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Cardiac injections of AntagomiRs as a novel tool for knockdown of miRNAs during heart development

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Abstract

Background:

Studying microRNA networks during heart development is essential to obtain a better understanding of developmental defects and diseases associated with the heart and to identify novel opportunities for therapeutics. Here we highlight the advantages of chicken embryos as a vertebrate model for studying intermediate processes of heart development. Avians develop a four-chambered heart closely resembling human anatomy and they develop *ex utero*, which makes them easily accessible. Furthermore, embryos are available all year with a steady supply.

Results:

In this report we established a novel method for the knockdown of microRNA function by microinjecting AntagomiRs into the chicken heart *in ovo*. Our approach enables the targeted delivery of antagomirs into a locally restricted area and is not impacted by circulation. After further embryo development the successful miRNA knockdown was confirmed. Loss of function phenotypes can be evaluated rapidly, compared to more time-consuming genetic ablation experiments. The local application avoids potential systemic effects of microRNA knockdown, therefore allowing the assessment of impacts on heart development only. The method can be adjusted for different stages of chicken embryos (HH13-HH18) as well as for knockdown or targeted overexpression of coding genes.

Conclusion:

In conclusion our method allows targeted and locally restricted delivery of Antagomirs to the heart leading to successful knockdown of microRNA function. This method enables rapid phenotypic assessment, for example by gene expression analysis of multiple cardiac genes.

Keywords: chicken embryo, micromanipulation, microinjection, heart development, microRNA, antagomiR

Introduction

The study of heart development contributed important insights into the normal process of organ patterning and growth control but also revealed factors involved in pathological development leading to a better understanding of congenital heart disease. MicroRNAs are small non-coding RNA molecules of approximately 23 base pairs in size that regulate gene expression (Bartel, 2009). It has been demonstrated previously that they play a significant role in both cardiac development and disease (Chen and Wang, 2012; Liu and Olson, 2010), however the details of how individual microRNAs regulate gene networks remain to be elucidated. Thus, further work is needed to acquire

new insights and to potentially translate this knowledge into new avenues for medical care. Cardiac microRNA networks have been studied in a number of model organisms each of them with different advantages for analysis. Two models closely resembling human heart anatomy, with a four-chambered heart comprising two atria and two ventricles, are the mouse (*Mus musculus*) and the chicken (*Gallus gallus*). The fact that mice develop *in utero* whereas chicken embryos develop *in ovo* has a strong impact on accessibility and therefore applicable methods. Commonly used approaches for altering gene expression in mice include Cre-mediated tissue specific (conditional) recombination of a selected target gene, CRISPR-mediated genome editing, or transgenic overexpression of genes of interest, allowing for permanent and/or inducible genetic alterations (Bouabe and Okkenhaug, 2013; Singh et al., 2015). Furthermore, lineage tracing experiments that reveal cell relationships have been used extensively in mice by applying genetic labelling methods (Kalhor et al., 2018; Kraus et al., 2014). In chicken, tissue grafting, electroporation and microinjections are the most commonly used techniques. The embryo is highly accessible *in ovo* and allows stage-specific manipulations with subsequent read out (Bellairs and Osmond, 2014; Stern, 2005). Even though a different subset of techniques is used in chicken embryos lineage tracing has also been employed to study cell fate (Kretzschmar and Watt, 2012), for example the contributions of cardiac neural crest to outflow tract septation and of the second heart field to the right ventricle were first identified in the chicken (Wittig and Münsterberg, 2016).

Genetic approaches in mice have significantly increased our understanding of heart development, however, these are time-consuming and their spatio-temporal control is limited by the ability to control the activity of the Cre recombinase, thus making the study of specific events during heart development more challenging. The precise local administration of pharmacological agents to the mouse heart is difficult due to in utero development and therefore lack of access to the embryonic heart. Injections into the vasculature lead to systemic delivery and thus an impact on tissues other than the heart cannot be excluded (Goehringer et al., 2009; Krutzfeldt et al., 2005). In chicken, the effective delivery of viral based expression constructs needs high concentrations (Lambeth et al., 2014), leading to potential DNA toxicity effects. A general complication is that due to heart contractions, injected compounds are quickly washed out into circulation (Davidson et al., 2001). Virus based transduction is slightly less affected by this since the virus binds to cell surface receptors. Effective binding can be improved by slowing the heart temporarily through embryo cooling. However, nucleic acid-based constructs or synthetic compounds can quickly disperse and their successful delivery and uptake requires steady local administration and/or temporary heart arrest, which could have non-specific effects on development.

