The effect of miR-21 on wound healing in mammalian systems

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Introduction

Limb Regeneration – Many species throughout the animal kingdom have the capacity of some form of regeneration[1]. Mammals, including humans, only have the capability of regenerating distal digit tips[2], whereas organisms from other phyla such as zebrafish (*Danio rerio*), bichir (*Polypterus senegalus*) and axolotl (*Ambystoma mexicanum*) can regenerate whole fins or limbs[1,3]. These animals have the capability to form a blastema, or a mass of cells that may develop into various body parts[4], as an adult, though the genetic basis for adult blastema formation has not yet been elucidated. The possibility that adult humans might have similar capability is of particular interest as the field of regenerative medicine grows.

miRNA and Regeneration – miRNAs, or microRNAs, are noncoding RNA sequences that have the capability to downregulate gene expression by binding the 3'UTR of gene transcripts, preventing their translation. These highly conserved sequences typically act on many targets, aiding in gene regulation[5]. Five upregulated and five downregulated miRNAs have commonly been found to be involved in blastema formation in three species capable of regeneration: zebrafish, bichir and axolotl. Three of these miRNA (miR-21, miR-181c and miR-31) are upregulated in concert in these species, with miR-21 the most expressed and most upregulated during blastema formation[3]. The mir-21 ortholog in humans, hsa-mir-21, has been linked with many types of cancer[6]. A knockdown of miR-21 in human breast cancer cell lines inhibits the cell's proliferation, migration and growth[6], suggesting miR-21 as a possible driving force in limb regeneration. The human miR-21 ortholog is so similar to that of axolotls that LNAs against hsa-mir-21 can be used to detect the axolotl orthologs in Northern blot assays. Hsa-mir-21 may even be identical

to axolotl miR-21[7]. The direct impact of miR-21 in wound healing in mammals has not yet been studied. This experiment aims to investigate further applications of miR-21 in humans, perhaps positive ones in wound healing.

Relevance and Impact

Skin-piercing wounds are often associated with high morbidity and mortality, especially in potentially immunocompromised patients such as the elderly. Infection of surgical wounds post-operation (surgical site infection, or SSI) results in longer and more likely hospitalizations, as well as higher mortality[8]. A potential way to limit incidence of SSI would be to improve the wound healing without the use of antibiotics which help the spread of antibiotic resistance[9]. Through using microRNA product to enhance wound healing, assuming a positive correlation between the upregulation of various miRNAs and quality/timing of wound healing, there could be a viable option to limit SSI through an alternative pathway to antibiotics. The quicker and better wounds heal, the smaller the chance of the patient developing an infection or a reliance on pharmaceuticals is, opening up many resources both socially and economically.

Establishing a relationship between these miRNA and positive wound healing would open more doors in miRNA therapy research. Establishing a positive correlation between limb regeneration in other species and healing in humans could show that humans still have the capability for regeneration and would lead to further research, perhaps into the exact pathways of the 10 miRNA that are found to be differentially expressed. More specifically, the gene targets of miR-21, miR181c and miR31 could be determined, and through these the gene pathways that trigger blastema formation could be discovered.

Hypothesis and Prediction

Research Question: Can miR-21, commonly upregulated and present in regeneration sites in species that are capable of limb regeneration (zebrafish, axolotls…), aid in healing of cutaneous wounds in humans (with a model system of mice) such as lacerations and incisions by decreasing healing time?

Hypothesis: MiR-21 is sufficient to affect wound healing in mouse cutaneous wounds. This is based on known facts that miR-21 is significantly and commonly upregulated in axolotl, bichir and zebrafish blastema sites, indicating that it is strongly involved in these organisms' regeneration. miR-21 also has been shown to be active and upregulated in human cancer cells, implying that it may be associated with cell growth and proliferation, though uncontrolled. Since miR-21 is linked in several ways to cell growth, the addition of it would encourage cell growth.

Prediction: Cell proliferation and growth around mouse wounds will increase, leading to shorter time of wound healing compared to the control population, and greater tensile strength of the wound compared to wild type mice. MiR-21 knockdown through RNAi will lead to longer wound healing time compared to the control population.

