Knockdown of the ERK pathway using siRNA in cultured chicken cardiomyocytes

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Abstract

The ancient South American birds called tinamous (Tinamidae) have the smallest hearts known among birds and their cardiomyocytes have previously been shown to express significantly lower levels of the mitogen-activated protein kinase ERK compared to the more modern chicken (*Gallus gallus*). ERK is a well-known mediator of growth signalling in the heart, especially in hypertrophy. The aim of this project was to assess the effect of ERK knockdown on proliferation in cultured chicken cardiomyocytes. By transfecting these cells with a lipoplexed siRNA, ERK mRNA levels were knocked down to approximately half (45%, SD: 27%) compared to cells transfected with a negative control siRNA. The knockdown was coupled with a decreased proliferative response to insulin-like growth factor 1 (IGF-1) and foetal bovine serum (FBS). In conclusion, the ERK pathway was confirmed to be instrumental also in proliferative signalling. The results also support the notion that ERK itself is the rate-limiting step of this MAPK cascade. The low native expression of ERK in tinamou cardiomyocytes is expected to impose a strict limit on proliferative growth in response to various stimuli in these hearts. The genetic changes leading to higher expression levels, and with it the potential for larger hearts, in modern birds would have led to greatly increased evolutionary fitness by way of an increased aerobic scope and the ability to sustain flight.
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1 Abstract

The ancient South American birds called tinamous (Tinamidae) have the smallest hearts known among birds and their cardiomyocytes have previously been shown to express significantly lower levels of the mitogen-activated protein kinase ERK compared to the more modern chicken (Gallus gallus). ERK is a well-known mediator of growth signalling in the heart, especially in hypertrophy. The aim of this project was to assess the effect of ERK knockdown on proliferation in cultured chicken cardiomyocytes. By transfecting these cells with a lipoplexed siRNA, ERK mRNA levels were knocked down to approximately half (45%, SD: 27%) compared to cells transfected with a negative control siRNA. The knockdown was coupled with a decreased proliferative response to insulin-like growth factor 1 (IGF-1) and foetal bovine serum (FBS). In conclusion, the ERK pathway was confirmed to be instrumental also in proliferative signalling. The results also support the notion that ERK itself is the rate-limiting step of this MAPK cascade. The low native expression of ERK in tinamou cardiomyocytes is expected to impose a strict limit on proliferative growth in response to various stimuli in these hearts. The genetic changes leading to higher expression levels, and with it the potential for larger hearts, in modern birds would have led to greatly increased evolutionary fitness by way of an increased aerobic scope and the ability to sustain flight.

2 Introduction

2.1 Project background

Previous gene expression studies carried out in my supervisor’s research group found that two mitogen-activated protein kinases (MAPKs) were expressed at significantly different levels in the hearts of chickens compared to two different species of South American tinamou: the ornate tinamou (Nothoprocta ornata) and the Chilean tinamou (Nothoprocta perdicaria). The two MAPKs were p38, which was expressed at higher levels (10-fold in N. p. and 14-fold in N. o.), and ERK, which was expressed at a considerably lower level (14%) in the tinamous (not yet published). Tinamous are primitive birds with significantly smaller hearts in terms of relative heart mass than chickens and most other birds. As MAPKs are involved in the transmission of both proliferative and hypertrophic cell signalling (Hefti et al., 1997; Sundgren et al., 2003b), this difference in expression patterns may help explain the difference in heart size and could reflect an evolutionary change necessary for the appearance of modern birds that are capable of sustaining flight for extended periods of time. The goal of this project was therefore to determine the effects of ERK knockdown on proliferation in cultured chicken cardiomyocytes using RNA interference.

