Epione: Mir-140 production and exosome delivery to the chondrocytes to halt the progression of Osteoarthritis

Jordi Kontis[1], Daniel Kotsi[1], Christos Mantis[1], Nicholas Romanidis[5], Michalis Sarris[4], Giannis Thimianidis[1], Alkmini Zania[6], Melina Zikou[3], Nefeli Zikou[2],

1. Democritus University of Thrace, Department of Molecular Biology & Genetics
2. Aristotle University of Thessaloniki, Department of Physics
3. Aristotle University of Thessaloniki, Department of Computer Science
4. National and Kapodistrian University of Athens, Department of Medicine
5. National and Kapodistrian University of Athens, Department of Biology
6. University of Macedonia, Department of Economics

Introduction

The team NOUS is a Greek team competing in the GOGEC competition with 9 undergraduate students from different Universities from all over Greece. The goal of the project Epione, is to propose a new treatment method to combat Osteoarthritis. Osteoarthritis affects 7% of the world population. It is one of the top 10 causes of paralysis in developed countries, with a prevalence of 9.6% among men and 18% among women over 60 years old. As the average life expectancy rises, the percentage of people with some type of osteoarthritis will rise by 45% until 2050, managing to affect approximately 90 million people while leaving 40 million people severely disabled. There are several commercially available solutions for people with osteoarthritis, but most of them do not solve the problem, they simply eliminate some of the symptoms. Osteoarthritis is a joint disease which is characterized by progressive deterioration of the articular cartilage. Articular cartilage destruction is caused by degeneration of extracellular matrix, mainly composed of type II collagen and aggrecan. Key matrix degrading enzymes that should be inhibited include matrix metalloproteinases (MMPs), namely MMP13, and metalloproteinase with thrombospondin motifs (ADAMTSs), namely ADAMTS4 and ADAMTS5. Cartilage matrix homeostasis is disrupted by proinflammatory cytokines and chemokines that stimulate the collective production of proteases, nitric oxide (NO), and eicosanoids such as prostaglandins and leukotrienes. The action of these inflammatory mediators results in the induction of the catabolic pathways, inhibition of matrix synthesis, and promotion of cellular apoptosis. Key proinflammatory cytokines secreted in OA onset are IL-1β and TNF-α, and drive the inflammatory cascade independently or in collaboration with other cytokines. IL-1β interferes with the production of structural proteins, affects MMPs' synthesis by chondrocytes and induces the production of reactive oxygen species, for example, nitric oxide (NO). The nuclear factor-kappa B (NF-κB) transcription factor plays a central role in the pathogenesis of osteoarthritis. It is triggered by proinflammatory cytokines and ECM degradation products. The activated NF-κB modulates the expression of several cytokines, chemokines and matrix-degrading enzymes. miR-140 is a micro-RNA that regulates cartilage development and homeostasis. The expression of mir140 is significantly decreased in OA, while its external administration and

overexpression have been shown to halt the progression of the disease and promote the regeneration of the cartilage. While the exact targets of mir140 are not yet fully known, they include MMP13 and ADAMTS5 that participate in the degradation of the extracellular matrix. Exosomes are extracellular vesicles (EVs) that are produced in the endosomal compartment of most eukaryotic cells. They are natural messengers in cell−cell communications and can therefore be modified to deliver an intended cargo to specified cells. In this approach, the goal is to halt this inflammatory cycle by inserting specifically transfected cells in the osteoarthritic joint. The cells produce synthetically designed exosomes that contain miRNA-140, due to a genetic circuit which is activated by NF-kB. The exosomes are also designed to carry a Chondrocyte Affinity Peptide (CAP) in their membrane which guides them to deliver their cargo specifically towards chondrocytes. The whole process is supported by 2 computational models concerning the function of the Genetic Circuit and the exosomes production. In addition, the entrepreneurial prospect of the project was analyzed and brought to life by participating in a business accelerator and receiving a letter of intent from a European capital investor company while at the same time many human centered activities were held in order to make an impact that matters to the community, breaking down the narrow boundaries of a laboratory.

Materials & Methods

Genetic Circuit

As mentioned, osteoarthritis is characterized by an intense inflammatory cycle, in which the Transcription Factor(TF) NF-kB plays a central role. NF-kB is highly expressed in chondrocytes during the duration of the disease.

