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Investigating the Role of circCdr1as in Macrophage Polarisation and Scar-Free Regeneration in Acomys

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Abstract. Most mammals heal injured tissue by forming scar tissue, which differs in structure and function from the original tissue. In contrast, the African spiny mouse, *Acomys cahirinus*, can regenerate ear tissue after injury with limited scar formation. Macrophages are important regulators of tissue repair, regeneration and fibrosis, and differences in macrophage phenotype may contribute to the different healing responses observed in *Acomys* and conventional laboratory mice. Macrophage polarisation can be regulated by several molecular mechanisms, including non-coding RNAs. Circular RNAs (circRNAs) can regulate gene expression by binding microRNAs (miRNAs) and reducing them. Previous studies have shown that circCdr1as can regulate macrophage phenotype through the circCdr1as/miR-7/KLF4 pathway. CircCdr1as contains multiple conserved binding sites for miR-7, suggesting that this interaction may also be relevant in *Acomys*. We therefore hypothesise that circCdr1as promotes M2 macrophage polarisation and VEGFC expression through the miR-7/KLF4 pathway, contributing to scar-free regeneration in *Acomys*. This project will first examine the relationship between circCdr1as, macrophage polarisation and VEGFC during regeneration. CircCdr1as will then be knocked down and overexpressed to determine its functional role in the regenerative response. This study may provide insight into whether a circRNA-mediated regulatory mechanism is conserved across mammalian species and whether circCdr1as contributes to scar-free regeneration in *Acomys*.

Keywords: circCdr1as, macrophage polarisation, scar-free regeneration, Acomys cahirinus, VEGFC

1. Introduction

1.1. Tissue repair and regeneration

After tissue injury, mammals usually repair the damaged area through wound healing. However, severe injury often results in fibrotic scar formation rather than complete regeneration. Scar tissue can restore structural integrity but does not fully reproduce the structure or function of the original tissue [1]. In contrast, some mammals have a greater capacity for tissue regeneration. The African spiny mouse, *Acomys cahirinus*, is able to regenerate 4 mm ear-hole punches, including cartilage, adipocytes, hair follicles and muscle [2]. This makes *Acomys* a useful model for studying how scar-

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free regeneration differs from fibrotic healing. Tissue repair and regeneration depend on the coordinated activity of many cell types. Among these, inflammatory monocytes and tissue-resident macrophages are key regulators of tissue repair, regeneration and fibrosis [3].

1.2. Macrophage polarisation

Following injury, macrophages can change their phenotype in response to signals from the local tissue environment. Although macrophage activation exists as a spectrum, macrophages are often broadly described as pro-inflammatory M1 or anti-inflammatory M2 states [3]. M2 macrophages are associated with tissue repair and regeneration, whereas a prolonged pro-inflammatory response may contribute to persistent injury and fibrosis [3]. Therefore, tissue repair may depend not only on macrophage recruitment but also on how macrophages change their phenotype after injury. This difference is particularly relevant to *Acomys* as both *Acomys* and laboratory mice recruit macrophages following ear injury, but *Acomys* shows a stronger and more sustained increase in CD206, a marker associated with pro-regenerative M2 macrophages [1]. This suggests that macrophage polarisation may contribute to the different healing outcomes between the two species.

An important question therefore remains: **which molecular factors regulate macrophage polarisation during regeneration?**

1.3. Circular RNAs and macrophage regulation

Circular RNAs (circRNAs) are non-coding RNAs with a covalently closed structure. This structure can make them more stable than many linear RNAs [4]. One well-established function of circRNAs is their ability to act as miRNA sponges. CircRNAs can contain binding sites for specific miRNAs. By binding these miRNAs, circRNAs can reduce their availability to repress their target genes [4, 5]. CircRNAs have also been linked to immune regulation and macrophage phenotype [4, 6]. This raises the possibility that circRNAs may act upstream of macrophage polarisation during tissue repair.

1.4. circCdr1as and miR-7

circCdr1as is one of the best-characterised circRNAs and contains numerous binding sites for miR-7 [5]. By binding miR-7, circCdr1as can reduce the activity of miR-7 and affect the expression of miR-7 target genes. Previous work has linked circCdr1as to macrophage phenotype. In bone marrow-derived macrophages, circCdr1as expression differed between pro-inflammatory and anti-inflammatory macrophage states. Overexpression of circCdr1as increased anti-inflammatory markers and CD206-positive macrophages, whereas knockdown produced the opposite effect [6].