2.2 Mechanisms of cardiac growth and development

Cardiomyocytes make up about 80% of the heart’s volume and are the main cell type responsible for heart function (Hefti et al., 1997; Sundgren et al., 2003a). Cardiomyocytes can grow in two different ways: hyperplasia or hypertrophy. During hyperplastic growth the cells divide actively, thus increasing cell number. During hypertrophic growth cell numbers remain the same, but the cells grow in size. The canonical view has been that cardiomyocyte hyperplasia is the predominant mode of vertebrate heart growth during embryonal development, followed by a terminal differentiation event soon after birth leading to loss of proliferative potential. All further heart growth is then dependent on cellular hypertrophy of pre-existing cardiomyocytes. This limited regenerative capability is also the reason why the postnatal heart is so sensitive to injury, such as in the case of a heart attack (Poss et al., 2002). While this model still holds mostly true for mammalian hearts, it does not appear to be true for all vertebrates. Notably, the hearts of zebrafish regenerate fully even after 20% of the muscle has been removed (Poss et al., 2002). Subsequent experiments have shown that this is mainly the result of already differentiated cardiomyocytes re-entering the cell cycle rather than the contribution from stem or progenitor cells (Jopling et al., 2010). The
cardiomyocytes of birds and amphibians have a somewhat different phenotype than their mammalian counterparts, and the lack of some distinguishing structures present in mammals may be interpreted as a lesser degree of differentiation in adult animals (Li et al., 1997).

Binucleation is one of the hallmarks of mature mammalian cardiomyocytes and has been linked to hypertrophic growth patterns in sheep cells (Sundgren et al., 2003b). In humans approximately 25% of cardiomyocytes are binucleated, and most nuclei develop tetraploidy during childhood (Bergmann et al., 2009). In chicken, however, a study found that only 18% of myocytes were binucleated 15 days after hatching and that these cells were still undergoing mitosis. The investigators examined the proliferative potential of chicken cardiomyocytes until 42 days post-hatch, and could not find any indication that it was decreasing. As amphibian cardiomyocytes had previously been shown to proliferate in vitro but rarely do so in vivo, the authors suggested that these less differentiated vertebrate cardiomyocytes may retain their potential for hyperplastic growth indefinitely, and that their low proliferative rate in vivo is probably the result of lack of hyperplastic stimuli under normal circumstances. (Li et al., 1997).

### 2.3 The role of ERK

Mitogen-activated protein kinase (MAPK) cascades are intracellular pathways that regulate gene expression by a series of phosphorylations in response to external stimuli such as growth factors and mitogens. The three pathways are typically named after their terminal kinases: p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). Each cascade consists of a three-level hierarchy where a MAPK kinase phosphorylates a MAPK kinase, which in turn phosphorylates the MAPK itself. In the case of the ERK cascade, ERK is phosphorylated by MEK (a MAPKK) after it has been phosphorylated by Raf (a MAPKK). ERK finally phosphorylates transcription factors in the nucleus, as well as components of the cytoskeleton in the cytoplasm (Hefti et al., 1997). The initial signals converging on the ERK pathway can come from diverse receptor types, such as G-coupled receptors and receptor protein-tyrosine kinases (RPTKs) (Glennon et al., 1996).

The ERK pathway has been implicated as a major mediator of hypertrophic stimuli in cardiomyocytes. For example, well-known hypertrophic agonists such as phenylephrine and endothelin-1 stimulate phosphorylation of ERK, and their effects are entirely inhibited by pharmacological inhibition of MEK in mammals (Yue et al., 2000; Rolfe et al., 2005). Transgenic mice with chronically activated MEK develop cardiac hypertrophy with a consistent 2.5-fold increase in ERK phosphorylation levels, which may indicate that ERK itself is the rate-limiting step of the cascade (Bueno et al., 2000). ERK knockdown by antisense oligonucleotide transfection has previously been performed in neonatal rat cardiomyocytes in culture, and produced a 90% reduction in ERK protein after 72 h with attenuation of hypertrophic responses to phenylephrine (Glennon et al., 1996).

