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CRISPR and its potential application to Limb Regeneration : A Literature Review

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Abstract

In this article, we explore the fascinating potential of limb regeneration in humans, inspired by the remarkable abilities of salamanders and axolotls (*Ambystoma mexicanum*). These amphibians possess the extraordinary capacity to regenerate entire limbs- a process driven by complex biological mechanisms. By leveraging advancements in CRISPR-Cas9 technology, scientists are beginning to unravel the genetic pathways involved in this regenerative process, opening the door to the possibility of inducing similar regenerative capabilities in humans. We will delve into the theoretical framework, current research, and potential future applications of CRISPR in limb regeneration, drawing parallels to the natural regenerative processes observed in these remarkable creatures.

1 What is CRISPR-Cas9?

CRISPR-Cas is an adapted immunity mechanism prevalent in bacteria. It was discovered about three decades ago in *Escherichia Coli* and afterwards in other bacteria and archaea. CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. It is integrated in the DNA of the bacteria as repeated units between the non-homologous spacers that protect the host from attacks from foreign elements. Upon attack by foreign genetic elements, the CRISPR-Cas immune system is activated, wherein Cas proteins act as scissors to cut the foreign DNA into shorter fragments which are then integrated as spacers in the host DNA. In the defence system of the bacteria the CRISPR-Cas works when the Cas protein is bound to two different RNA molecules CRISPR RNA (crRNA), that guides the Cas enzyme to the DNA to cleave it, and a transactivating CRISPR RNA (tracr RNA), that binds to the Cas itself. This process ensures that the further attack by the foreign gene is recognized and the host is protected [26][16].

CRISPR-Cas system is presently most reliable and widely used mechanism for genome editing and engineering. The variant Cas9 of the Cas enzyme was

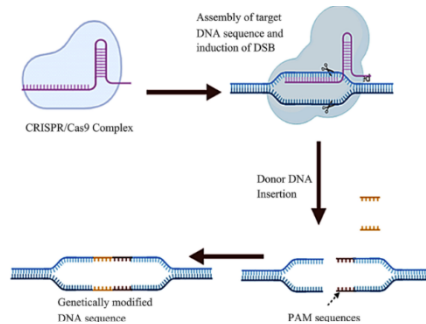


Figure 1: Mechanism of the CRISPR-Cas9 system [15]

obtained by isolating it in the bacterium called *Streptococcus pyogenes* (Sp-Cas9). The CRISPR-Cas9 tool is mainly composed of two components: guide RNA (gRNA) and Cas9. While using it as a tool for gene editing, the crRNA and tracrRNA are fused together to form a single RNA and is called the gRNA. It is thus made up of a programmable part consisting of exactly 20 nucleotides and a constant part. The programmable part of the gRNA inherits information about the target DNA as in its nucleotide sequence is complementary to that of the DNA. This ensures target specificity. The constant part of gRNA, on the other hand, are recognized by the Cas9 protein and they combine to form Cas9-gRNA complex. This complex identifies the PAM (Protospacer Adjacent Motif - 3 nucleotide of the form NGG, i.e., any nucleotide N followed by two Gs) in the target DNA. It unwinds the double-stranded DNA and identifies the complementary sequence to gRNA. Cas9 then employs its two nuclease domains, called RuvC and HNH, which independently cut the complementary strand and the non-complementary strand of the DNA, respectively. Afterwards, the gene repair process, called Homology Directed Repair is employed, which can potentially repair or even create a completely new gene changing its characteristic fundamentally. This process takes place when CRISPR-Cas9 is employed for a double stranded break in the DNA. This in turn causes the HDR cell to identify a homologous sequence and substitute it in the place of removed sequence.

The CRISPR-Cas9 gene editing tool has been widely employed in stem cell research [25], cancer treatment [28], advancement of cell therapies [22], mitochondrial diseases and gene replacement therapy [10] among various other potential treatments and uses. In this article we aim to study the potential possibility of using this genome-editing technique for limb regeneration by taking inspiration from the natural process that takes place in salamanders.