More recently, circCdr1as was shown to directly bind and sponge miR-7. Increasing miR-7 promoted a more pro-inflammatory macrophage phenotype, whereas inhibition of miR-7 increased anti-inflammatory markers [7]. These findings suggest that miR-7 may act as an important intermediate between circCdr1as and macrophage polarisation.

1.5. KLF4 as a downstream target

miR-7 can repress the expression of downstream target genes. One proposed target is Krüppel-like factor 4 (KLF4), a transcription factor involved in the regulation of macrophage phenotype [7]. In macrophages, circCdr1as-mediated reduction of miR-7 activity was associated with increased KLF4

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expression. Manipulation of KLF4 also changed macrophage phenotype, supporting the proposed circCdr1as/miR-7/KLF4 regulatory pathway [7].

Together, these studies provide a possible mechanism:

circCdr1as → miR-7 sequestration → reduced repression of KLF4 → changes in macrophage polarisation.

1.6. Knowledge gap

Previous studies show that *Acomys* displays a strong pro-regenerative M2 macrophage response during tissue regeneration, while circCdr1as has been shown to regulate macrophage phenotype through the miR-7/KLF4 pathway [1, 7]. However, these two findings have not yet been connected in the *Acomys* regeneration model. It is therefore unknown whether circCdr1as contributes to scar-free regeneration in *Acomys* through the regulation of macrophage polarisation.

We hypothesise the following pathway:

Injury → circCdr1as ↑ → miR-7 sequestration → KLF4 ↑ → M2 macrophage polarisation ↑ → VEGFC ↑ → scar-free regeneration

The proposed relationship between M2 macrophages and VEGFC will be tested experimentally. The complete circCdr1as–VEGFC pathway in Acomys therefore remains a hypothesis.

2. Project design

This project investigates whether circCdr1as contributes to scar-free regeneration in *Acomys* through macrophage polarisation and VEGFC expression. Aim 1 will examine the relationship between circCdr1as, macrophage polarisation and VEGFC during regeneration. Aim 2 will test the effect of circCdr1as knockdown on macrophage polarisation and regeneration, while Aim 3 will examine whether circCdr1as overexpression enhances the regenerative response.

2.1. Relationship between circCdr1as, macrophage polarisation and VEGFC during regeneration

2.1.1. Rationale

Aim 1 provides the first step in testing this hypothesis by establishing whether circCdr1as changes during regeneration and whether its expression follows the same pattern as M2-associated macrophages and VEGFC. This will provide a basis for the functional experiments in Aim 2.

2.1.2. Method

An *Acomys* ear-punch injury model will be used. Ear tissue will be collected before injury and at Day 3, Day 7 and Day 14 after injury. circCdr1as expression will be measured by RT-qPCR. Total RNA will first be extracted from ear tissue and reverse-transcribed into cDNA. Primers targeting the circCdr1as back-splice junction will be used to preferentially detect the circular transcript. For macrophage analysis, ear tissue will be enzymatically digested to generate a single-cell suspension. Cells will be stained with fluorescent antibodies and analysed by flow cytometry. Macrophages will first be identified within the cell population using F4/80 and CD11b. The macrophage population will then be further analysed using CD86 and CD206 as M1- and M2-associated markers, respectively. VEGFC protein levels will be measured using ELISA. Protein will be extracted from ear tissue, and samples will be added to wells containing a VEGFC-specific capture antibody.

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Following binding of VEGFC, a detection antibody and enzyme-linked detection system will be used to generate a measurable signal. The signal will be compared with a standard curve to estimate VEGFC concentration.

The temporal profiles of circCdr1as expression, M2-associated macrophage proportion and VEGFC levels will then be compared. A coordinated increase or similar temporal pattern would identify an association between these factors during regeneration (Figure 1).