To exploit the abundance of NF-kB in the osteoarthritic chondrocytes, we chose the Genetic Circuit designed by Smole et al., since its activation happens through the binding of NF-kB. This circuit is composed of 5 parts:

- A Sensor that can detect inflammation and in particular the transcription factor NF-kB (even on small concentrations), to activate the secretion process of the proteins.
- An Amplifier that can amplify the sensor's signal.
- An Effector activated by the sensor and the amplifier, which initiates the transcription of the microRNA and Lamp2b
- A Thresholder which acts as a threshold to avoid overexpression of the effector.
- A Safety Switch which deactivates the genetic circuit, by administering a circuit inhibitor, doxycycline (Dox).

The goal when the circuit is activated, is to produce exosomes that will carry the mir-140 and eventually release it inside the Osteoarthritic Chondrocytes. For this reason, the mir-140 sequence should be incorporated in the Genetic Circuit. To ensure that the produced exosomes would specifically target the Chondrocytes, a Chondrocyte Affinity Peptide (CAP) should be fused to the membrane of the exosomes. To achieve that the CAP was fused to the Lamp-2b protein. The lysosome-associated membrane glycoprotein 2b (Lamp2b) is located in the membrane of exosomes. In our design, we use a modified lamp2b that includes a chondrocyte-affinity

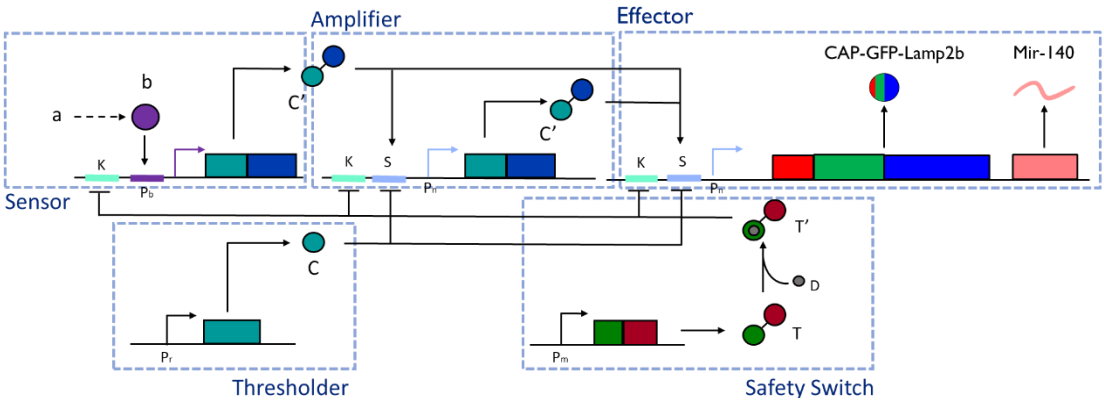

Figure 1. Genetic Circuit

peptide (CAP) at the N-terminus of lamp2b that will promote binding to the membrane of chondrocytes. To enable monitoring of the exosome production, we added a Green Florescent Protein (GFP) domain between the CAP and Lamp2b (Figure 1).

Cell type decision

A suitable cell candidate for our approach are Mesenchymal Stem Cells (MSCs) for two reasons: (a) they are already being studied as a potential therapy of osteoarthritis, as they can differentiate to chondrocytes and support cartilage regeneration and (b) they can produce exosomes.

However, MSCs are difficult to handle, let alone to transfect. Considering the limited time span available for the competition, we opted to work with HEK293T cells, which also have the same ability to produce exosomes while they are easier to cultivate and transfect. Still, the genetic circuit consists of 5 different parts, each one of them is incorporated into a separate plasmid. Which means that 5 different plasmids need to be transfected into a single cell, making the implementation of the experiments difficult.

Genetic Circuit Computational simulation

The genetic circuit was created, utilizing the CellDesigner v4.4 software, aiming to investigate the feasibility of our proposed implementation and develop an approach to tackle the "low transfection" problem the Wet Lab team encountered. It was used to determine how our results would have looked like, if we had included it in the Wet Lab experiments.