Insulin-like growth factor 1 (IGF-1) stimulates hypertrophy, hyperplasia and/or cell differentiation depending on the type and maturation state of the treated cells (Sundgren et al., 2003a). While it regulates many intracellular pathways, ERK and PKC are the ones mainly affected (Li et al., 2001). These two pathways work in unison to produce many of the effects of IGF-1, as demonstrated by a microarray study that found 70% of the gene regulation effects exerted by IGF-1 within two hours of treatment to be dependent on both pathways (Liu et al., 2001).

In adult mammalian hearts IGF-1 is produced in response to pressure overload and induces hyper trophy. However, an increased availability of IGF-1 in the hearts of foetal sheep led to larger hearts with a lower percentage of binucleation in cardiomyocytes compared to the control (Sundgren et al., 2003a). These results were in agreement with a previous study where IGF-1 was transgenically overexpressed in the hearts of postnatal mice and led to progressively larger hearts with higher cell counts and no sign of cellular hypertrophy (Reiss et al., 1996). Sundgren et al. (2003a) concluded that IGF-1 induces hyperplasia in the mitotically competent mononucleated cardiomyocytes, and suggested that the level of cellular differentiation determines whether IGF-1 acts as a hypertrophic or hyperplastic agonist in cardiomyocytes. Considering the relatively low level of differentiation
in avian cardiomyocytes that has already been mentioned, a hyperplastic response is expected in chicken myocytes.

### 2.4 RNA interference

Antisense oligonucleotides have long been used to inhibit the corresponding endogenous RNA in gene expression studies. The effect was thought to result from simple stoichiometric base-pairing and subsequent inhibition of protein synthesis, but in the late 90’s one research group found that double-stranded RNAs (dsRNAs) were remarkably more efficient RNA-inhibitors than their single-stranded counterparts (Fire et al., 1998). The phenomenon was termed RNA interference, or RNAi for short, and has been found to function endogenously as both a transcriptional regulator and as an antiviral mechanism (McManus & Sharp, 2002; Li et al., 2013). Knockdown of gene expression is initiated by a short interfering RNA (siRNA) associating with the cytoplasmic RISC complex, which then cleaves the homologous RNA upon basepairing with the siRNA. The siRNA-RISC complex, however, is kept intact and is recycled for many more rounds of RNA cleavage. Only with the dilution of consecutive cell divisions does the effect appear to be lost (McManus & Sharp, 2002; Sato et al., 2004).

The canonical siRNA is 21 nucleotides in length with 3’ overhangs of 2 nucleotides. The sequence of the overhangs is not important for basepairing, and so dTdT-overhangs have become popular as they are cheap to synthesize and may provide an increased resistance towards nucleases (Elbashir et al., 2001). While eukaryotic cells themselves possess the ability to generate siRNAs from longer sequences of dsRNA, gene expression studies have come to rely primarily on siRNA directly. This bypasses other steps in the antiviral response of the cells, such as the interferon response which is activated by dsRNA >30 nucleotides long and leads to a general down-regulation of all gene expression and a stop in protein synthesis (Elbashir et al., 2001).

Because of their large size and negative charge, nucleic acids can rarely enter cells unaided (Felgner et al., 1987). Several methods of improving transfection rates are available. Larger nucleic acids such as plasmids can be difficult to transfect efficiently, especially in primary cells. In these cases electroporation is often the most efficient method (McManus & Sharp, 2002). When transfecting with siRNA, the gentler method of lipofection is often used. While free liposomes can be harmful to the cells, toxicity is generally much lower than with electroporation (Djurovic et al., 2004).