For our study we chose the chicken embryo as model organism, its developmental stages are well-defined (Hamburger and Hamilton, 1951) and the heart is readily accessible, particularly the early and intermediate stages, which closely resemble human anatomy (Wittig and Münsterberg, 2016). Embryos are cheap to obtain and are incubated to the desired stage in controlled conditions. This facilitates *in ovo* manipulations with precise temporal and site-specific targeting and permits the study of different phases of intermediate heart development, including S-shape (heart) looping, chamber remodelling, trabeculation and septa formation. Following manipulation and further embryo development subsequent visual assessment and gene expression analysis is possible and does not require sacrificing the parent animal. Therefore, the use of chicken embryos complements the analysis of genetically modified embryos of other model species (Bellairs and Osmond, 2014).

The injection technique into the chicken heart illustrated here can be applied to different subsections of the heart and thus overcomes some of the above-mentioned drawbacks. Furthermore, the targeted injections could be performed with plasmid constructs – combined with

Lipofectamin (England et al., 2017), or with virus particles, synthetic compounds, antisense morpholinos or microRNA mimics and inhibitors. Delivery of microRNA inhibitors, AntagomiRs, is the primary focus of this report as it represents a new tool for studying the functions of individual or multiple microRNAs during intermediate heart development, during which the heart tube is remodelled into a mature chambered heart. Once mastered, this method can be used for heart-specific knockdown and gain-of-function studies and allows medium-throughput screening of multiple genes of interest.

Results

Protocol for cardiac injections:

The whole procedure is shown step by step in Movie 1: Cardiac injection procedure.

1. Incubate fertilized chicken eggs
    a. Fertilized chicken eggs are stored at 17°C for up to a week prior to incubation
    b. Eggs are incubated with the blunt end up in a humidified incubator at 37°C
        i. Depending on stage of interest the length of egg incubation has to be adjusted. To obtain HH15 embryos we incubated fertile eggs for approximately 66h (Hamburger and Hamilton, 1951)
2. Preparation of injection setup:
    a. For 30 eggs prepare 25 ml 1:500 PBS/ Pen Strep (PS) (Gibco Life Technologies) solution and 25 ml PBS/PS-solution with Indian ink (Winsor & Newton)
    b. Prepare Syringes with needles
        i. 1 ml syringe with 25Gx 5/8” needle for ink injection
        ii. 1 ml syringe with 25Gx 5/8” needle for incision of the extra embryonic tissue (EET)
        iii. 10 ml or 2 ml syringe with 21Gx2” needle for albumen removal
    c. Forceps and tweezers for removal of the shell and outer/inner membranes
    d. Adhesive tape for egg sealing
    e. Small table-top incubator for usage during procedure
3. Prepare injection apparatus
    a. Pull needle for microinjection (borosilicate glass capillaries 1.00 mm O.D. x 0.78 mm I.D.)
    b. To enable easy heart manipulation, we recommend shorter needles with a very sharp tip, since long flexible ones will not penetrate the heart and simply bend.
        i. Needle pull settings for Sutter Instruments P-97: P=300, Heat=ramp test value, Pull=150, Vel=80, Time=130.
    c. Load needle with ~1.5 µl AntagomiR at 1µM (or other) using Microloader™ (Eppendorf) and mount onto microinjector
4. Prior to procedure spray eggs with 70% Ethanol to kill surface germs
5. Remove single egg from incubator (Figure 1A)
6. Crack it open at the blunt end using tweezers and remove shell pieces until embryo is easily accessible (Figure 1B)
7. Using syringe (i) in a shallow angle inject ink-mix beneath the embryo for contrast and apply 2 drops PBS/PS-solution on top using a plastic Pasteur pipette to increase elasticity of EET (Figure 1C)
8. Place egg under a stereo-dissecting microscope and use syringe needle (ii) to cut open the vitelline membrane (Figure 1D)

   a. Cut EET around heart until it is freely accessible (Figure 1E)
9. Perform multiple injections in areas of interest (Figure 1F)
   a. Depicted is conus, primitive ventricle and primitive atrium (Figure 1F)
10. Remove needle and visually confirm injection (Figure 1G)
   a. Injected compound (AntagomiR) is conjugated to a fluorophore
11. Slide the needle of the large syringe (iii) down along the inside of the egg shell and remove albumen until the embryo is sufficiently lowered and will not touch the sealing tape (Figure 1H)
   a. If embryo adheres to the egg shell, flush it gently using albumen until it detaches
   b. Apply a few drops PBS/PS-solution
12. Seal the egg using clear adhesive tape
   a. Combine two strips of tape together (parallel) and apply them to the egg. Use a third strip and cross the other two (perpendicular) (Figure 1I)
13. Return the egg to an incubator immediately (for convenience this could be the desk-top incubator).