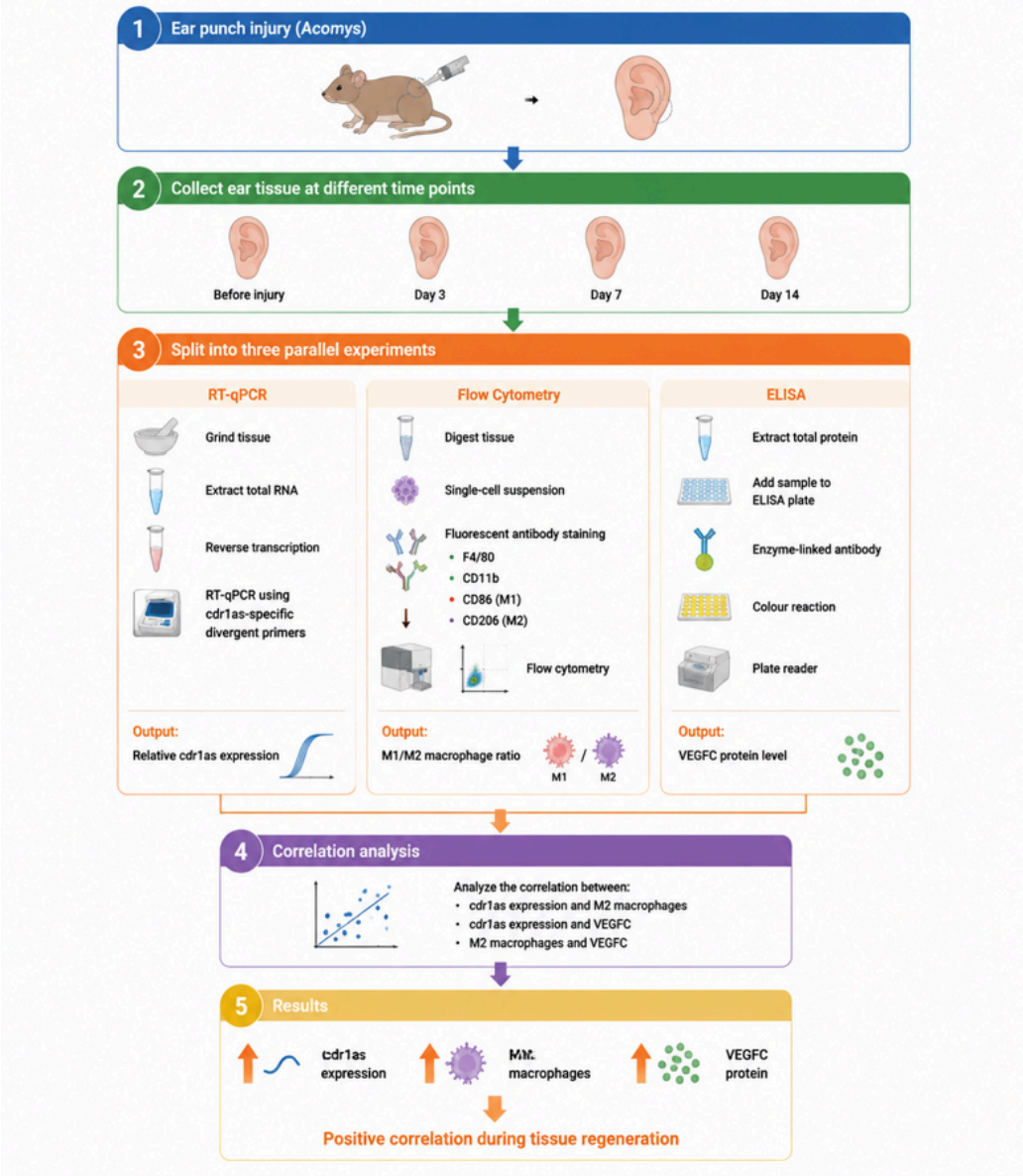


Figure 1. Experimental design for Aim 1: Relationship between circCdr1as, macrophage polarisation and VEGFC during regeneration. Ear tissue will be collected before injury and on Days 3, 7 and 14 after ear-punch injury in Acomys cahirinus. RT-qPCR, flow cytometry. Figure created with BioRender

2.1.3. Expected outcome

circCdr1as expression is expected to increase after injury alongside an increase in M2-associated macrophages and VEGFC. A similar temporal pattern would support an association between

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circCdr1as and the regenerative response. This would provide a basis for directly manipulating circCdr1as in Aim 2.