Experimental Approach

Part I – miR-21 isolation/RNAi creation: To be able to insert or knock down miR-21 in vivo, miR-21 oligonucleotide synthetic product must be produced, along with dsRNA that will allow for RNAi. By using miR-21 consensus sequences determined by Holman et. al[7], forward and reverse primers are created in order to amplify miR-21 RNA using RT-PCR. RNA is extracted from axolotl blastemas as described by Holman et. al[7-], and RT-PCR performed following the Wellcome Trust Sanger Institute RT-PCR protocol[10]. The resulting miR-21 DNA transcript is then translated in vitro using Invitrogen's Retic Lysate IVT Kit, leaving miR-21 in a functional mRNA transcript form (Fig 1). The transcript is isolated and purified, then lyophilized to a dry format. RNAi is created using chemical

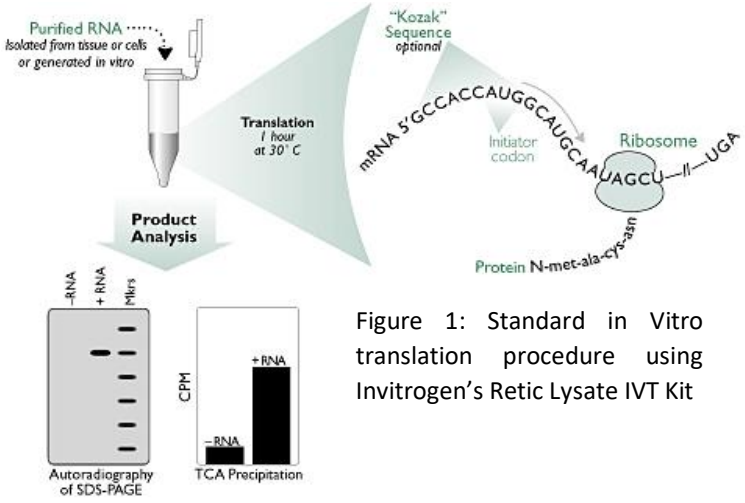

Figure 1: Standard in Vitro translation procedure using Invitrogen's Retic Lysate IVT Kit

synthesis to prepare siRNAs in the form of dsRNA, which would be cleaved in the cell to form siRNAs that will target to miR-21 (Fig. 2). These miRNAs complement a 23bp consensus sequence in mammalian miR-21 and are prepared in vitro using Ambion's *Silencer*TM siRNA creation techniques. A positive *Silencer*TM GAPDH siRNA control and a negative *Silencer*TM Negative Control #1 siRNA are also obtained.