2 Limb Regeneration in Salamanders

Although tissue regeneration after injury is widely observed in many mammals, the regeneration of a wide range of tissues is observed in very few organisms

restricted to only a few special parts. In vertebrates, however, salamanders show remarkable capacity to regenerate a broad range of tissues, ranging from complete limbs to parts of the brain or heart. The main four stages of limb regeneration in salamanders after the amputation are wound healing, formation of blastema, blastema patterning, and lastly cell differentiation [11]. Immediately after the amputation, the first step is to stop the bleeding which is achieved by the formation of fibrin clot followed by the migration of non-proliferative keratinocytes, characterizing the wound healing step [3]. The wound healing process is accompanied by epithelialization, wherein the cells migrate to form a wound epithelium. Followed by the formation of wound epithelium, keratinocytes begin to proliferate leading to the formation of Apical Epithelial Cap (AEC), that in turn produces molecules essential for the formation of blastema cells. It must be noted that for an unhindered regeneration process, a scar free wound healing is necessary which essentially involves regulation of gene expression achieved by differentiable cells which also produce proteins that leads to the formation of blastema cells, that eventually differentiate to form the necessary cells to regenerate the lost limb.

An important factor in the process of wound healing is TGF- β 1. It stands for Transforming Growth Factor Beta 1, which is responsible for cell growth division, tissue repair, and most importantly it influences cell differentiation, which is one of the key processes in wound healing for limb regeneration in salamanders. Interestingly, this gene is found in both salamanders and human beings, but slight differences such as

3 Difference in TGF- β 1 in Humans And Salamanders

The TGF- β 1 gene although present in both human beings and salamanders, plays different roles in both the cases. In salamanders TGF β -1 is used to maintain a regenerative, rather than fibrotic environment [12] due to its cellular response causing the activation of progenitor cells and regenerative fibroblasts. They can effectively regenerate without the excessive formation of scar tissue, while in contrast in humans it often leads to fibrosis, due to proliferation of cells, causing them to produce a large amount of collagen, which in turn forms scars, hence forbidding the formation of a blastocyst [21]. In order to understand this difference in function of the gene in different organisms, we study the difference in structure of the TGF- β 1 protein in the two cases [13]-

1. **Segment 1:** Query sequence to position 57 Both sequences have similar patterns of loops (L) and helices (H). The DSSP (Define Secondary Structure of Proteins) annotations match with the query and subject sequences, showing conserved structural features.
2. **Segment 2:** Position 117 to position 170 Similar patterns of alternating helices (H) and loops (L) are present in both sequences. Both show consistent structural elements.

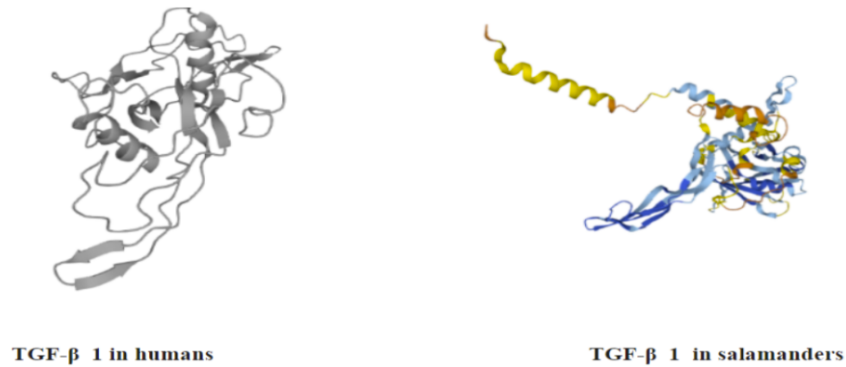


Figure 2: Structure of TGF- β 1 protein in humans (left) and salamanders (right)[2]

3. **Segment 3:** Position 170 to position 228 There are long stretches of helices (H) and extended regions (E) in both sequences, indicating conserved structural motifs.
4. **Segment 4:** Position 228 to position 286 Both sequences exhibit similar patterns of extended regions (E) and loops (L), with some regions of helices (H), suggesting shared structural domains.
5. **Segment 5:** Position 286 to position 341 Similar patterns of loops (L) and helices (H) are evident in both sequences, indicating conserved secondary structures.
6. **Segment 6:** Position 341 to end of sequence Extensive regions of helices (H) and extended regions (E) are present in both sequences, showing high similarity in structural arrangement.