2.1.4. Potential pitfall and alternative

circCdr1as may not show the expected correlation with M2 macrophages or VEGFC. This could occur if circCdr1as acts at a different stage of regeneration or primarily functions in another cell population. Additional time points could therefore be included. circCdr1as expression could also be examined in isolated macrophages or other relevant cell populations.

2.2. circCdr1as knockdown and its effect on regeneration

2.2.1. Rationale

Aim 1 can identify an association between circCdr1as and the regenerative response, but an association alone cannot show whether circCdr1as has a functional role. A loss-of-function experiment is therefore needed to test whether circCdr1as is required for macrophage polarisation and regeneration. If circCdr1as is an upstream regulator of the proposed pathway, reducing its expression should alter downstream miR-7/KLF4 signalling, macrophage phenotype and regeneration. CRISPR/Cas9 provides a widely used approach for targeted genome editing, while AAV vectors can be used for in vivo delivery. These tools provide a possible strategy for testing circCdr1as function in *Acomys*.

2.2.2. Method

The genomic sequence and genomic locus of the *Acomys* circCdr1as-producing region will first be identified using available genome resources, such as NCBI or Ensembl, if an appropriate annotated *Acomys* sequence is available. Because circCdr1as is produced by back-splicing, the targeting strategy must be designed carefully. A dual-sgRNA strategy will be used to target genomic regions flanking the circRNA-producing locus, with the aim of disrupting circCdr1as production. Candidate sgRNAs will be designed using a CRISPR design tool and selected based on predicted editing efficiency and low off-target potential. Candidate guides may first be tested in vitro. Editing can be assessed by PCR amplification of the target region followed by T7E1 assay or Sanger sequencing. Validated sgRNAs will be inserted into an AAV vector containing the sgRNA expression cassette. The vector will also contain the CRISPR editing components required for the proposed experiment. Following cloning, the construct will be verified by restriction analysis and sequencing before viral production. AAV particles will then be produced, purified and quantified to determine viral titre. The AAV construct will be delivered locally to the adult *Acomys* ear using the proposed local injection method. A non-targeting sgRNA vector will serve as a negative control, while the uninfected contralateral ear will provide an internal control. After a sufficient period for editing to occur, ear tissue will be collected. Genomic DNA will be extracted and the target region amplified by PCR. Sanger sequencing and ICE or TIDE analysis will be used to estimate editing efficiency. For the dual-sgRNA strategy, gel electrophoresis may additionally be used to identify larger deletions. circCdr1as expression should also be measured directly by RT-qPCR to confirm that genomic editing produces a reduction in the circular RNA. The macrophage analysis described in Aim 1 will then be repeated to compare M1- and M2-associated macrophage proportions following circCdr1as knockdown. VEGFC protein levels will be assessed by ELISA, and ear regeneration will

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be evaluated by measuring wound closure and/or remaining ear-hole size over time. Histological analysis may also be used to assess the regeneration of tissue structure.

To examine the proposed mechanism more directly, miR-7 and KLF4 expression could also be measured after circCdr1as knockdown. An increase in miR-7 activity together with reduced KLF4 expression would support the proposed signalling pathway (Figure 2).

