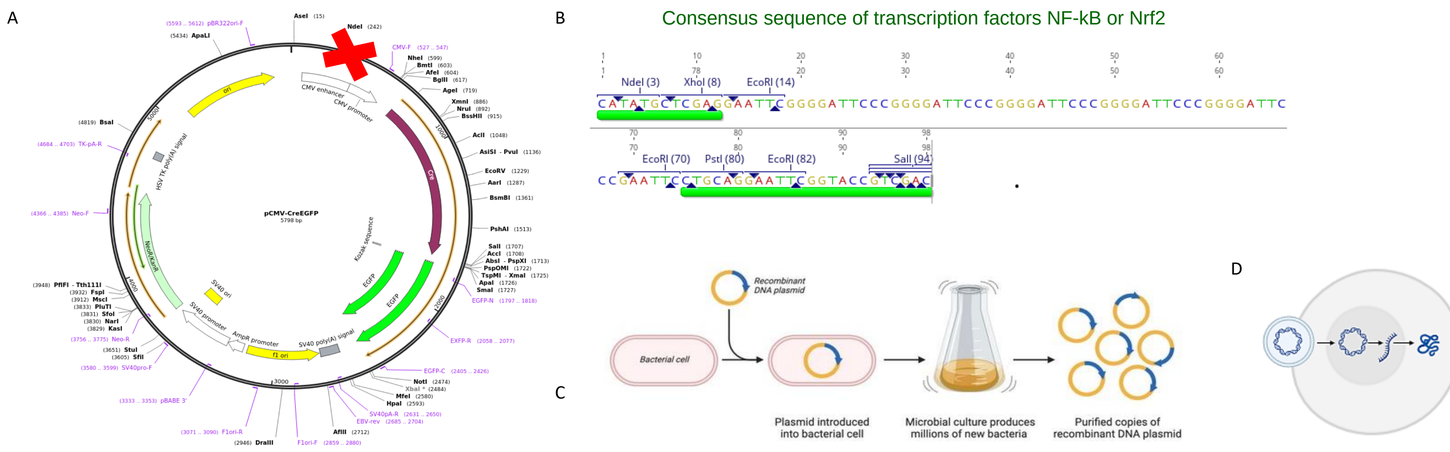
C.H. Emmerich al., Nature, 2020

Figure 2. Pipeline of development of an RNA drug. The different steps are represented in different colors. When a target is identified, a preclinical study is started, which turns into Clinical trials. Different and new technologies are necessary to study new types of drugs and identify off-targets.

METHODS

We generated reporter-based plasmids that express GFP and YFP fluorescent proteins under the control of consensus sequences recognized by the main transcriptional factors involved in inflammatory response (NF-kB) and oxidative stress response (Nrf2) (**Figure 3A-C**). These constructs were then transiently transfected into several lines derived from different tissues, including myoblast (the C2C12 and C2SOD1 lines[1]), hepatocyte (HepG2 and HUH7), thyroid cell lines (Nthy-ori, K1, BCPAP) (**Figure 3D**).

Figure 3. The fluorescent constructs.



RESULTS

Firstly, we verified the correct functioning of the constructs in 2D cell cultures by inducing cellular stress with hydrogen peroxide and analyzing the levels of NF-kB and Nrf2 using Real-Time PCR and western blotting. **(Fig.1).** Subsequently, we cultured transfected cells in the extracellular matrix and obtained 3D systems with distinct structures.