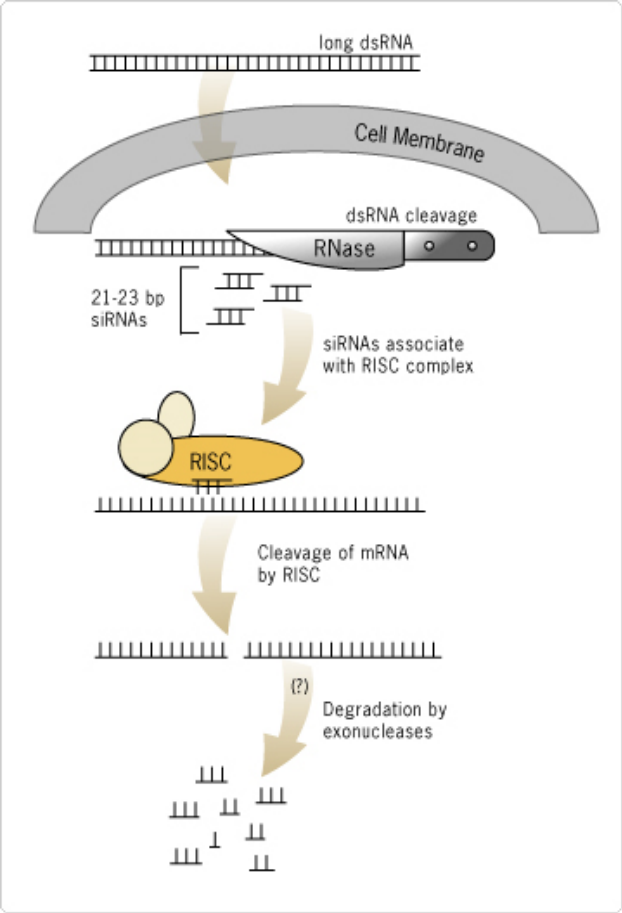

Figure 2: An overview of RNA Interference (RNAi). dsRNA is used to transport into the cell and is then cleaved into siRNA. siRNA then combine with the RISC complex to target and degrade mRNAs.

Part II – Wound Creation and miR-21 treatment:

Mus musculus is chosen as the model system for this experiment because mice are fairly genetically similar to humans due to the homology of their genomes, and because the development of vertebrates is especially conserved in mammalian lineages[11]. Three treatment groups of *Mus musculus* are created, all wild-type mice with no significant differences in genome. To determine how much miR-21 was naturally active in wound healing, some mice are given 3 cm long incisions under anaesthesia, following the procedure outlined in Mulisa, Asres and Engidawork[12] (Fig. 3). RNA from the incision site is extracted and quantified at 6, 12 and 24 hours post-incision, and RT-PCR performed using to quantitatively determine the amount of miR-21 present in wild type wound healing. The first experimental group (with increased miR-21) would be given sufficient miR-21 to show a 10-fold increase in cells as compared to WT concentrations. Wounds are created using the same method as before, and the excess miR-21 administered in a TRIS solution by injection into the wound site at 6, 12 and 24 hours post-incision. For the second experimental group, the RNAi product is administered in

sufficient quantity to inactivate all miR-21 at 6, 12 and 24 hours post-incision, based on WT mir-21 concentrations. Another control group also receives an injection at 6, 12 and 24 hours of just TRIS solution. A positive siRNA control group is given injections at the same time intervals of *Silencer*™ GAPDH siRNA, and the results quantified after 7 days using qRT-PCR. A negative siRNA control group is injected at the same time intervals with *Silencer*™ Negative Control #1 siRNA, and similarly quantified. Wound healing time for all mice is measured in days, with a wound considered fully healed when no scabbing exists. Tensile strength of the wound on day 7 of healing is tested using the protocol outlined in Mulisa, Asres and Engidawork[12] for all mice to determine quality of wound healing (Fig. 3).

The controls in this experiment serve several purposes. The first is to determine the concentration of miR-21 naturally occurring in cells to allow us to increase the amount present, creating a miR-21 addition experimental group with miR-21 concentrations based on biological data. The second purpose is to provide a baseline to which we could compare the wound healing of mice in each experimental group. It also serves to show that the TRIS injection does not have any significant effect on wound healing, and that it would not impact our data. The RNAi negative control using *Silencer*™ Negative Control #1 siRNA is used to control for effects of using siRNA in the cell, and the *Silencer*™ GAPDH siRNA is used as a positive control to ensure siRNA function. Its effects are viewed by using qRT-PCR to follow GAPDH expression in the cell[13]