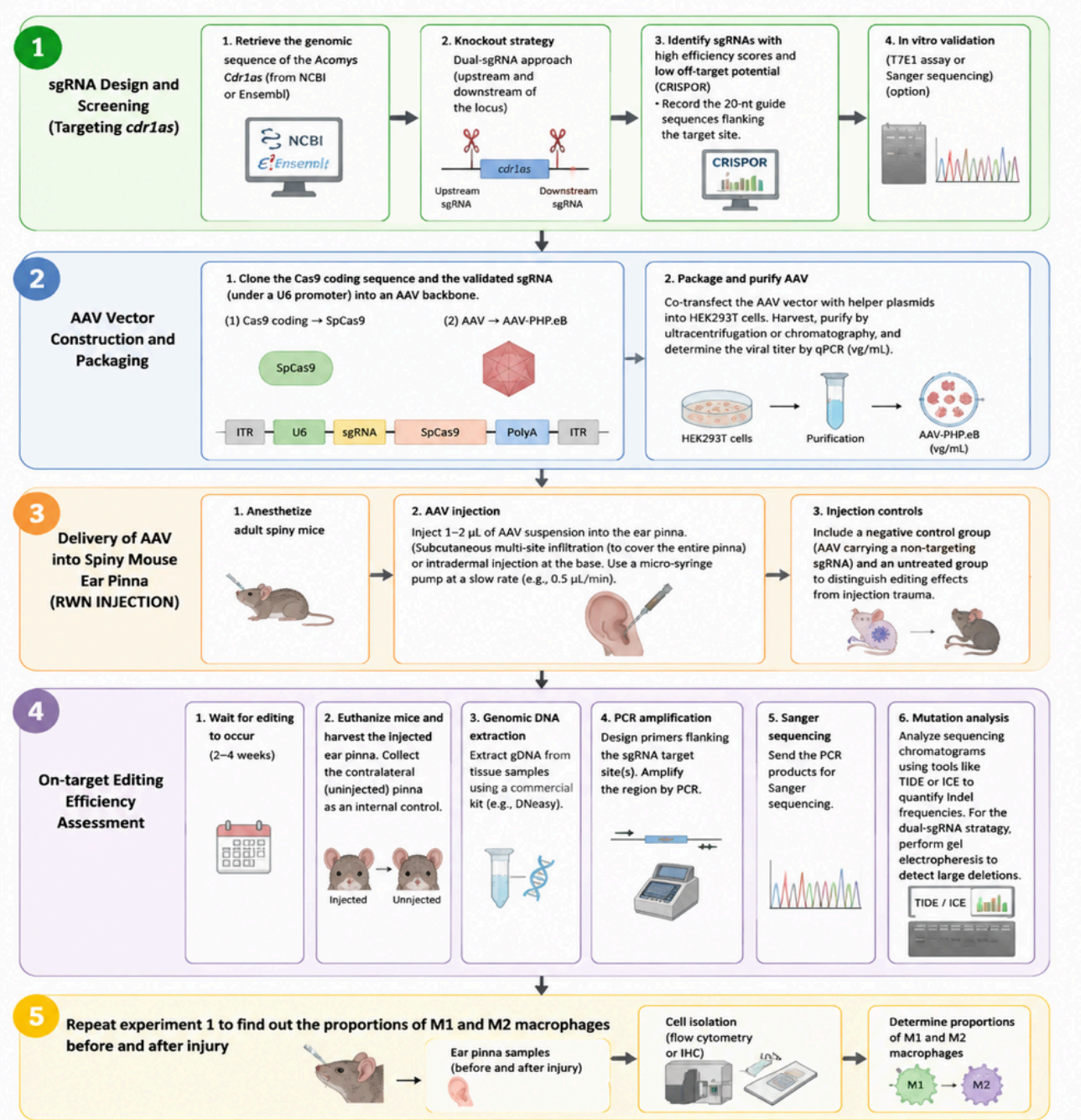


Figure 2. Experimental design for Aim 2: circCdr1as knockdown and its effect on regeneration. CRISPR/Cas9-mediated targeting will be used to reduce circCdr1as expression in the Acomys ear-punch model. A non-targeting sgRNA vector will serve as a negative control. Figure created with BioRender

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2.2.3. Expected outcome

circCdr1as knockdown is expected to reduce circCdr1as expression and alter the macrophage phenotype towards a less pro-regenerative state. If the proposed pathway is correct, circCdr1as reduction may be associated with increased miR-7 activity, reduced KLF4 expression, a lower proportion of M2-associated macrophages and reduced VEGFC levels. These changes are expected to be accompanied by impaired ear regeneration.

2.2.4. Potential pitfall and alternative

Low AAV delivery efficiency or incomplete editing may lead to insufficient circCdr1as reduction. Therefore, both genomic editing and circCdr1as expression should be confirmed before interpreting downstream effects. Off-target editing may also affect the phenotype. Multiple sgRNAs and appropriate non-targeting controls could improve confidence in the specificity of the result. A further limitation is that genomic CRISPR targeting may affect linear transcripts produced from the same locus. If specific disruption of the circular RNA cannot be achieved, an alternative approach targeting the circCdr1as back-splice junction, such as shRNA or an RNA-targeting system, may provide greater transcript specificity. This is particularly important for interpreting a circRNA-specific phenotype.

