Effect of microRNA miR-21 on wound healing in mammalian systems

Biol 463 – Gene Regulation in Development

Introduction

Limb Regeneration – Many species throughout the animal kingdom have the capacity of some form of regeneration to varying extents[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*) are able to 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 humans somehow have the capability for adult blastema formation but has somehow silenced it 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 to the 3'UTR of gene transcripts, preventing their translation. They're highly conserved sequences, and commonly act on many targets, aiding in gene expression regulation[5]. Five commonly upregulated and five commonly downregulated miRNAs have 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) were commonly upregulated in all three organisms, and not just a subset of the three. Of these three, miR-21 was found to be 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, and it has been shown that a knockdown of miR-21 in human breast cancer cell lines inhibits the cell's proliferation, migration and growth[6], indicating that it plays a role in encouraging cell growth and proliferation, even to the point of uncontrolled growth. This characteristic makes miR-21 a possible driving force in limb regeneration, as it appears to activate cells to grow, and is common to several organisms capable of limb regeneration. The human miR-21 ortholog is so similar to that of axolotls that using LNAs against has-miR21 can be used to detect the axolotl orthologs in Northern blot assays and may even identical to that of axolotl miR-21[7]. To investigate if miR-21 is active in mammalian wound healing, a RNAi knockdown could be constructed. As of yet, the direct impact of miR-21 in wound healing in mammals has not been studied, only its oncogenic effects. This experiment aims to begin to discover 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 process in some way, 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. This in turn could lead to less reliance on pharmaceutical treatments of wound healing, instead favouring a more natural direction. The quicker and better wounds heal, the chance of the patient developing an infection or a reliance on pharmaceuticals is, as well as the duration of time spent in hospital recovering, opening up many resources both socially and economically.

More science-specific, 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 such as zebrafish and axolotls and healing in humans could show that humans still have the capability for regeneration, just that it somehow needs to be reactivated would lead to further research, perhaps into the exact pathways of the 10 miRNA that are found to be differentially expressed, as well as if they can be traced back to a specific source in the human genome. More specifically, the gene targets of miR21, miR181c and miR31 could be determined, through that 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 was significantly and commonly upregulated in axolotl, bichir and zebrafish blastema sites, indicating that it is strongly involved in the healing process in these organisms. miR-21 also has been shown to be active and upregulated in human cancer cells, showing that it may be associated with cell growth and proliferation, though that may be in a more uncontrolled manner. 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 were created in order to amplify miR-21 RNA using RT-PCR. RNA was extracted from axolotl blastemas as described by Holman et. al[7-], and RT-PCR then performed using the two primers and the RNA extract, following the Wellcome Trust Sanger Institute RT-PCR protocol[10]. The resulting miR-21 DNA transcript was then translated in vitro using Invitrogen's Retic Lysate IVT Kit, leaving miR-21 in a functional mRNA transcript form. The transcript was isolated and purified, then lyophilized to a dry format.

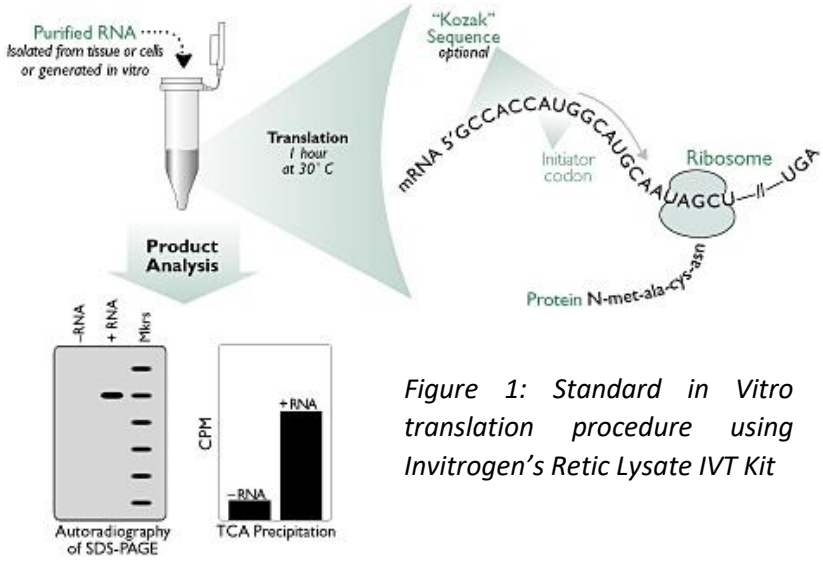

Figure 1: Standard in Vitro translation procedure using Invitrogen's Retic Lysate IVT Kit