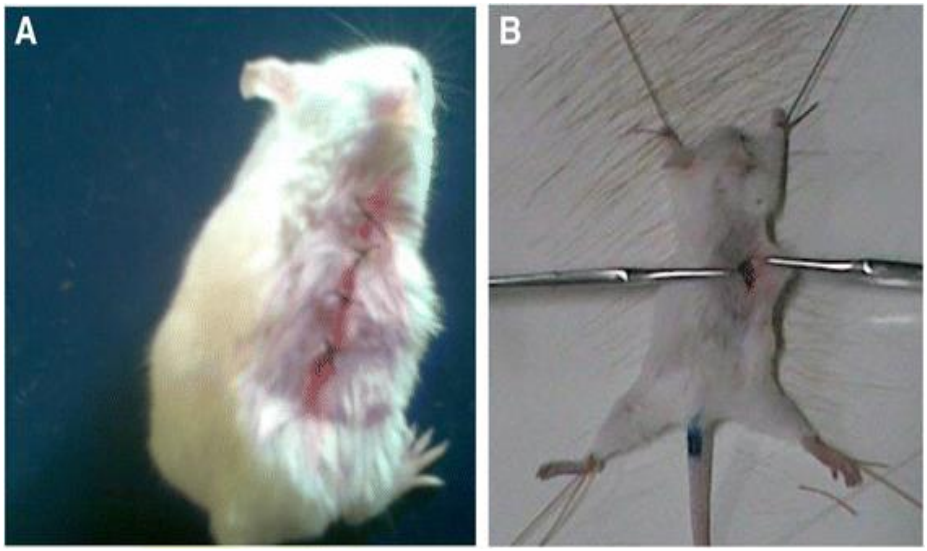

Figure 3: Incision (a) and tensile strength measurement (b) in mice. The incision was created under anesthesia. The tensile strength was measured after takedown using a continuous water flow technique and two forceps.

Possible Outcomes

1) No significant increase in wound healing (tensile strength or time)

Observations: All treatment groups do not differ significantly in healing times or wound tensile strengths.

Conclusions: MiR-21 doesn't have any significant impact on wound healing, and therefore is not necessary for the process. It can be inferred then that miR-21 does not play a role in mammalian wound healing, assuming that other miRNAs cannot compensate for or be supressed by miR-21. This inference fits with the current understanding that miR-21 is cancer linked, as opposed to functioning in native cells that are tightly regulated. However, this result would be surprising as in other organisms such as axolotls miR-21 was very upregulated in blastemas. Further studies could investigate the role miR-21 plays in axolotl limb regeneration as well as see how the regeneration process differs from mammalian wound healing.

2) Increase in wound healing with miR-21 between control and +mir-21

Observations: The experimental group with addition of miR-21 is observed to have significantly lower healing times and/or higher tensile strength as opposed to both the control group and the knockdown group. The knockdown group is observed to have significantly longer healing times and/or lower tensile strength than that of the control group.

Conclusions: It can be concluded that miR-21 has a positive effect on wound healing. It can then be inferred that miR-21 acts to encourage cell growth and proliferation and does this by functioning quantitatively in some pathway, where an increase in miR-21 can be directly linked to better wound healing. This inference fits with the idea that the upregulation of miR-21 in blastemas is related to its regenerative qualities and begins to elucidate how exactly miR-21 functions in pathways. It would show that miR-21 is responsible for the cell proliferation and growth in blastemas and would also show that it

does have the ability to encourage cell growth in humans as well, outside of oncogenic circumstances. Further research could elucidate the exact pathway that miR-21 functions in, showing what it works to inactivate in this context to allow for such proliferation and growth.

3) Decrease in wound healing with RNAi knockdown

Observations: The experimental group with a miR-21 RNAi knockdown is observed to have either longer healing times or less tensile strength in their wounds, or both, as compared to both the control group. There is no significant difference in both categories of wound healing between the control group and additional miR-21 experimental group.

Conclusions: MiR-21 is necessary for wound healing, but the quantity in wild type is sufficient for optimal wound healing, and the addition of miR-21 has no effect. It can be inferred that presence of miR-21 in any amount is enough to promote wound healing, and that miR-21 acts in a more qualitative than quantitative manner. This inference fits with the idea that miR-21 is active in wound sites. Further studies could investigate why this is, and how miR-21 acts to promote wound healing, and its target genes in this circumstance, as opposed to its function in oncogenic cells.

Other potential results

There are many other possible results, most of which would not be expected given previous data. For example, miRNA addition could *decrease* wound healing/increase duration, leading to the conclusion that there is perhaps a dosage balance of mir-21. Another result could be that the addition of mir-21 could cause oncogenic growth in the area, as mir-21 in humans has been linked to cancer. This would lead to the conclusion that mir-21 has a serious negative effect on wound healing, and the inference that mir-21 may in fact play an important part in cancer development. This in turn could lead to further research on the influence of mir-21 in cancer development, showing how scientific results in one study may in fact provide insight into separate issues.

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Figure Sources

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