2.3. circCdr1as overexpression and its effect on regeneration

2.3.1. Rationale

Aim 2 will test whether circCdr1as is required for the regenerative response. However, loss of circCdr1as cannot determine whether increasing its expression is sufficient to promote regeneration. A gain-of-function experiment will therefore test whether circCdr1as overexpression can increase M2-associated macrophage polarisation, VEGFC expression and ear regeneration. Previous studies have used circCdr1as overexpression systems containing flanking sequences to promote circularisation, and RT-qPCR using back-splice junction primers can be used to confirm expression of the circular transcript [6].

2.3.2. Method

An *Acomys* ear-punch injury model will be established with a control-vector group and a circCdr1as-overexpression group. The circCdr1as overexpression construct will contain a promoter to drive transcription and appropriate flanking circularisation elements to promote back-splicing and formation of the circular transcript. The construct will be delivered locally to the injured ear using an AAV-based delivery system. circCdr1as overexpression will first be confirmed by RT-qPCR using primers targeting the back-splice junction. Linear Cdr1 expression may also be measured to determine whether the manipulation preferentially increases the circular transcript. Macrophage polarisation will then be analysed by flow cytometry using the same marker strategy as Aim 1. VEGFC protein levels will be measured by ELISA. Ear regeneration will be assessed by comparing wound closure or ear-hole size over time between the control and circCdr1as-overexpression groups. Histological analysis could further assess the restoration of tissue structure (Figure 3).

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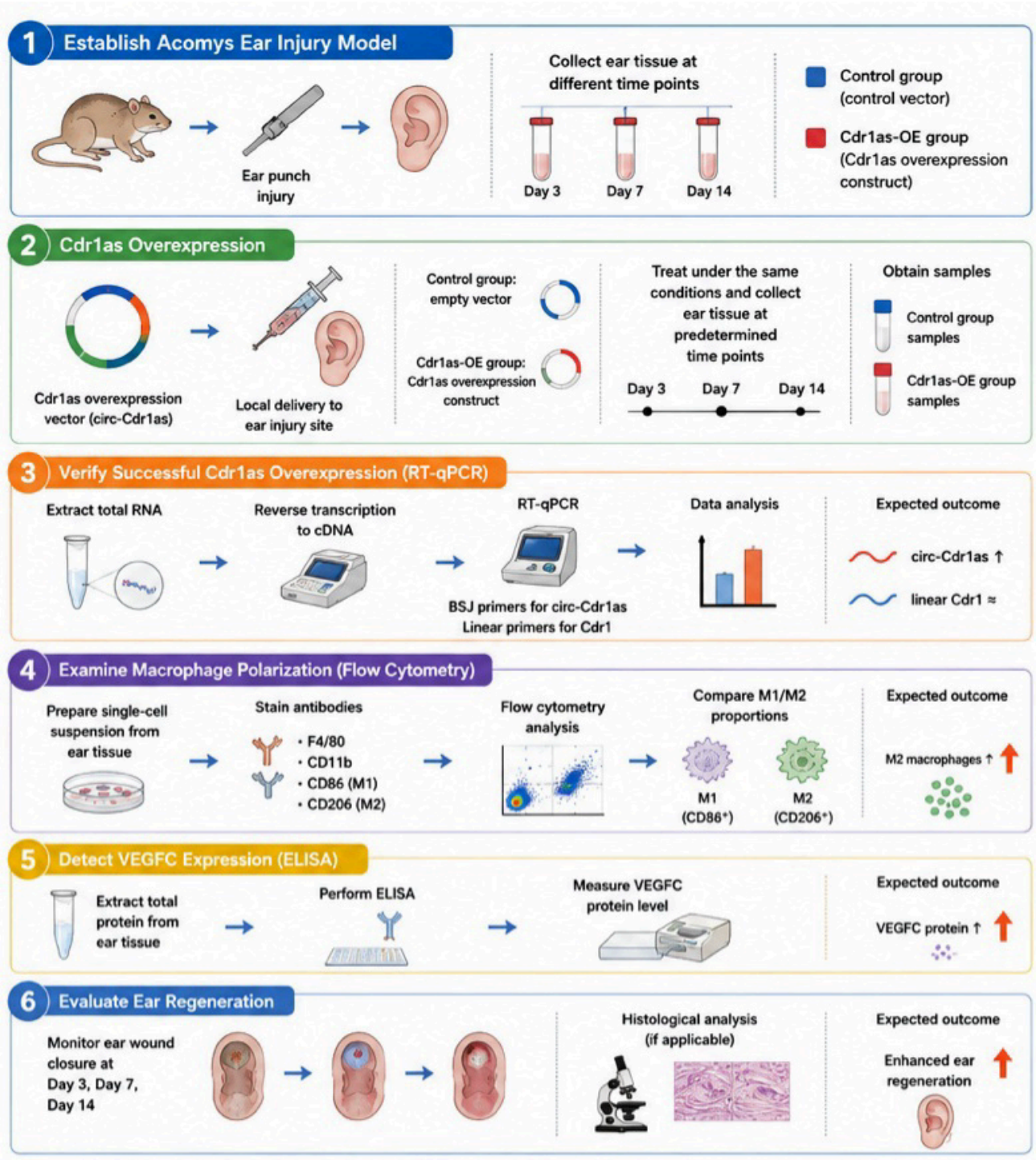