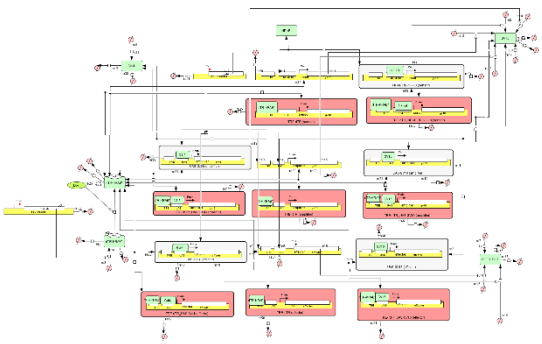

Figure 2:The Genetic Circuit Model in CellDesigner v4.4

How the Genetic Circuit Model works
The red and white rectangles are complexes created from the combination of genes, transcription factors and/or inhibiting proteins. The correct combination of a gene and a transcription factor enables the gene to be expressed and synthesize the corresponding protein.

Complexes that contain the activated rtTR-KRAB* protein are considered inactive but have a small synthesis rate due to leaky protein production.

The Thresholder and Repressor genes are not activated by a protein of the genetic circuit. They are constantly expressed utilizing transcription factors native to the cell, in order to maintain their restrictive function.

Finally, there is the degradation of the proteins into aminoacids (aa) and the disassembly of the gene array due to cell dilution.

After careful thought and examination of the simulation results, the conclusion was that no less than 4 plasmid constructs can be used and therefore the genetic circuit must be abandoned. This is how the genetic circuit model contributed to the aid of the Wet Lab, as well as the big picture of the proposed solution.

Plasmid Design

After the indications from the computational simulation of the Genetic Circuit that the genetic circuit would be adequately functional in our proposed implementation, we concluded to only test the Effector of the circuit and incorporate it into one plasmid. We had assembled all the sequences that we wanted on Geneious Prime. We wanted this part to be transfected in HEK293T cells and be expressed under constant expression in order to be able to measure the outcomes to which it leads to, meaning the exosomes production and the presence of miRNA-140 inside the exosomes.

Our genetic construct should be easily selected when amplifying it in bacteria (DH5alpha E.coli) and when transfected in HEK293T cells. We also wanted our insert to be expressed constantly. For these reasons, our plasmid backbone should have:

- Ampicillin-resistance Gene (for bacteria cultures)
- Kanamycin/Neomycine – resistance gene (for HEK293T selection)
- Origin of Replication
- CMV promoter
- PolyA_signal

According to these, pcDNA3 GFP LIC cloning vector (6D) was chosen and ordered from addgene.

Figure 3:Signal Peptide-Chondrocyte Affinity Peptide-GFP-Linker-Lamp-2b-mir140

Our insert has 2 main subparts. The miRNA-140 and the modified Lamp-2b protein. The sequence of precursor mir-140 was found on miRbase and added to the insert. The sequence of Lamp-2b was found on Uniprot and added to the insert in silico.

As Lamp-2b is a transmembrane protein though, it has a Signal-Peptide which needs to be in the beginning of the polypeptide chain, in order to achieve the membrane localization. Also, the chondrocyte Affinity Peptide needed to be in the outer surface of the modified protein in order to guide the produced exosomes to the chondrocytes. So the sequence was as follows.

SP Lamp2b – CAP – GFP- Lamp2b

The sequence of the precursor mir-140 followed (so that the mir-140 is cut off in the cells).

The backbone we chose already had a GFP gene, which we did not need as the modified Lamp-2b was equipped with a GFP by itself. So, the idea was that the plasmid would be double digested and the GFP gene would be excluded. For this purpose, as we had to ligate the insert inside the plasmid we had to choose wisely the restriction enzymes that we would use. The plasmid backbone already had a HindIII restriction site right before the GFP gene. Consequently, a HindIII restriction site was added to our insert as well. We needed to find a restriction site right in the end of the GFP gene in our plasmid backbone which would suit our experiments, while making sure that we don't encounter any problems in the ligation digestion with the insert and our vector. In the end of the GFP gene in our plasmid there was an XbaI restriction site. It is a commonly used enzyme, but the problem was that our insert already had an XbaI restriction site in it. But, a NheI restriction site was added in the end of our insert.

