The Effect of Near-Anoxic Conditions on KGF Production

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Abstract

The amount of oxygenation at sites is a key determinant of the outcome of healing because oxygen is crucial to the healing process and for resistance to infection. The non-healing skin ulcers that are formed from diabetic gangrene tend to be hypoxic; hypoxia is a condition not favorable to tissue repair. According to numerous studies, topical treatments of growth factors and peripheral blood mononuclear cells can help treat ulcers non-invasively in gangrene patients. It is posited that the growth factors promote angiogenesis, the physiological process by which new blood vessels form from pre-existing vessels. It is also a vital process in growth and development as well as in repair of damaged tissue. Furthermore, angiogenesis can render greater oxygen delivery to wounded areas, further enhancing growth and recovery to the areas suffering from chronic ischemia. In order to promote angiogenesis in gangrenous sites, and in turn treat the ulcers, growth factors such as fibroblast growth factor (FGF), vascular endothelial growth factor (VGF), and keratinocyte growth factor (KGF) can be produced in elevated quantities. To address the issue of inadequate oxygen levels during the healing process, we coupled the VHb gene promoter, which encodes for the Vitreoscilla hemoglobin molecule, to a downstream gene encoding KGF. The VHb promoter is most activated at an oxygen threshold of 2%, allowing for maximum transcription of the KGF gene and production of the growth factor in response to conditions of hypoxia. We also wanted to tested the production of another essential growth factor, FGF, under the influence of various constitutive promoters. This oxygen-sensing device is part of an array of growing applications targeted towards the promotion of angiogenesis as a means of healing in patients with severe, diabetic gangrene.

OBJECTIVES

- To apply concepts of synthetic biology to the design and implementation of an optimization device
- To connect oxygen deficiency and depletion (hypoxia) to growth factor production
- To spread awareness of synthetic biology and GMOs through outreach programs and social media
- To abide by safety procedures when handling potentially hazardous biological agents in the laboratory
- To familiarize team members with standard procedures used in molecular biology for the manipulation of plasmid DNA and microorganisms

Materials & Methods

Spread bacteria on agar plates
- Spread bacteria in a zigzag pattern. Bacteria containing the separate parts (KGF and VHb) were plated on agar with the corresponding antibiotic they had resistance to.
- Spread NEB-beta culture on agar plates for use in the transformation reaction
- Incubated plates overnight

Grew liquid cultures
- Created three 100 ug/ml Amp-LB solutions
- Swirled in colonies from their respective plates using an inoculating loop
- Incubated overnight in a shaker

Miniprep
- Centrifuged each liquid culture to pellet cells
- Resuspended each pellet in Buffer P1, Buffer P2, and Buffer N3 to isolate the DNA and pelleted out other cellular materials via centrifugation
- Passed the DNA-containing supernatants through a QIAprep column
- Eluted the DNA from each column using Buffer EB into fresh 1.5 ml centrifuge tubes
- Labeled each tube with the corresponding part it contained

Restriction Digest
- Transferred 10 ul of DNA from each tube into fresh tubes labeled VHb or KGF
- Added 2ul of 10x buffer to each tube
- To the VHb tube, added 1 µl each of EcoR1 & Spe1.
- To the KGF tube, added 1 µl each of EcoR1 & Xba1.
- Using a thermal cycler, incubated the restriction digests at 37 degrees Celsius for 30 minutes, then at 80 degrees Celsius for 20 minutes.

Ligation
- Thawed T4 DNA Ligase buffer at room temperature.
- Obtained one small tube (specific to the thermal cycler) and labeled it KGF + VHb.
- To the tube, added 5 µl of distilled water, 2 µl of T4 DNA Ligase Buffer, 1 µl of T4 DNA Ligase, and 6 µl of each of the digested parts.
- Gently mixed by pipetting up and down slowly
- Using a thermal cycler, incubated the restriction digests at 16 degrees Celsius for 30 minutes, then at 80 degrees Celsius for 20 minutes.

Transformation
- Labeled two small eppendorf tubes “Ex” and “Control”
- Pipetted 200 µl of CaCl2 into each eppendorf tube and placed the tubes on ice.
- Used a sterile inoculating loop to scrape up a patch of cells from the NEB-beta culture grown earlier and swirled the cells into cold CaCl2.
- Resuspended the cells by vortexing them briefly.
- Made one 5 µl aliquot of the ligated parts.
- Pipetted 75 µl of bacteria into the tube with the aliquot for the experimental and into a clean tube for the control.
- Incubated both tubes on ice for 5 minutes
- Heat shocked them for 90 seconds; moved them to a rack at room temperature.
- Pipetted experimental and control transformation mix to the tubes.
- Spread the mixes across their given plates.
- Incubated the petri dishes with the agar side up at 37 degrees Celsius overnight.

Testing and Results

We did not have the time and resources to test our transformed bacteria for their KGF production under anoxic and near-anoxic conditions. The following explains how we would test our bacteria and the expected outcomes of the testing.

Testing

We would use the bacterial cell culture procedure on OpenWetWare to create our liquid cultures. We would use the spectrophotometer to measure our cell culture density at OD 600 and will use this information to adjust the cell culture density so that all of the tubes we use for the different trials are at the same density.