4 CRISPR-Cas9 for editing TGF- β 1 in Homo Sapiens

1. The most significant part of gene editing using CRISPR-Cas9 is the guide RNA. In order to modify the TGF β -1 gene in homo sapiens, it is imperative to create a gRNA such that it can form a complex with Cas9 protein to execute the relevant gene editing using the process of homologous repair, inspired from the relevant sequence in ambystoma mexicanum.

Similarities between the TGF- β 1 structure in homo sapiens and ambystoma mexicanum: We first must look at similarities between the gene sequence of homo sapiens (humans) and ambystoma mexicanum (axolotls), to form the custom guide RNA which should essentially allow

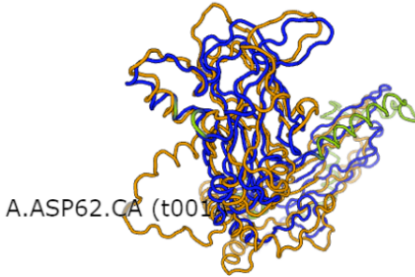


Figure 3: Combination of TGF β -1 sequence in Ambystoma Mexicanum and Homo Sapiens [14]

us to remove the unneeded sequence i.e. The TGF- β 1 sequence which results into fibrosis in humans, by using Cas9 and fixing it with the needed sequence found in salamanders using a process of homologous repair [7].

Using this information we can use the similar sequence on a guide RNA and cut the gene which controls fibrogenesis of wounds and instead use the needed RNA sequence of salamanders which leads to differentiation and blastocyst formation.

2. **Design gRNA:** Ensure the gRNA is highly specific [1] by using a unique sequence which is not similar to any other sequence in the genome (i.e., only a certain significant part is altered) to minimize off-target potential such as cancers [20]. We can also developed a high-throughput genome-wide CRISPR/Cas9 screening platform to develop the correct transforming growth factor (TGF)- β -induced gRNA [27]. The specific part of the TGF BETA-1 gene that controls the fibrosis/regenerative properties in both species is called Latency-Associated Peptide (LAP) [24]. Both homosapiens and Ambystoma mexicanum contain this. In homosapiens this causes fibrosis of a particular wound while in Ambystoma mexicanum it causes the formation of blastocyst leading to regeneration. In this specific process we can use LAP from an axolotl sequence, combined with Cas9 to alter the sequence found in humans.
3. **Cloning gRNA:** Synthesize the gRNA oligonucleotides which consists of crRNA and tracrRNA [23]. Clone the gRNA into a CRISPR-Cas9 expression plasmid using standard molecular cloning techniques. Initially insert the above mentioned gRNA into the plasmid vector (small, circular DNA molecule used to carry and express genes in cells). It then can be into the body by first removing the[5] blood stem cells from the body, modifying with CRISPR to create the gRNA and then combining it back into the body via gel, creams, food or drinks, even skin grafts and injections [17]

can be used which was seen during human trial conducted on people with a rare hereditary blindness disorder by Dr. Chan, thought in the aforementioned case the efficiency of the experiment was rather low with only two out of the 7 patients claiming to have gotten a positive response thus, we can not determine the efficiency of the gRNA without suitable trials or even human trial.