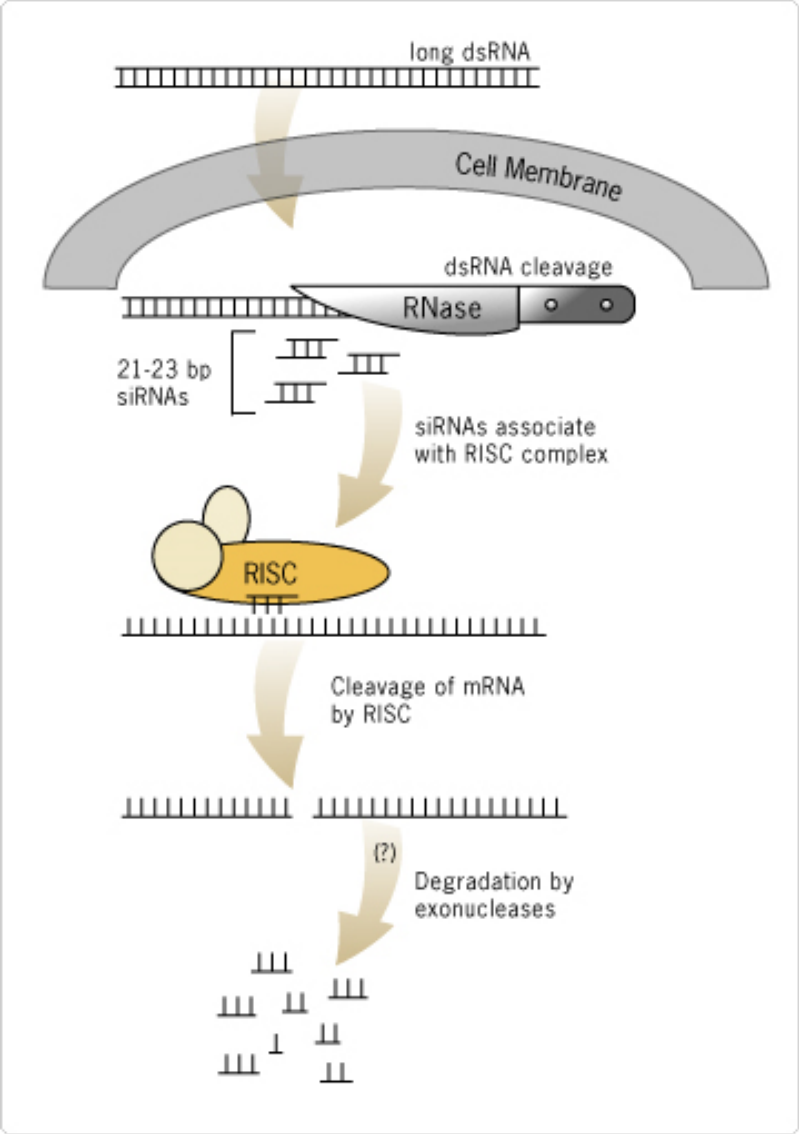

Figure 2: An overview of RNA Interference. dsRNA is used to transport into the cell, and is then cleaved into siRNA. siRNA then combine with the RISC complex to target and degrade mRNAs.

RNAi was created using chemical 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. These miRNAs complemented a 23bp consensus sequence in mammalian miR-21 and were 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 were also obtained.

Part II – Wound Creation and miR-21 treatment: *Mus* musculus was chosen as the model system for this experiment, as they are fairly genetically similar to humans due to the relative homology of their genomes, and the development of vertebrates is fairly conserved, especially those both in mammalian lineages[11]. Three treatment groups of *Mus musculus* were created, all wild-type mice with no significant differences in genome. To determine how much miR-21 was naturally active in wound healing, a subset of the control group was given 3 cm long incisions under anaesthesia, following the procedure outlined in Mulisa, Asres and Engidawork[12]. RNA from the incision site was extracted and quantified at 6, 12 and 24 hours post-incision, and RT-PCR performed using miR-21 primers to quantitatively determine the amount of miR-21 present in wild type wound healing. From that it was determined that the first experimental group (with increased miR-21) would be given miR-21 as to show a 10 fold increase in cells as compared to WT. Wounds were created using the same method as for the control, and the excess miR-21 was administered in a TRIS solution by injection into the wound site of the mouse at 6, 12 and 24 hours post-incision. For the second experimental group, the RNAi product was administered in sufficient quantity to inactivate all miR-21 at 6, 12 and 24 hours post-incision, as determined by the control group. Another control group also received an injection at 6, 12 and 24 hours of just TRIS solution. A positive siRNA control group was given injections at the same time intervals of *Silencer*TM GAPDH siRNA, and the results quantified after 7 days using qRT-PCR. A negative siRNA control group was injected at the same time intervals with