Embryo survival and possible timescales of experiments

Several parameters have an impact on survival of the injected chicken embryos. In this study we analysed survival of embryos, injected with AntagomiR or with a scrambled sequence, 24 hours post injection (hpi), 48hpi and 72hpi (Krutzfeldt et al., 2005; McGlinn et al., 2009) (representative photos, Figure 2A). For both the non-specific scrambled control, AntagomiR-SCR, and the specific AntagomiR-499-5p embryo survival declined with longer incubation periods (Figure 2B). After 24 hours post injection around 90% of embryos had survived and developed normally, this dropped to 40% and 35% at 48hpi and 72hpi. The almost identical survival rates observed with control and experimental AntagomiRs suggests that the decrease in survival is due to the manipulation itself. The data presented for AntagomiR-SCR represents the baseline for survival and we assume that survival beyond 72hpi is possible, potentially even up to hatching. In experiments where other AntagomiRs were injected the embryos showed a steeper decline in survival (up to 100% before 48hpi, not shown), suggesting a biological impact affecting survival resulting from this particular knockdown.

Several factors during the procedure should be considered to promote embryo survival. During egg opening, it is vital to avoid damaging the embryonic tissue and the vasculature. Bleeding indicates damage and these embryos cannot be used. In addition, following the microinjection embryos can sometimes adhere to the egg shell and their detachment can result in damage. Incubation with rocking prior to injection may reduce the occurrence of adherence to the shell and it has been reported that agitation during incubation improves hatchability and should therefore promote healthier development (Randles and Romanoff, 1954). In the final step of the procedure, when lowering the embryo after injection, it is essential to avoid contact with sealing tape. To avoid these confounding issues, we have excluded egg shell adhering embryos in our survival analysis.

To make the heart accessible for injection the vitelline membrane and some of the EET surrounding the heart need to be partially removed. During this, caution is necessary to avoid wounding vessels or the heart to prevent bleeding. The same applies to the actual injection step, since the injection needle is sharp enough to cause small incisions in the heart. Finally, the procedure was optimized by returning the embryos to a heated incubator at 37°C immediately after injection, this improved survival rates.

To determine if the injection procedure had an impact on heart function, we analysed heart rates (HR) of control-injected embryos compared to wildtype (non-manipulated) embryos as a

representative variable for cardiac health (Figure 2C top panel). The data revealed no significant differences between the two groups for the first two timepoints analysed, 24hpi and 48hpi (unpaired t-test, $p>0.05$). This also shows that the injected AntagomiR has no non-specific toxicity. The HR becomes faster with increasing age, therefore, to ensure accurate comparison, the same stages of embryo development were used (Figure 2C bottom panel). The quantification of HR requires the heart to be visually accessible and this makes analysis of older embryos (>5 days) difficult. After 5 days of development the allantois is covering the heart and this made HR-quantification challenging even though still possible for some embryos (Spurlin and Lwigale, 2013). However, electrophysiological methods for HR- quantification may still be suitable (Shi et al., 2013). We have also found that injection of some AntagomiRs can induce HR changes and the complete analysis of such phenotypes require detailed follow-up analysis.