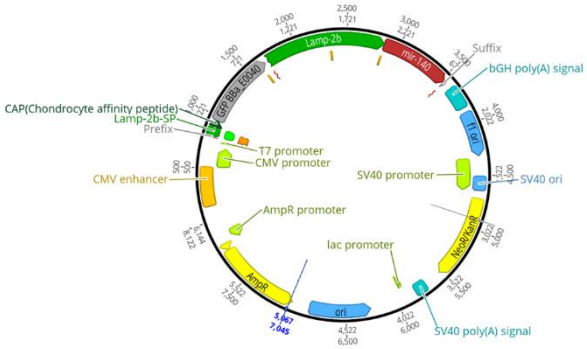

Figure 4: NOUS designed Plamsid

The digestions and ligation were performed in silico on Geneious to make sure that everything was well designed and we ended up with our whole plasmid designed.(Figure 4).

Wet lab experiments

The plasmid was ordered from Addgene in a bacterial stab and was amplified. The bacterial stab was streaked in LB-agar+Amp(100μg/ml) plates and incubated overnight at 37oC. Colonies were selected and inoculated in LB broth+Amp(100μg/ml) overnight at 37ºC, 200 rpm. The plasmid purification was performed using the QIAprep Spin Miniprep kit. The concentration of the isolated DNA was measured on Nanodrop.

The Gene Fragment that we received from IDT which was our insert was resuspended in 100μl of Water for Injection, as the company suggests.

5μl of the plasmid (550ng) were digested by HindIII and XbaI at 37oC, overnight, in a total reaction volume of 50μl. Gel extraction was performed using QIAquick Gel Extraction Kit to isolate the desired part of the vector.

42μl of the insert (250ng) were digested by HindIII and NheI at 37oC, overnight, in a total reaction volume of 50μl.

SpeedVac Vacuum Concentration was performed to the samples to increase the DNA concentration for the ligation.

After the Speedvac Vacuum Concentration ligation was performed approximately 50ng of vector (8μl) and 108ng (9μl) of insert were ligated with 2μl of T4 DNA Ligase buffer, 1 μl of T4 DNA ligase and 1μl of Water for injection reaching a total volume of 20μl and the desired ratio of 1:4 vector to insert, overnight at RT(25ºC).

DH5a E.coli bacteria were transformed with the ligation mix and cultured in LB-agar+Amp(100μg/ml) overnight at 37oC. Single colonies were selected, inoculated in LB broth+Amp(100μg/ml), cultured overnight at 37ºC, 200 rpm. The Plasmid DNA was purified using QIAprep Spin Miniprep kit, went under diagnostic digestions with HindIII and KpnI and a gel electrophoresis to visualize whether the genetic construct was assembled.

Exosome Production Model

Two models were created to calculate the levels of exosomes carrying miRNA to chondrocytes of the cartilage. The process of building each model is described below and is mainly based on literature data known to date.

Approach A

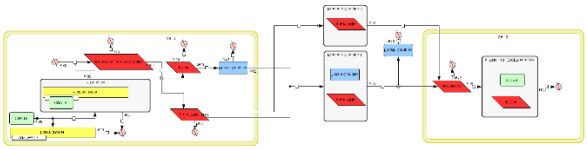

Figure 5: The Exosome Production Model in CellDesigner v4.4.

Assumptions:

a) The mRNA – pre miRNA always splits into its respective RNA molecules.
b) The production rate of exosomes is constant.
c) Exosomes have a constant endocytosis rate.

d) DICER concentration in a cell is high enough that the cell will not run out.

How the Model Works

The model begins with the plasmid of the transfected HEK-293 cells binding with the appropriate transcription factor via the CMV promoter.

The RNA complex is split into the mRNA molecule that codes the Lamp2b-CAP protein and the pre miRNA. The mRNA will then be translated and synthesize the Lamp2b-CAP protein. After the premiRNA & the Lamp2b-CAP membrane protein is synthesized, they need to be transferred to the produced exosomes.

Two kinds of exosomes can be synthesized. The first has both the premiRNA and the Lamp2b-CAP protein in its membrane. We will call this type of exosome a "CAP exosome". The second, only contains the premiRNA molecule. These two types of exosomes are produced at different rates. More accurately, it has been measured that the number of CAP exosomes is 2.42 larger than the ones who do not have it. Therefore, we assumed that the production rate of the CAP exosomes is 2.42 times the one of the regular exosomes.