Lipofection relies on a cationic lipid which spontaneously forms complexes with the anionic nucleic acid. The resulting lipoplexes can form in different ways, and the structure they adopt appears to affect their mode of entry into the cells. In multimellar lipoplexes nucleic acid fills the spaces between layers of lipid, while honeycomb lipoplexes consists of hexagonal sets of lipid tubes encapsulating the nucleic acid molecules. Because of an excess of positive charge, both structural types associate electrostatically with the negatively charged cell surfaces. Honeycomb lipoplexes then appear to merge with the cell membrane, effectively releasing the nucleic acid into the cell (Tros de Ilarduya et al., 2010). This was the mechanism originally proposed for lipofection by Felgner et al. (1987), but it has since been found that multimellar lipoplexes are more likely to enter the cells through endocytosis. Once inside the cell, the endosome undergoes an internal drop in pH which triggers a flip-flop translocation of negatively charged phospholipids from the outside to the inside of the endosomal membrane. The positively charged lipoplex lipids then merge with the endosomal membrane, simultaneously freeing the DNA or RNA from the complex and destabilising the endosome so that the nucleic acid is released into the cytoplasm (Tros de Ilarduya et al., 2010).

The functionality of RNAi in birds was first shown by Sato et al. (2004), who achieved a reporter gene knockdown of approximately 80% in embryonic chicken cells. However, the researchers co-transfected siRNA and plasmid, only achieved successful transfection in about 40% of cells and cell types were not characterised. Toyota et al. (2008) successfully used siRNA lipoplexes to transfect embryonic chicken cardiomyocytes in culture and reported a transfection efficiency above 80% as determined by a fluorescent control, as well as near-complete inhibition of the targeted protein.
3 Material and methods

3.1 Cell culture and transfections

Chicken hearts were collected at embryonic day 19 and either proceeded directly to cell isolation or were frozen at -80°C until needed. Cells were then isolated enzymatically and fibroblasts removed by 1 hour of pre-plating. Cardiomyocytes were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% foetal bovine serum (FBS), sodium pyruvate, non-essential amino acids and penicillin/streptomycin, and were split 1:4 approximately once a week. Cells used for qPCR assay were seeded in either 35-mm petri dishes or 6-well plates and allowed 48 hours to adhere before any experimental procedure was carried out, then washed twice with PBS, switched to antibiotic-free DMEM without serum and transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) at a final concentration of 2 µg/ml. Three candidate siRNAs with the sense sequences CAAGAAGAUUUGAAUGAUA (siRNA 1), GAAAUCAUGCUGAAUUCUA (siRNA 2) and GAUAUUAUCAGAGCUCCAA (siRNA 3) were tested at final concentrations of 40 nM. All had 3’ dTdT-overhangs and were ordered from Sigma Aldrich (Saint Louis, MO). siRNA sequences correspond to nucleotides 769-787, 613-631 and 286-304 respectively of the *Gallus gallus* ERK mRNA (see Appendix 1). MISSION siRNA Universal Negative Control #1 (Sigma Aldrich, Saint Louis, MO) was used as a negative control at equivalent concentration.

3.2 Fluorescence microscopy

Cells used for fluorescence microscopy were seeded in 8-well glass chamber slides (Thermo Fisher Scientific, Waltham, MA) that had been pre-coated with 0.2% gelatin. The cells were transfected using the procedure mentioned above and subjected to 40 nM final concentration of BLOCK-iT Alexa Fluor Red Fluorescent Control (Life Technologies, Carlsbad, CA). After 24 h incubation they were washed twice with PBS, fixed with 4% PFA in room temperature for 20 min, then washed twice more with PBS. They were then stained with 50 µg DAPI for 5 minutes, washed, stained with [conc] Bodipy phallacidin for 1 h and finally washed again before the cover slip was mounted. Imaging was performed in a LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

3.3 qPCR

Cells destined for qPCR were washed twice with PBS and lysed in TRI Reagent (Sigma Aldrich, Saint Louis, MO) 24 hours after transfection, with the exception of the cells used for qPCR in parallel with the MTS assay which were lysed at the same time as the other assay was performed. Samples either proceeded directly to RNA isolation, or were frozen at -80°C until isolation could take place. Extracted RNA was frozen at -80°C, thawed and quantified by spectrophotometry at 260 nm using a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). 1 µg of RNA was used for mRNA-specific cDNA synthesis using oligo-dT(18) primers (Thermo Fisher Scientific/Fermentas, Waltham, MA). Quantitation relied on SYBR Green I dye (Roche Diagnostics, Basel, Switzerland) used according to the manufacturer’s protocol. GAPDH and β-actin were used as reference genes. The following qPCR primer sequences were used:

