Construction:

Three modules in our project are constructed separately and later assembled together to function as a whole in our project.

1. Target binding:

   In order to target colon tumor cells, colon tumor recognition peptide RPMrel is displayed on bacillus cell wall through lytC cell wall displaying system. lytC gene encodes N-acetylmuramoyl-L-alanine amidase, a type of autolysin on cell wall. It contains a 24 amino acid signaling peptide and three repetitive cell wall binding regions on its N terminus which facilitate the translocation and insertion of LytC to cell wall while the catalytic domain is located at C terminus. In our project, we only introduce the first 318 animo acid of LytC in our construct to signal the secretion and retention in cell wall. A helical linker with three repetitive sequence ,*EAAAK, is* fused to the C terminus of lytC, separating cell wall binding domain and RPMrel peptide in order to maintain the structure and function of both part. RPMrel peptide is then fused to the C terminus of linker, hanging on cell wall, pointing to extracellular environment, to recognize colon tumor.

   The expression of this fusion protein is driven by a high efficient constitutive promoter pVeg. An endogenous ribosome binding site *spoVG*, is linked to the downstream of pVEg promoter, stimulating translation.

   Two parts were built for this module. While in one part BBa_733007 RPMrel is fused to lytC cell wall binding system, another part (BBa_733008) in which lytC system is linked with a Flag tag after helical linker is constructed for the confirmation of correct expression and translocation of lytC cell wall binding system on cell wall.

   To build our parts, we utilized the biobrick BBa_K316037 built by 2010 imperial college of London iGEM team. Fusion protein was obtained by adding RPMrel or Flag tag coding sequence to the C terminus of helical linker through PCR.

   For detailed information, please refer to the page:…….

2. Antitumor molecule synthesis and secretion:

   In this module, two main parts with 5 intermediate parts were constructed. To synthesize and secrete active BMP2 from cytosol to extracellular environment, mature BMP2 is expressed with a secretory signal peptide fused to its N terminus. Composed of a positively charged lysine or arginine residue in N terminus, a hydrophobic H domain and type I Spase cleavage site A-X-A at its C terminus, sec-type signaling peptide can carry BMP2 to the cytoplasma membrane and cleaved at A-X-A site, releasing BMP2 to the extracellular environment. While BMP2 is secreted out and folded to its functional form, the retained signaling peptide in cytoplasma membrane will be degraded by SPPase.

   Since the secretion efficiency and cleavage accuracy for secreted BMP2 in prokaryotic system has never been investigated, we built two construct with BMP2 fused with two different signal peotide, ybdN and ydjM respectively based on previous studies in signallung peptides in B. subtilis.

   Considering the lack of protease in B. subtilis which used to modify BMP2 after translation, we directly amplify the DNA sequence encoding mature BMP2 from mouse genome. We further fuse signaling peptide to its C terminus and linked the fusion protein with constitutive promoter pVeg and a strong ribosome binding site for further characterization.

   Detailed construction ways are shown in diagrams and can be referred to information of each

biobrick.

In addition, due to the existence of EcoRI cutting site in BMP2 coding region, a point mutation is done to eliminate this illegal cutting site without changing the amino acid sequence.

3. Regulatory system and cell growth inhibitory device:

   To regulate the timing of BMP2 expression and the dosage of BMP2, two regulatory systems are built in this module. 9 biobricks are constructed to meet this need or to further engage in characterization.

   pTms promoter (BBa_K733001) which is used to driven the expression of antitoxin, YdcD, is constructed through Gibson assembly. With a very low transcription efficiency, the amount of antitoxin accumulated in bacteria can provided a threshold supporting certain amount of BMP2 expression while over-induction from xylose can also lead to the inhibition of growth when toxin outrange antitoxin.

   Xylose inducible promoter (BBa_733002) which used to controlling BMP2 synthesis time is obtained from integration plasmid 'pAX01' from BGSC. Two XbaI and one EcoRI cutting site are mutated in order to meet the requirement of bio-brick.

   Antitoxin (BBa_K733003) and toxin (BBa_K733004) coding gene which are further used in cell growth inhibition device are obtained from B. subtilis genome directly through PCR.

   In order to build the Cell growth inhibition device (BBa_K733012), toxin (BBa_K733004) is ligated after Xylose inducible promoter (BBa_733002) to generate biobrick (BBa_K733011) while antitoxin (BBa_K733003) is linked to the downstream of pTms promoter (BBa_K733001) to obtain BBa_K733010. Finally, BBa_K733011 and BBa_K733010 were assembled together to generate the cell growth inhibition device and further characterized.

   While characterizing the cell inhibition device as a whole, we further investigate the transcription efficiency of constitutive promoter pTms and xylose inducible promoter through the construction fo BBa_K733009 and BBa_K733018. Biobrick BBa_E0240, which including a ribosome binding site, a GFP reporter gene and double terminator, is ligated to the downstream of pTms and xylose inducible promoter respectively.

Although biobricks for each module were built separately, they will be assembled together in an integration plasmid pDG1661 and function together in our B. Hercules. To know detailed assembly method, please 'Assembly'.