Approach B

The second approach is developed in C++ code language using the Visual Code editor. It uses Ordinary Differential Equations (ODEs) to describe the experimental design.

Assumptions:

a) The cell is cultivated in ideal conditions, so exosome production is stable

b) The loading of mRNA into exosomes can be ignored because of its great length and because it lacks sequences which would lead to loading

c) The time interval between the premiRNA synthesis and the loading of the exosomes is too short to take into account

d) The premiRNA that is loaded into exosomes is divided evenly in them

e) The packaging of protein to exosome and end of translation happen simultaneously because of a short time interval that can be ignored.

f) The diffusion of the protein in the other membranes of the cell is ignored

How the Model Works

The model simulates the behavior of one cell, transfected with one plasmid. With simple calculations it shows the effect more cells would have, if the treatment was implemented in real life conditions.

There is an equation describing the rate of change of the concentration of every substance partaking in this process: miRNA, protein, exosomes, DNA, mRNA, miRNA in exosomes, protein loaded to exosomes, miRNA that reaches the cells.

Multiple functions described the rate of each substance while the current value of every element is stored. Setting 1 minute as a fraction of time, the current rate of change is added to the value that was calculated the previous moment. This equals the total concentration up to that moment.

All values and hypothesis were found and corroborated with literature findings, and all equations were constructed based on similar projects and data about basic biology functions.

The program successfully, and without delay, iterates these equations for a certain period of time, adjustable by the user. The model begins with the plasmid of the transfected HEK-293 cells binding with the appropriate transcription factor via the CMV promoter.

You can find our C++ code in executable form in our github page.

Aegle: Osteoarthritis Risk Factor Calculator

Current trends in health reflect an important contemporary shift towards citizen engagement for health and prevention, as opposed to mere disease management.

However, broadcasting generic health messages has limited effects unless there is a convincing, easily perceived and personally customized body of evidence to back healthy choices.

Based on this evidence we created a new OA risk calculator that uses medical evidence from recent high evidence level medical publications, AEGLE.

The NOUS OA Risk Calculator is expandable and can include new risk evidence when this is published. The tool is based on the CARRE health risk ontology and expands the CARRE health risk database (CARRE is an EU FP7 ICT project, Contract No. 611140).

In this project the risk factor conceptual model and ontology developed in the CARRE EU FP7 ICT project and available via the NCBO BioPortal is used.

Data Sources

The risk factors included in Aegle were retrieved from scientific publications found in PubMed.

8 publications produced 7 risk factors:

knee injury --> knee osteoarthritis

occupational exposure --> knee osteoarthritis

occupational exposure --> hip osteoarthritis

soccer playing --> knee osteoarthritis

metabolic syndrome --> knee osteoarthritis

hypertension --> knee osteoarthritis

smoking --> knee osteoarthritis

Software Design

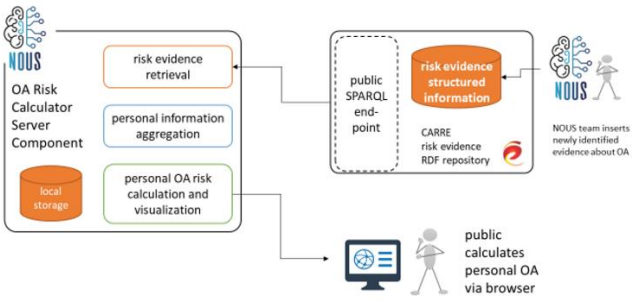

Figure 6: High level system architecture.

Adapted from CARRE D.2.2, 2014 https://www.carre-project.eu/project-info/deliverables-2/

The application is uploaded and ready for use in this link, while the software can be downloaded from our Github repository.

Results

Genetic Circuit Computational Simulation

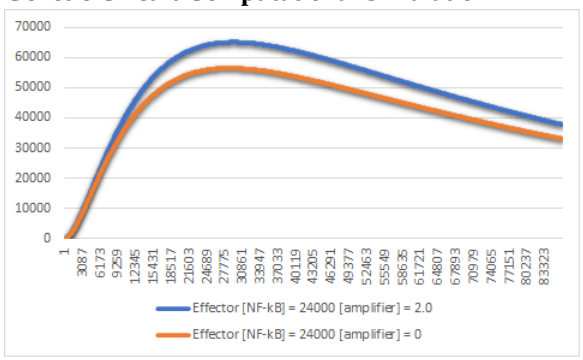

Figure 7:Effector expression levels in different values of the amplifier construct.