Validation of AntagomiR administration

The injected compounds in this study were AntagomiRs, complementary sequences to microRNAs designed to inhibit their function (Krutzfeldt et al., 2005; McGlinn et al., 2009). The AntagomiRs contained two modifications, a 3' cholesterol group to allow cell uptake and a 5' Fluorescein group for tracing purposes. The Fluorescein enables easy localization of AntagomiRs during the procedure, which is helpful for targeting and to avoid injection into the bloodstream, potentially causing systemic rather than heart specific effects. Furthermore, the fluorescent tag enables detection post-injection at 24hpi and 48hpi (Figure 3A). However, fluorescence intensity decreased over time and was no longer detectable at 72hpi. Signal strength may also depend on local retention of the AntagomiR. The AntagomiR-SCR used as a control in this study has no binding partner in the chicken embryo, we found that its signal intensity was reduced, compared to an AntagomiR that is directed against a microRNA expressed in the heart, such as miR-499-5p (Figure 3Bi) (van Rooij et al., 2009). After Antagomir-499-5p injection a much stronger signal was consistently detected using enzyme catalysed immunohistochemistry (IHC) against the FITC-tag. We propose that this is most likely due to binding of the endogenous complementary microRNA, miR-499-5p. In addition to IHC on dissected hearts (Figure 3Bi), we examined whole embryos to confirm specific targeting of the heart only (Figure 3Bii). Specific staining was restricted to the heart shown by two purple stripes. Some faint staining was visible in the body due to trapping, which is a commonly observed drawback of methods involving chromophore conversion (Antin et al., 2007; Lufkin, 2007). However, if this diffuse signal is due to leakage of AntagomiR into other tissues, it would indicate that there was very little if any.

In mice, AntagomiRs were detected for up to three weeks after systemic injection (Krutzfeldt et al., 2005). Consistent with this, IHC against Fluorescein detected AntagomiRs for periods longer than 48hpi (Figure 3B).

Furthermore, Knockdown of miR-499-5p can be confirmed by Northern-Blot (Figure 3C, Supplement 1) and qPCR (Figure 3D). Hearts were dissected post incubation, pooled (six for 24hpi & three for 48hpi) and RNA was extracted. Both methods show a significant reduction of miR-499-5p expression at both timepoints, indicating that AntagomiRs are effective and stable in an *in vivo* environment (unpaired t-test, Northern Blot, n=3, 24hpi and 48hpi $p<0.01$, qPCR, n=5, 24hpi and 48hpi $p<0.001$). The miR-499-5p bands on Northern Blots were normalised to U6 and qPCR data was normalised to the expression of other microRNAs, miR-451 and miR-126-3p. These microRNAs were not targeted and showed stable expression. We have found that detection of U6 by qPCR was inconsistent, especially when using different RNA amounts for cDNA synthesis.

In addition, we found that the molecular verification using Northern Blot and qPCR is dependent on the chosen RNA extraction method. If the extraction method included Trizol, a frequently used solution, the knockdown could not be demonstrated (Supplement 2A, B). Initially we wondered whether the lack of observable knockdown could be explained by a feedback mechanism, involving the upregulation of miR-499-5p synthesis, however qPCR detection of the longer precursor microRNA in Trizol extracted samples did not support this idea (Supplement 2C). Another explanation might be, that Trizol releases the stable duplex of AntagomiR and microRNA and thus, more microRNA can be detected in these samples even though microRNA function is still inhibited. Furthermore, Trizol has a known bias regarding GC-content, particularly when extracting microRNAs (Kim et al., 2012). Taking this into consideration, we do not recommend extraction methods that involve Trizol when examining microRNAs.

Discussion

In conclusion, our method can be used for AntagomiR injections and to achieve successful knockdown of microRNA function in the heart. The injected compound can easily be replaced and thus the technique is also suitable for delivery of morpholinos, or plasmid constructs, or viral particles to achieve transient knockdown or area specific expression of genes of interest. For example, in preliminary experiments injection of LNA modified microRNA mimics, usually used for tissue culture experiments to promote miRNA mediated silencing, showed target RNA expression changes (data not shown). Also, it is possible to combine multiple AntagomiRs into a single injection to study synergistic, antagonistic or additive effects of microRNA inhibition. The method can be applied to variety of stages of chicken development and thus allows the study of different processes of heart development. Most suitable are HH13 to HH18, after turning of the chicken embryo and before the heart encapsulating tissue becomes too rigid for dissection to gain access (Martinsen, 2005; Wittig and Münsterberg, 2016). Injections at older developmental stages will allow the specific targeting of only the atria or ventricles, similarly at younger stages it is possible to restrict injections to parts of the primitive heart tube. Strikingly, following delivery the administered AntagomiR was locally restricted to the heart (Figure 1F and Figure 3A, B) and not affected by the circulating bloodstream. Therefore, this procedure allows studying the impact of microRNA-inhibition on the heart only, which presents an important advantage to approaches used previously (Davidson et al., 2001; Goehringer et al., 2009). This spatial restriction is helpful to avoid effects on other organs caused by systemic microRNA knockdown and will improve the analysis of phenotypes.