To control the oxygen concentration of the liquid culture, we will use an oxygen controller to keep the oxygen level in the liquid medium at a certain level. We plan to set the chamber at 0, 1, 2, 3, 4, and 5 percent dissolved oxygen for the different oxygen levels. We will have multiple trials for each level. After two hours at a given oxygen level, the cultures, which will be in tubes, will be centrifuged and frozen at -80 degrees Celsius to stop protein expression and cell growth.

The frozen cells will be resuspended in lysis buffer and the suspension will be incubated at room temperature for 20 minutes. The suspension will then be sonicated to shear DNA and reduce turbidity. The suspension will then be centrifuged at 40,000 x g for 20 minutes at 4 degrees C.

We will quantify how much KGF is expressed under anoxic and hypoxic conditions using a human KGF ELISA kit, which uses a double antibody sandwich technique. Since growth factors are soluble, KGF and bFGF should be in the supernatant. We will pipette the supernatant out and into the wells of the ELISA plate. We will run the ELISA exactly according to the protocol given and will measure the amount of KGF captured and therefore expressed using a microplate reader at the given wavelength, which measures the color produced due to the enzymatic reaction.

Anticipated Results

We expected our bacteria to be able to respond to changes in oxygen levels by creating a maximum level of growth factor at a 2% oxygen threshold. When the oxygen levels are lower than 2%, we expected to observe a sharp decrease in production, and when oxygen levels are higher than 2%, we expected to observe a decrease in production in between the 2% and 3% threshold, with a linear increase in production for all thresholds above 3%. Oxygen thresholds below 2% should result in a sharp decrease in production of growth factor because the oxygen-dependent control mechanisms used by the promoter cause production to be maximally induced under hypoxic but not completely anaerobic conditions in E.coli. 2% is the ideal oxygen threshold according to the control mechanisms, but slightly higher thresholds should also produce significant yields.

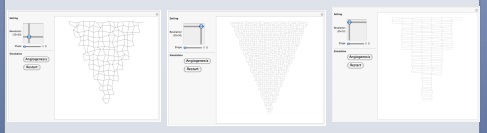
Modeling

We developed a program in Wolfram Mathematica that simulates the growth of vein angiogenesis in soft tissue. The simulation is made in such a way that the creation of new veins is impacted by proximity to other veins, and by the size of neighbor veins. Additionally, the creation of veins in paths that trace the shortest distances from a target point to the origin of the system ensures, the most efficient growth of a vascular system and results in a realistic simulation for natural growth.

```
buildGraph[MODEpairs_, rootNode_] := Module[
  {ruleList},

  ruleList = Map[{#[[1, 1]], #[[2, 1]]} -> EuclideanDistance[#[[1, 2]], #[[2, 2]]] &, MODEpairs];
  ruleList = Flatten[{#, Reverse@#[[1]] -> #[[2]]} & /@ ruleList, 1];
  Return@SparseArray[ruleList, {rootNode, rootNode}]
  ];
```

Every time the program is run, the lines along which veins can “grow” are randomly skewed, but kept nearly evenly spaced, as to provide a chaotic element that is present in all biological systems. The lines can also be modified so that the “resolution” of the simulated image is high or low, or the vascular system is stretched vertically or horizontally.



Due to realistic rules of vein growth (the system must reach every point in the grid, while using the least amount of “vascular tissue”), and the chaotic but fairly ordered paths along which the “veins” can grow, our simulation results in a realistic representation of true angiogenesis.

Conclusions

To further study the relationship between oxygen levels and growth factor production using the constructed part, it is recommended that bacteria transformed with this part be tested at actual wound sites, so as to determine whether topical treatment using this application is feasible. In addition, more research regarding the chassis organism must be done before transformation in order to prevent an unnecessary immune system reaction against the membrane of the bacteria. Since the iGEM team was not able to apportion the time appropriately this year, it is recommended for future years that more planning and foresight be involved before experimentation. A good rule of thumb is that 90% of results in science are futile, so to maximize the chances of yielding satisfactory results, it is essential that everything is timed accordingly, everyone is aware of and experienced with protocols, and that no short-cuts are taken to obtain results. Also, the iGEM team plans to make trouble-shooting protocols for next year to use time more efficiently in case one of the steps is not performed correctly.

We hope that our research will contribute to the development of “smart” wound dressings that accelerate healing and prevent infection. By combining synthetic biology with current research on the wound healing process and chronic wounds, we explored how bacteria could be used to produce growth factors in near-anoxic conditions. This result could be extremely useful for many facets of wound treatments. Of particular importance are chronic, hypoxic wounds such as gangrene. Research has shown that basic fibroblast growth factor (bFGF) has successfully treated gangrene in cases of both collagen disease and diabetes. A major cause of this success is bFGF’s promotion of angiogenesis in surrounding areas. Therefore, our bacteria could be useful in maximally producing angiogenesis-promoting growth factors in wounds that need it the most, i.e., in near-anoxic wounds. Our work could also be useful in acute wounds under near-anoxia, since it is important for growth factors to be produced early on in the healing process. In the future, these bacteria could be applied to many areas of medicine, ranging from acute wound healing to treatment of gangrene and other chronic, hypoxic wounds.

We were ultimately unsuccessful in our efforts to create a genetically engineered bacteria that would create growth factor in anaerobic conditions to aid in wound healing. However, our team gained powerful insight into our subject area. Our teacher and mentor, Dr. Burnett, once said that when you first start something, you should make as many mistakes as quickly as possible because that’s the best way to learn. We have certainly accomplished this and look forward to next year, when we can use what we learned to create a fantastic and impactful iGEM project!

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