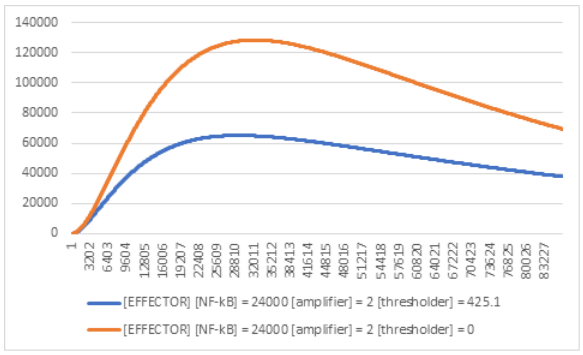

Figure 8: Effector expression levels in different values of the thresholder construct.

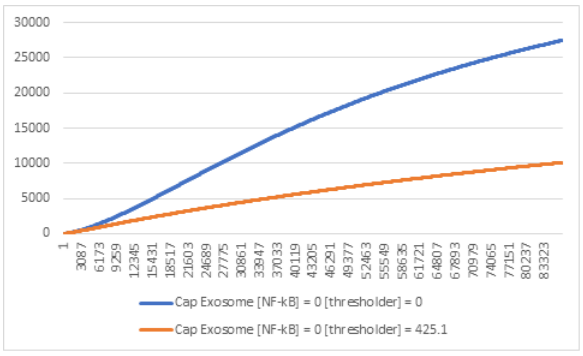

Figure 9: Effector expression levels in different values of Thresholder.

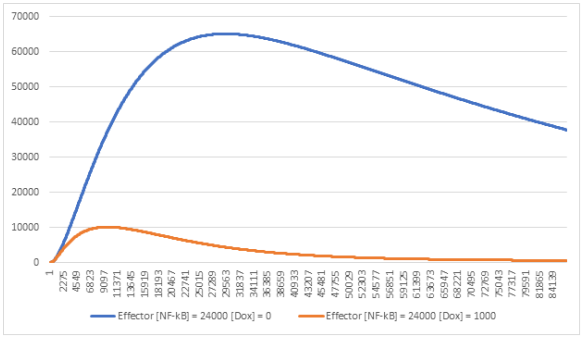

Figure 10: Effector expression levels in different values of Doxycycline (Dox)

As it is clear from Figures 2 and 7-10, the only exclusion that will not affect the correct function of the genetic circuit, is that of the Amplifier construct. Removing any other construct, will result in loss of function of the circuit and is therefore undesirable.

Exosome Production Model

Approach A

The simulation was executed for a time span of 24h.

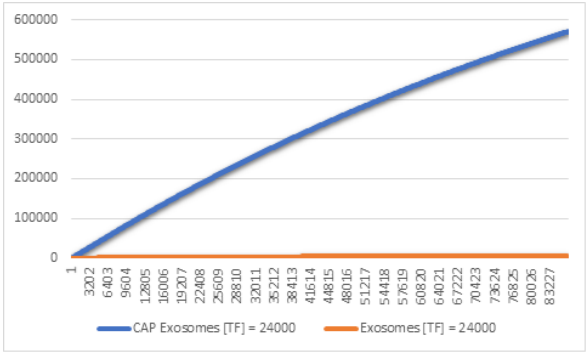

Figure 11: CAP and regular exosomes synthesized with [TF] = 24000

The model produces both CAP exosomes and regular exosomes. In 24h the amount of CAP exosomes synthesized is far greater than that of regular exosomes (Figure 11). This is a desired result, because it has been proven that CAP exosomes have higher affinity for chondrocytes, they get absorbed quickly by the cartilage cells and rarely end up entering the blood stream.