The method allows the downstream analysis of different parameters to assess the phenotype, including morphology, histology, gene expression changes and changes in physiology. Here, we quantified the heart rate of injected embryos and compared it to wildtype siblings. Quantification of zebrafish heart rates has been done using a high frame rate camera (Musso et al., 2014), this was adopted for this study. Video recordings of the heart allow automated heart rate quantification using Fiji software and furthermore allow calculation of input and output volumes. The latter requires visual access to both, atria and ventricles (Musso et al., 2014), which is rarely the case past 48hpi since at that stage the embryo is covered by the allantois (Spurlin and Lwigale, 2013). However, by selecting recorded videos where all heart chambers are fully visible, it is possible to determine cardiac input and output volumes. Alternatively, the heart rate can be determined with electrodes producing an electrocardiogram as shown by Shi et al. (2013) in the chicken embryo. Electrophysiological experiments surrounding the conduction system of the heart could involve patch clamp, pulse wave, action potential and/or ECG measurements (Borghetti et al., 2018; Shi et al., 2013). Morphological changes in the heart post-injection can be analysed by serial sectioning and

3D reconstruction (Wang et al., 2015), optical projection tomography (Sharpe et al., 2002) or histological staining procedures (Alturkistani et al., 2015).

To assess successful administration and resulting knockdown of miR499-5p, we employed Northern Blot analysis and quantitative RT-PCR. Such analysis can be further expanded for genes of interest to determine differential expression and thus dissect pathways relevant for the targeted microRNA. In order to understand the functional relevance of miRNAs and their corresponding targets and to decipher the miRNA network in heart development, the samples can be further processed for RNAseq to obtain a broader picture of differentially expressed genes. We showed that the RNA extraction method used, column-based or Trizol based, affects the ability to verify the knockdown. The reasons for this are still unclear, but we feel that it is an important observation to report. For further molecular analyses, non-Trizol extracted samples are preferable. This extraction method enables confirmation of microRNA knockdown after AntagomiR administration (Fig. 3C, D)(Krutzfeldt et al., 2005; Velu and Grimes, 2012) and avoids the reported GC-bias of Trizol for microRNAs (Kim et al., 2012).

Taken together the cardiac injection method presented here facilitates investigation of microRNA functions during intermediate heart development in the chicken embryo. Advantages of this knockdown approach are the targeted local delivery at specific stages of development and the short timeframe in which results can be obtained.

Material and Methods

AntagomiR design

AntagomiRs are fully complementary to the microRNA of interest and include a 5' Fluorescein and 3' Cholesterol addition. Further the backbone consists of 2-O-methyl RNA and contains phosphorothioate bonds at the three most 3' and two most 5' bases (McGlinn et al., 2009).

Table 1: AntagomiRs used in this study

AntagomiR name	sequence
AntagomiR-SCR	5'-(FL)mC(*)mA(*)mUmCmCmAmUmCmAmCmUmCmAmCmUmCm CmAmU(*)mC(*)mA(*)mU(3'-Chl)-3'
AntagomiR-499-5p	5'-(FL)mC(*)mU(*)mAmAmAmCmAmUmCmAmCmUmAmCmAmAm GmUmCmU(*)mU(*)mA(*)mA(3'-Chl)-3'

Embryos

Fertilized Dekalb White chicken eggs (Henry Stewart & Co.Ltd, Fakenham, UK) were incubated at 37°C until needed. Stage HH14-16 embryos (~66h) were used for the cardiac injection procedure (Hamburger and Hamilton, 1951).

Fluorescence microscopy

AnatagomiR delivery was confirmed using a LEICA MZ16F microscope with a LEICA DFC 300FX camera to track the fluorescence of the Fluorescein tag. Brightfield and green channel images were taken and merged in Adobe Photoshop CC.

Heart Rate Assessment

To asses heart rates, a single injected egg was removed from the incubator and a circular opening was cut in the tape seal. The egg was immediately placed under the microscope and a 15-30 sec clip at 1080p/120fps was recorded (RIBCAGE H5PRO). Subsequently the embryo was dissected and

processed for other methods. Collected video clips were played in slow motion using Apple QuickTime and heart beats were counted manually by J.G.W. and M.R.G. Beats per minute were calculated accordingly $BPM = \frac{n_{beats}}{t_{recording}} * 60s$.