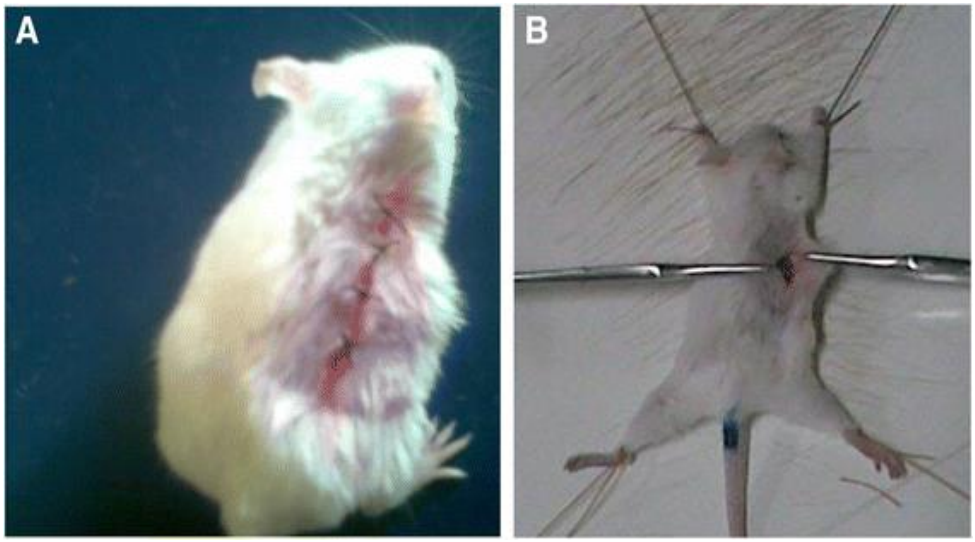

Figure 3: Incision (a) and tensile strength measurement (b) in mice. The incision was created under anesthesia. The tensile strength was measured after takedown using a continuous water flow technique and two forceps.

*Silencer*TM Negative Control #1 siRNA. Wound healing time for all mice was then measured in days, with a wound considered fully healed when no scabbing existed. Tensile strength of the wound on day 7 of healing was tested using the protocol outlined in Mulisa, Asres and Engidawork[12] for all mice.

The controls in this experiment served several purposes. The first was to determine the concentration of miR-21 naturally occurring in cells to allow us to increase the amount present. This allowed us to create a miR-21 addition experimental group with miR-21 concentrations that were based on biological data, rather than just a random injection concentration. The second purpose was 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 did not have any significant effect on wound healing, and that it would not impact our data. The RNAi negative control using *Silencer*TM Negative Control #1 siRNA was used to control for effects of using siRNA in the cell, and the *Silencer*TM GAPDH siRNA was used as a positive control to ensure siRNA function. Its effects were viewed by using qRT-PCR to follow GAPDH expression in the cell[13]

Possible Outcomes

One of three possible outcomes can be expected:

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

Observations: All treatment groups do not have significantly different healing times or wound tensile strengths from one another.

Conclusions: Here it can be concluded that the presence or lack of miR-21 doesn't have any significant impact on wound healing, and therefore is not necessary for the process. It can then be inferred that miR-21 likely does not play a role in mammalian wound healing. This is assuming that other miRNAs cannot compensate for the lack of miR-21 or be supressed by the presence of miR-21. This inference fits with the current understanding that miR-21 is very much 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, sites of severe injury in these organisms. Further studies could investigate the role miR-21 plays in axolotl limb regeneration as well as see how the regeneration process differs from the wound healing process in mammals.

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 begin to bridge the gap on 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: Here it can be concluded that 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. From this 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 manner than quantitative in this process. This inference fits with the idea that miR-21 is active in wound sites, and further displays the role it may play in wound healing. 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.

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Figure Sources

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