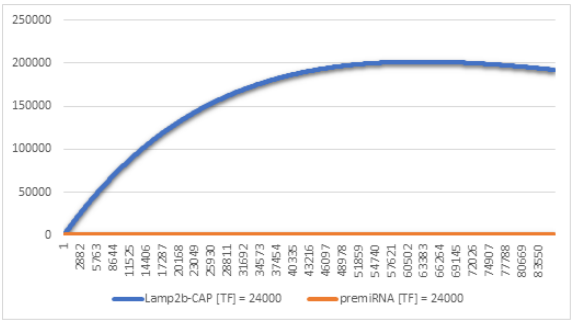

Figure 12: Lamp2b-CAP and premiRNA synthesis with [TF] = 24000

In Figure 12 there is indeed a higher amount of Lamp2b-CAP produced in the cells. This result makes sense, because when the gene is transcripted once, we get at most a single premiRNA molecule and an mRNA molecule which is likely translated more than once.

Approach B

For a total runtime of **2880 minutes (= 2 days)** the cell produced, **16153920** useful exosomes (that carry the protein and miRNA), the total concentration of miRNA in the cell is: **[miRNA] = 298,527 CPC**, a total concentration of miRNA- useful (miRNA that got into exosomes): **[miRNA useful] = 520,230,000 CPC** and a total concentration of protein **[protein] = 755,747 CPC**, were produced by one cell transfected with one plasmid.

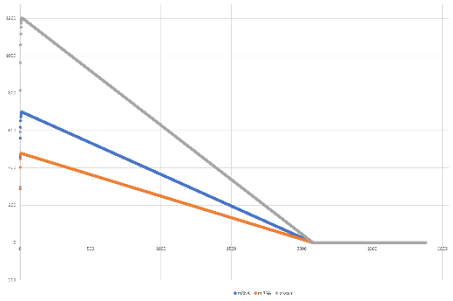

Figure 13: Graph of mRNA, miRNA and protein rate through time

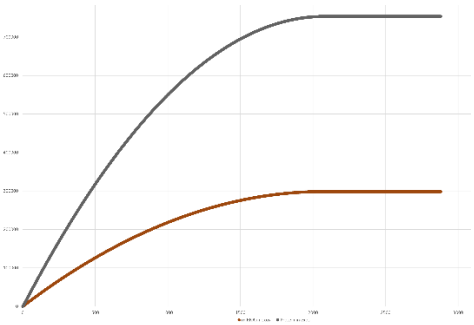

Figure 14: Graph of miRNA in exosomes and Protein in exosomes value through time.

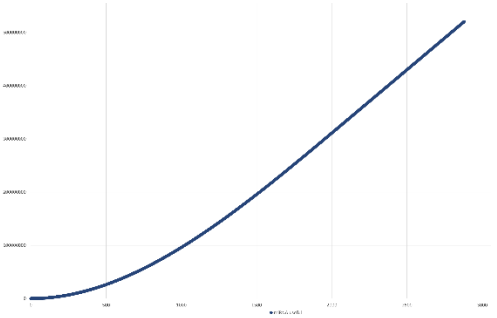

Figure 15: Graph of miRNA useful value through time.

The mean average of cells in a joint is 149,000,000 with a deviation of 46,000,000 cells. Along with the model results, how much concentration of miRNA would result in each cell can be calculated, considering that the exosomes would reach every cell equally. The average is **[miRNA] = 3.49148** copies per cell while using the deviation the best-case scenario is **[miRNA] = 5.05078** copies per cell and the worst-case scenario is **[miRNA]= 2.66785** copies per cell.

Total [miRNA useful] (CPC)	Average case (CPC)	Worst case (CPC)	Best case (CPC)
5.2023e+11	3491.48	2667.85	5050.78
1.04046e+12	6982.96	5335.7	10101.6
1.56069e+12	10474.4	8003.54	15152.3
2.08092e+12	13965.9	10671.4	20203.1
2.60115e+12	17457.4	13339.2	25253.9

Literature data has showed that exosomes with the guiding tag do not leave the target joint, while without, can be found in the whole organism.

For a hypothetical introduction of N cells transfected with the plasmid into a joint, the large-scale extent of idea is calculated, to simulate how it would work and what efficiency it would have in more real-life conditions and numbers. The calculations are similar to those above. The results are presented in the table below.