Immunohistochemistry

Endogenous phosphatases of dissected hearts have been inactivated at 67°C in HYB buffer overnight. Following several washes according to previously described procedures (Goljanek-Whysall et al., 2011) an antibody against Fluorescein (Anti-Fluorescein-AP, Fab fragments, Roche) was applied and incubated overnight. Again, following several washes colour was developed for all samples simultaneously using BCIP/NBT

RNA extraction and RT-qPCR

RNA and miRNA isolation from pools of dissected hearts was performed using Quick-RNA™ MiniPrep Plus (Zymo Research) according to the manufacturer's protocols. We used RNA Clean & Concentrator™-5 (Zymo Research) in the case of Trizol Reagent (Invitrogen™) treated samples. cDNA was synthesized from 200 ng RNA in a 20 µl reaction using a Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific). For miRNAs, cDNA was synthesized from 10 ng in a 10 µl reaction using miRCURY LNA RT Kit (Qiagen). RT-qPCR was performed on a 7500 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. MicroRNA specific primers have been designed by Qiagen (mir-449-5p: YP02111236, mir-451: YP02110557, mir-126-3p: YP02111065). Primers used for the miR-499 precursor are F: 5'-TTTGAGGGAGCGGCAGTTAA-3' and R: 5'-TGAGGAGAGAAGTAGCACAGACT-3' (Sigma-Aldrich). RT-qPCR was normalized to β-Actin for mRNA, and miR-451 and miR-126-3p for micro RNA. Analysis was done according Livak and Schmittgen (2001) and significance was determined by unpaired t-test using GraphPad Prism.

Northern blot

Northern blot analysis was performed as previously described (Pall and Hamilton, 2008). Briefly, 10 µg of total RNA was resolved on a 15% denaturing urea polyacrylamide gel. The RNA was transferred to a Hybond-NX nylon membrane (GE Healthcare) by semidry-blotting for 1h at 20 V and chemically crosslinked using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma Aldrich) at 60°C for 2h.

For Northern blot hybridization the membranes were pre-hybridized using Perfect Hyb buffer (Sigma Aldrich) and the antisense DNA oligonucleotides (Sigma-Aldrich) were end-labelled using 3000 Ci/mmol of [γ-^{32}P]-ATP (Perkin Elmer). The hybridization of the membranes with the 5'-^{32}P labelled DNA oligonucleotide was performed overnight at 37°C. The membranes were washed twice with 0.2x SSC and 0.1% SDS for 20 min and exposed to a phosphor imager screen for autoradiography. The membrane was imaged using the Typhoon Scanner and band intensities were quantified with the Image Quant Software (GE Healthcare).

For re-probing of the membranes with the loading control U6 the membranes were stripped with 0.1% SDS for 5h at 80°C.

Antisense DNA oligonucleotide probe sequences used were:
The miR499-5p miRCURY LNA miRNA detection (Qiagen) probe was used for the detection of miRNA 499-5p (5'-CTAAACATCACTACAAGTCTTA-3') and the membrane was re-probed with U6 snRNA as a loading control (5'-GCTAATCTTCTCTGTATCGTTCC-3').

Competing interests

The authors declare no competing or financial interests.

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Author Contributions

Conceptualization: J.G.W., E.L.V., A.E.M.; Methodology: J.G.W., E.L.V.; Validation: J.G.W.; Formal analysis: J.G.W.; Investigation: J.G.W., M.B., E.L.V., M.R.G; Writing: J.G.W., M.B., A.E.M.; Visualisation: J.G.W.; Supervision: A.E.M.; Project administration: A.E.M.; Funding acquisition: A.E.M.

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Movie 1: Cardiac injection procedure

The movie shows all necessary details for carrying out the cardiac injection procedure described in this study.