Figure 3. Experimental design for Aim 3: circCdr1as overexpression and its effect on regeneration. An AAV-based vector will be used to promote circCdr1as overexpression in the Acomys ear-punch model. A control vector will serve as a comparison. circCdr1as express. Figure created with BioRender

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2.3.3. Expected outcome

circCdr1as overexpression is expected to increase M2-associated macrophages and VEGFC levels, resulting in enhanced ear regeneration. Together with the loss-of-function experiment in Aim 2, these findings would provide stronger evidence that circCdr1as has a functional role in the regenerative response.

2.3.4. Potential pitfall and alternative

AAV-mediated overexpression may vary between animals. circCdr1as expression must therefore be confirmed before interpreting downstream effects. Overexpression may also produce non-physiological levels of circCdr1as and may not reproduce its natural expression pattern after injury. Dose optimisation or an alternative delivery strategy could be considered. circCdr1as may also act in cell types other than macrophages. Further experiments could therefore isolate specific cell populations to determine where circCdr1as is expressed and functions during regeneration.

3. Impact and significance

This project will investigate whether circCdr1as links circRNA regulation with macrophage polarisation and scar-free regeneration in *Acomys*. If the hypothesis is supported, the results may identify a circCdr1as/miR-7/KLF4 pathway that contributes to the pro-regenerative macrophage response in this model. The project may also provide evidence about whether a circRNA-mediated regulatory mechanism identified in other mammalian systems is conserved in *Acomys*. More broadly, understanding why *Acomys* undergoes regenerative rather than fibrotic healing may help identify molecular pathways involved in tissue repair. This could provide a basis for future work on circRNA regulation in fibrosis and impaired tissue repair.

4. Overall pitfalls and alternatives

The main limitations include uncertain delivery efficiency in *Acomys*, incomplete or off-target CRISPR editing, and difficulty in distinguishing effects on circCdr1as from effects on other transcripts produced from the same genomic locus. The proposed pathway may also be more complex than the model presented here. circCdr1as may interact with targets other than miR-7, and other circRNAs or miRNAs may compensate for circCdr1as loss. These issues will be addressed by confirming circCdr1as manipulation, including appropriate controls, examining miR-7/KLF4 where possible, and using alternative targeting strategies if genomic editing does not provide sufficient circRNA specificity.

5. Conclusion

This project will investigate whether circCdr1as contributes to scar-free regeneration in *Acomys cahirinus* through the regulation of macrophage polarisation and VEGFC expression. The study will examine the relationship between circCdr1as, macrophage phenotype and VEGFC during regeneration, followed by loss-of-function and gain-of-function experiments to assess its functional role. It will also explore the proposed miR-7/KLF4 pathway. If the hypothesis is supported, this study may identify a regulatory mechanism that links circRNA activity with the pro-regenerative macrophage response in *Acomys*. These findings may improve our understanding of the molecular

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differences between regenerative healing and fibrotic repair. They may also provide a basis for future research into circRNA-mediated regulation of tissue repair.

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