4. **Cell Transfection:** Culture target cells i.e., the cells of TGF-BETA1 [19] transfect cells with the CRISPR-Cas9 plasmid using a transfection reagent of any type of related genetic material or electroporation [18] which creates temporary pores in the cell membrane allowing nucleic acid to enter the cell, thus allowing the variation of the cell: This can be done via transfection (elaborated in greater detail above) or in the case of using purified CAS9 it will be combined in-vivo before entering the cells. Afterwards we must assess what occurs to the cells and check for negative side effects,[17] which is elaborated more in the last section on “Precautions”.

5 Commonly Asked Questions

1. **Is it feasible to unfold the protein to correct the amino acid sequence, as the new sequence may correspond to a different protein structure?** The protein folding does not need to be reversed as CRISPR already indirectly allows changes in protein sequences since the DNA sequence determines the amino acid sequence of proteins
2. **Can we add on the sequence from salamander to humans instead of removing it then adding it.** No, due to the nature of CRISPR as well as the nature of RNA for mutation of a gene the specific sequence must be removed for Homology Directed Repair to occur.
3. **Is it feasible for proteins?** While CRISPR does not directly modify proteins, it exerts its effects on them by altering the genes that encode them. By editing the gene responsible for a particular protein, CRISPR can induce changes in the protein’s amino acid sequence. This modification in the genetic code ultimately leads to the production of a protein with altered structure and function, highlighting CRISPR’s powerful role in gene editing and its downstream impact on protein expression and cellular processes.
4. **Can it be done ex vivo (i.e., in labs)** Yes, it can be done ex vivo but results would be better, and easier if it is done in vivo. It would also save time when it comes to physio and can avoid side effects such as phantom pain, and have a minute chance of rejection in comparison with doing it ex vivo.

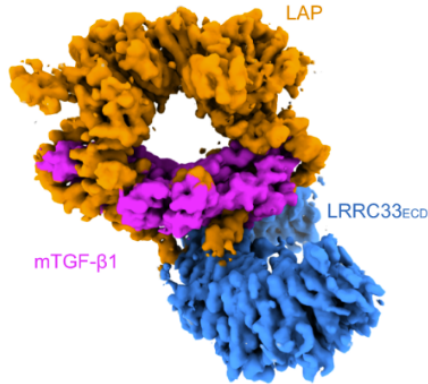


Figure 4: A Sequence schematic diagrams of L-TGF- β 1 and LRRC33. Chromatic bricks represent the protein regions used in our study, whereas gray bricks represent regions that are not included. Latency-associated peptide (LAP), mature TGF- β 1 (mTGF- β 1), and the extracellular domain of LRRC33 (LRRC33ECD) are colored in orange, magenta, and blue, respectively [9].

6 Conclusion

In this paper, we study the process of limb regeneration in ambystoma mexicanum and the mechanism of the potential for human beings. Specifically we compare the structure of TGF- β -1 gene that is present in both human sapiens and ambystoma mexicanum. On comparing the structure in the two cases, we discovered that the gene is different in its structure and therefore it performs different functions in homo sapiens and ambystoma mexicanum. We then studied the mechanism of gene editing tool CRIPR-Cas9 and proposed a general method which can be further specified to alter the gene sequence of TGF- β -1 in human beings. Although, theoretically possible, it must be noted that a further deeper study is required for its pragmatic employment. It must also be noted that this study is a result of pure inquiry into the nature of the process of limb regeneration and subsequent processes that are involved in it, and should in no way be taken as a method proposed to execute the practice as this may result in severe health hazards like artificially formed aggressive cancers [8], hemorrhages [6] due to the larger surface area of an open wound in homo sapiens in comparison to an open wound in ambystoma mexicanum which can easily be clotted using a “plasma clot” within a few hours to a day according to the size of the wound, while in homo-sapiens it can take up to 4 week to close a wound (i.e., an amputated leg) [4]. Another notable risk is, any organism which has modified genes will not only contain it within one’s body, but it will also be passed down to its progeny much like a genetic disorder though in short, replacing the LAP gene is extremely risky it is theoretically possible and controlled rigorous laboratory testing must be done this infer the consequence as well as

the ability of the newly modified gene.

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