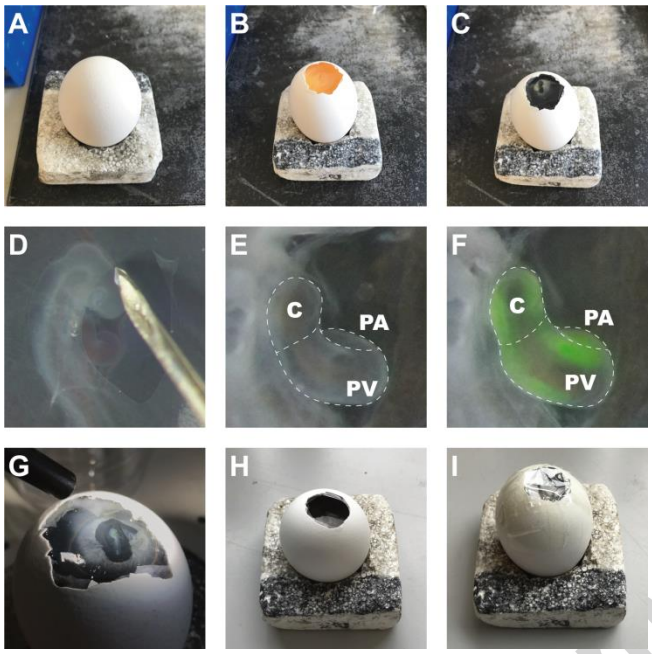
Figure 1: Injection Sequence. Image sequence of procedure steps. (A) Incubated egg for manipulation, (B) egg was cracked open and outer and inner membrane were removed, (C) ink was applied into the yolk sac below the embryo to provide contrast, (D) vitelline membrane and EET were cut open to access the heart, (E) accessible heart for injection, red blood cells are visible in the lumen, (F) injected heart along conus (C) downwards to primitive ventricle (PV) and on the right side the primitive atrium (PA), (G) injection verification, (H) removal of albumen and lowering of embryo, (I) sealing of egg and returning to incubation at 37°C.

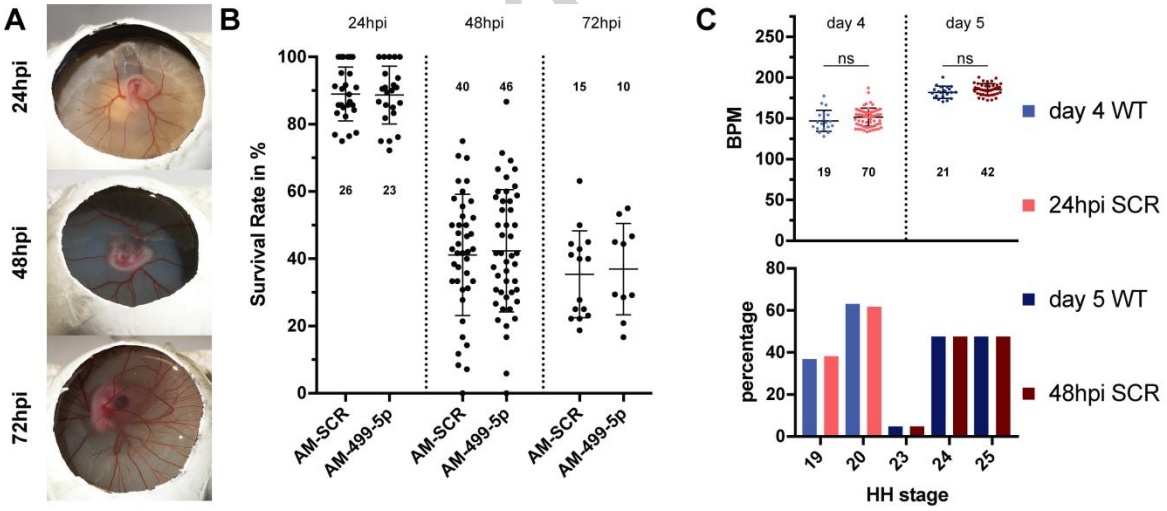
Figure 2: Representative results – embryo survival & impact on heart rate. Embryos survive until at least 72hpi and show no differences in cardiac health. (A) Representative photos of embryos post injection at the 3 time points analysed. (B) Survival analysis for embryos injected with AntagomiR-SCR (control) and AntagomiR-499-5p, a specific microRNA inhibitor. (C) Comparison of WT non-manipulated embryos vs control-injected embryos show no significant difference in heart rate. The bottom panel shows the distribution of HH stages that were analysed. We ensured the same proportion of HH19 and HH20 embryos were examined on day 4 (light blue and light red columns) and the same proportion of HH23, HH24 and HH25 embryos were examined on day 5 (dark blue and dark red columns). (N in (B) is number of experiments, each experiment comprised between 25 and 30 embryos, N in (C) represents the number of analysed hearts)