N cells	Total [miRNA] (CPC)	Total [protein] (CPC)
1000	2.98955e+08	7.55747e+08
2000	5.97911e+08	1.51149e+09
3000	8.96866e+08	2.26724e+09
4000	1.19582e+09	3.02299e+09
5000	1.49478e+09	3.77874e+09

Genetic Construct Assembly

15 selected colonies were run in a gel electrophoresis gel to spot the isolated DNA.

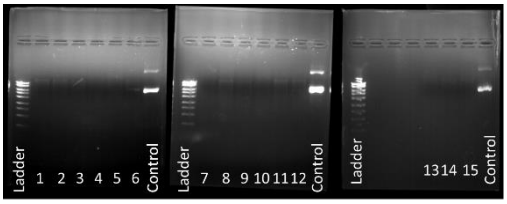

Figure 16: Electrophoresis Gel after plasmid purification from 15 colonies

Colonies 1, 8, 11 were further grown and went under diagnostic digestions with HindIII and KpnI.

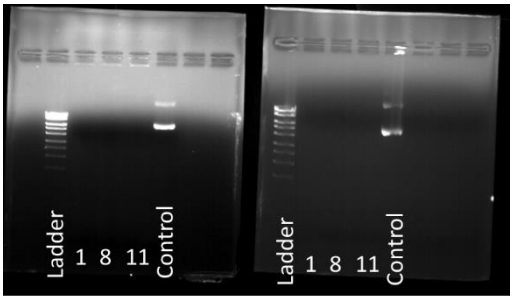

Figure 17: Diagnostic Digestions for colonies 1,8,11. HindIII digestions on the left. KpnI digestions on the right

No DNA is visible and therefore the Genetic Construct was not assembled.

Discussion

As it is obvious from the results, the genetic construct was not assembled. The bacteria survived in the LB even though Ampicillin was added in all the transformations that we tried. Theoretically, even in the case of an infection, the other bacteria should not be able to survive. Therefore, we should have chosen more wisely the kit we used or try some other kits for the plasmid purification step to isolate the plasmid DNA. To answer this question a plasmid backbone which allowed a blue/white screening at the stage of the colonies selection should be chosen. In that way, the colonies that were equipped with the insert could be discerned. In the case that there were no colonies with the insert some things concerning the ligation process should be reformed. The insert was ordered from IDT as a gene fragment. The problem with that is that a very low amount of DNA was available from the insert to use in the

ligations. The insert should have been ordered in a high copy plasmid which would enable a limitless amplification.

After the genetic construct assembly, some more experiments would follow. In particular, the construct would be transfected in HEK293T cells in an exosome-depleted FBS and Total Exosome Isolation reagent (TEI) would be used to isolate the produced exosomes. Afterwards, the miRNA content of the exosomes would be purified using Total Exosome RNA & Protein Isolation Kit. The miRNA-140 would be quantified by performing an RT-qPCR. Ideally, the isolated exosomes would be administered in an Osteoarthritic Chondrocytes culture and then Western Blot would be performed to quantify the effects in certain proteins such as the MMP-13 and ADAMTS-5.

Concerning the Approach A of the Exosome Production Model the results are promising and show that by introducing the therapy in its current design, cells will be affected in the whole target joint by a sufficient number of exosomes that will introduce a significant amount of miRNA in each cell since miRNA copies per cell typically vary between hundreds and 120000 copies per cell.

From literature it is known that chondrocytes in osteoarthritis have lower levels of miRNA140 and many positive effects of increasing it have already been documented by promoting cartilage formation and inhibiting its degeneration. Even though further correction of the model was not possible, due to lack of laboratory results, it shows that based on literature there is **sufficient therapeutic potential.**

In the future, all the computational models (Genetic Circuit, Exosome Production Model) can be used in tandem, to determine the number of transfected cells needed for each patient to effectively cure the cartilage. Also, by using different constant values, it can be used to generate useful information about other diseases or exosome delivery models. While Aegle can give useful indications for the patients concerning the possibility of suffering from Osteoarthritis in order to take the appropriate measures and prolong the joint health.

Conclusion

Although there are many promising indications emerging from the computational simulations, further experiments must be performed in order to practically prove the validity of our proposed treatment method. Also, a considerable progress was, and is to be made, concerning the research gap related to the therapeutic properties of the exosomes. Implementing Epione to the real world will be a long process, but all indications seem to point out the effectiveness and competitiveness of the team's approach.

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