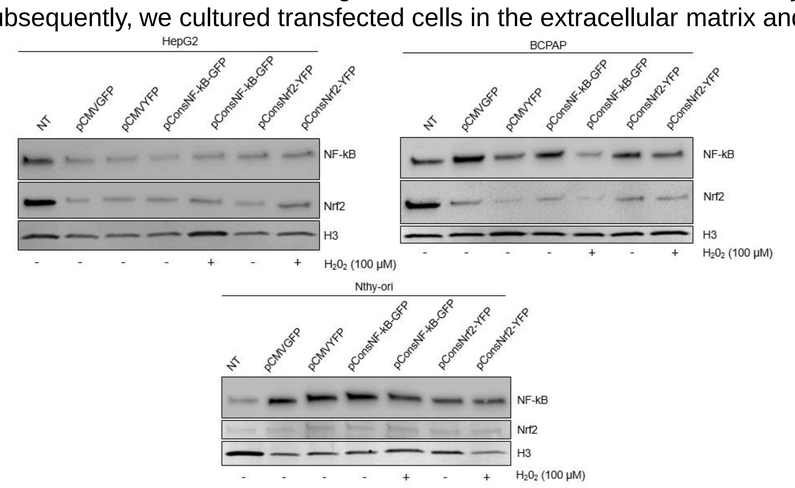
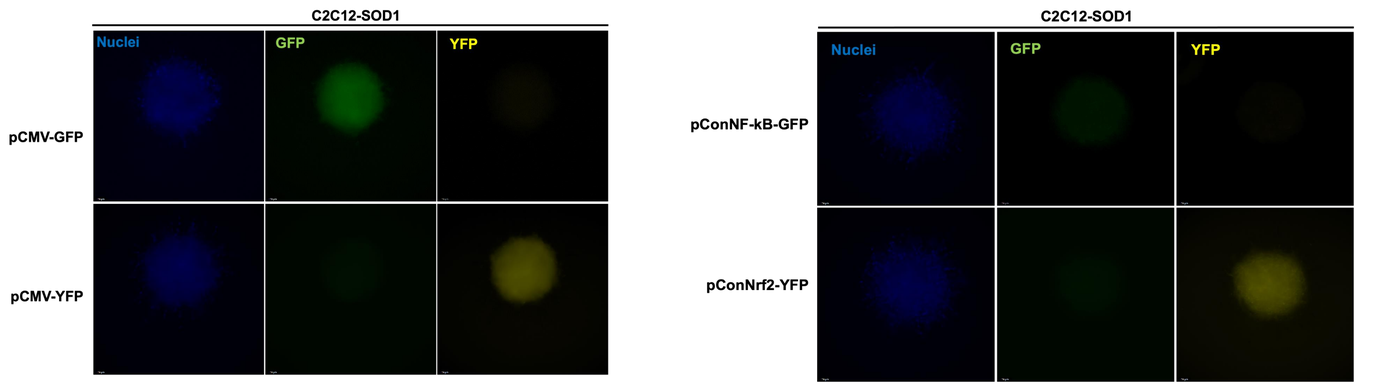
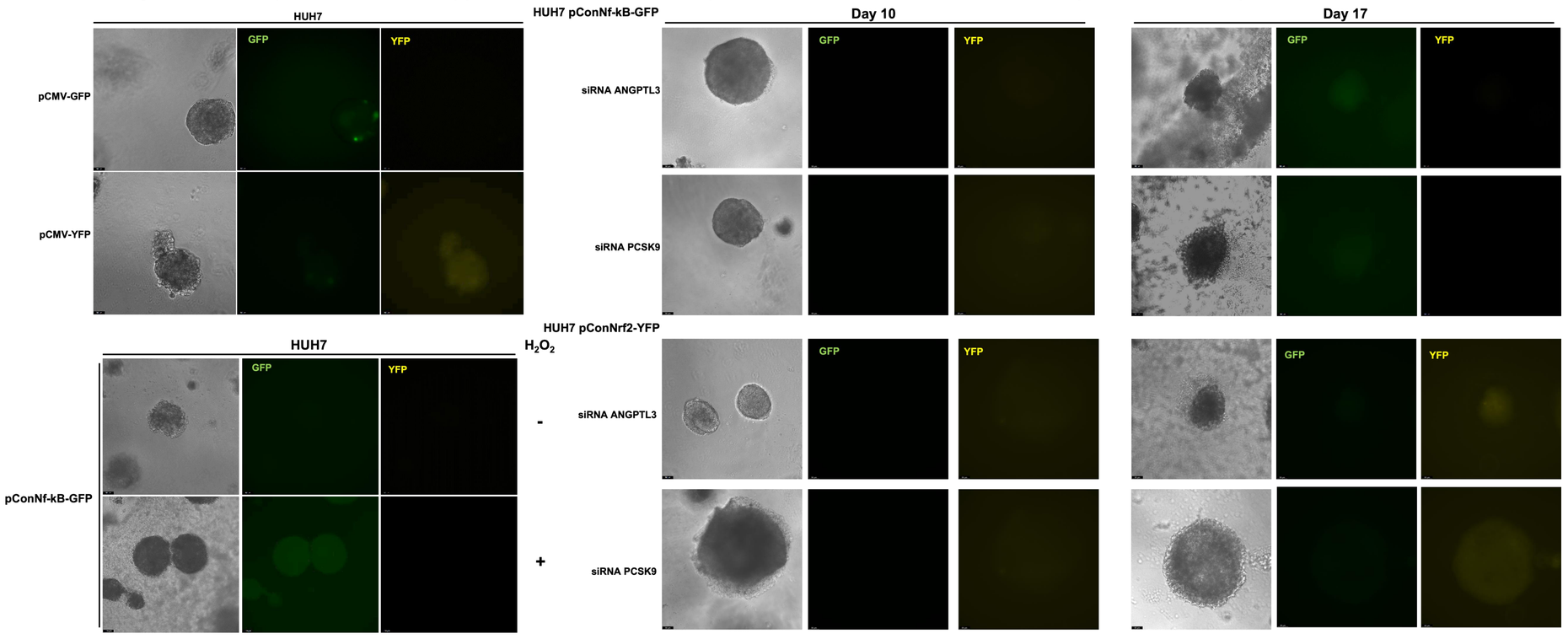


Fig. 1. NF-kB and Nrf2 expression was evaluated in HepG2, BCPAP and Nthy-ori cell lines after the induction of cellular stress with hydrogen peroxide.



Using immortalized cell lines from different tissues, we generated 3D reporter systems that activate the fluorescence in case of cellular stress. The organoid models were treated with different types of RNA molecules to monitor long-term effects. In detail, we focused on two RNA-approved drugs (the siRNA anti-ANGPTL3[2] and PCSK9[3]) and two miRNAs derived from different research projects in our laboratory (the miR-139-5p and the miR-335-5p). Following the triggering of stress, we compared some targets implicated in the RNA target molecules' signaling pathways in treated and untreated samples using Real-Time PCR. For example, we observed a significant reduction of PPARa and SREBP1-c levels following the siRNA anti-ANGPTL3 and PCSK9 treatment. Additionally, iodide transporter NIS levels appeared to increase, as expected, after treatment with miR-335-5p and miR-139-5p.



CONCLUSIONS and FUTURE PERSPECTIVES

Our research has led to the development and validation of a long-term monitoring system for the adverse effects of RNA drugs. The REP-ORG systems will allow us to identify aberrant gene expression in the cells and the long-term effects on non-specific targets of RNA-based drugs, thereby enhancing the precision of RNA-based therapies. Experiments with anti-RYR2 siRNA are currently underway, expanding our research scope.

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