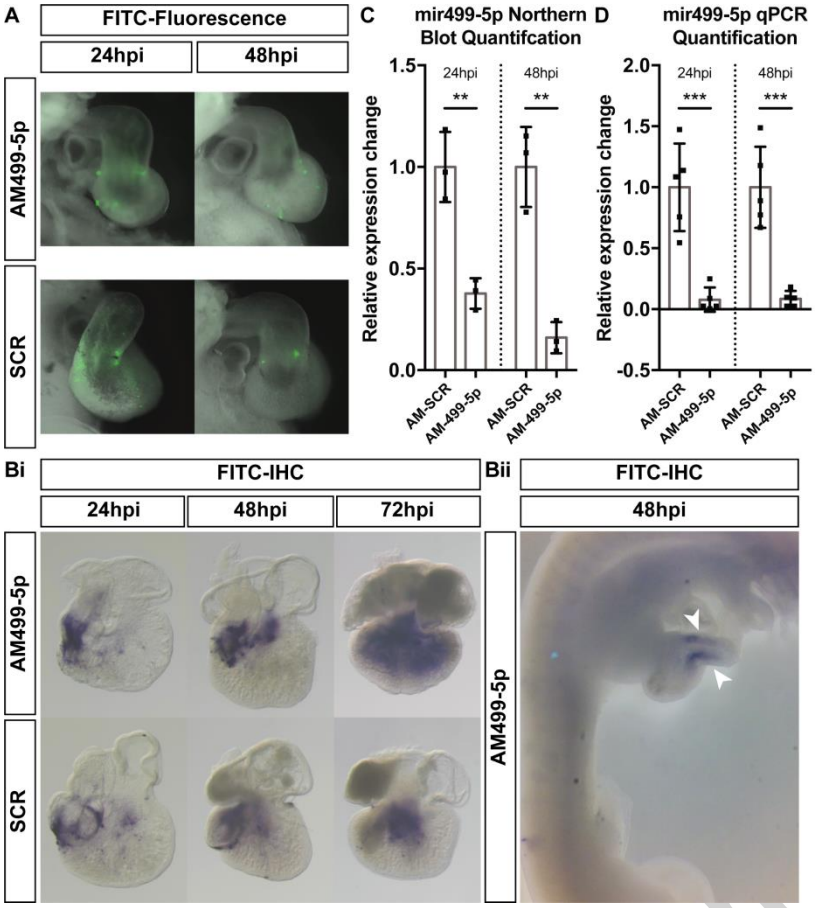
Figure 3: Confirmation of successful AntagomiR delivery. Visual and molecular verification of AntagomiR delivery and microRNA knockdown. (A) Tracing of FITC-tag fluorescence of AntagomiR-SCR and AntagomiR-499-5p at 24hpi and 48hpi. (B) Chromophoric detection of the FITC-tag by IHC. (Bi) AntagomiR-SCR and AntagomiR-499-5p are detected by IHC at 24hpi, 48hpi and 72hpi in dissected hearts. (Bii) Whole embryo IHC for AntagomiR-499-5p. Specific signal is detected in the heart (white arrowheads), faint background staining originates from trapping. (C) Northern Blot confirmation of miR-499-5p knockdown (unpaired t-test, n=3, 24hpi and 48hpi, $p<0.01$). (D) qPCR confirmation of miR-499-5p knockdown (unpaired t-test, n=5, 24hpi and 48hpi $p<0.001$).

Supplement 1: Extended Data Figure 3. Corresponding Northern Blot Images to Figure 3C three most left lanes AntagomiR-SCR injected samples and three most right lanes Antagomir-499-5p injected samples (3 lanes) (arrow indicates target).

Supplement 2: Difference in Results using Trizol based RNA extraction. MicroRNA-499-5p knockdown cannot be confirmed using RNA extraction involving Trizol. (A) Northern Blot analysis (n=3) shows a significant increase of mircoRNA-499-5p at 24hpi but no significant difference at 48hpi (unpaired t-test, $p<0.01$; $p>0.05$) (A2) Northern Blot raw images, three most left lanes AntagomiR-SCR injected samples and three most right lanes Antagomir-499-5p injected samples (arrow indicates target) (B) qPCR analysis confirms Northern blot results (unpaired t-test, 24hpi $p<0.05$, 48hpi $p>0.05$, n=4) (C) qPCR quantification of mir-499-5p precursor transcript shows significant reduction at 24hpi but not 48hpi (unpaired t-test, $p<0.01$; $p>0.05$, n=4).







Highlights

- Cardiac injections into live embryos facilitates the targeted and localized delivery of AntagomiRs
- Chick embryos enable rapid phenotype analysis
- Following AntagomiR injection the successful knockdown of the cognate microRNA was observed
- The choice of RNA extraction method is critical