**ERK:** CCAATGTGCTTCATCGCGACCT (forward) and CTGCAACACGAGCCAGTCCG (reverse).

**GAPDH:** GTCAAGGCTGAGAACGGGAA (forward) and GCCCATTTGATGTTGCTGGG (reverse).

**β-actin:** CACAGATCATGTTTGAGACCTT (forward) and CATCACAATACCAGTGTTACG (reverse).

3.4 MTS assay

Cells used for proliferation measurements were subjected to 20 µg MTS substrate per well after 24 h of treatment with either FBS or human recombinant IGF-1 (Sigma Aldrich, Saint Louis, MO).
The substrate is reduced by mitochondrial enzymes and forms a coloured product with maximal absorbance at 490 nm. This assay quantifies cell viability, and was used as an indirect measurement of cell proliferation. Absorbance at 490 nm was measured in an ASYS UVM 340 (Biochrom, UK) after 2, 3 and 4 hours, and the 3 hour measurement was used to calculate the results.

### 3.5 Data analyses

qPCR results were calculated using two reference genes (GAPDH and β-actin) and the comparative Ct method. Results were then normalised to the average value of control-transfected cells whenever possible. In some early tests on cell density, results were instead normalised to the average of non-transfected cells.

MTS assay results were normalised to untreated, co-transfected cells seeded from the same cell sample to correct for any unintentional differences in seeding density. Results are shown as relative proliferation as compared to the absorbances of untreated, control-transfected cells.

Statistical significance was tested using Student’s t-test and p<0.05 was considered significant.

Calculations were performed and graphs produced using Gnumeric Spreadsheet 1.10.16 (Gnome Project).

### 4 Results

#### 4.1 Establishing functional transfection

To establish a working transfection protocol and identify an efficient siRNA sequence, transfections were attempted at varying cell densities with three different siRNAs. While low to medium cell densities are often recommended for transfection, higher cell numbers are beneficial for qPCR preparations as they will produce higher RNA yields. Cells were lysed 24 hours after transfection and ERK expression levels were quantified by qPCR. At the first two densities tested, 500,000 cells and 300,000 cells per 35-mm well respectively, non-transfected, control-transfected and expected knockdown cells all showed similar levels of ERK expression. Transfections were attempted in cells with different passage numbers (1, 7 and 11) and from different isolations, but this did not appear to affect the results.

Cell density was then reduced to 150,000 cells/well and cell lysate from two wells pooled into each sample to yield sufficiently high RNA concentrations for cDNA synthesis. Figure 1 shows the different levels of ERK expression, where all three siRNAs appear to knock down ERK levels compared to control-transfected cells.

In parallel with the qPCR assays, transfection efficiency was also examined separately by using a fluorescent control. Cardiomyocytes were seeded on a gelatin-coated chamber slide at four different cell densities corresponding to 150,000, 300,000, 450,000 and 600,000 cells per 35-mm well and transfected with a fluorescent negative control siRNA. However, fluorescence microscopy revealed that transfection seems to be taking place at all four confluencies. It is unclear why efficient transfection did not appear to elicit a knockdown effect at higher cell densities.

#### 4.2 Knockdown of ERK

Transfections were repeated at 150,000 cells/well before a specific siRNA was chosen for further studies. All three siRNAs produced significant knockdown effects compared to the negative control (Figure 2). siRNA 3 achieved the most efficient average knockdown at 48% of control expression levels, although with higher variation (SD: 27%). siRNA 1 on the other hand was somewhat less effective at 55%, but showed the least variation (SD: 12%). siRNA 2 was the least interesting alternative at 58% (SD: 32%) ERK expression. However, all siRNAs had not been tested the same
amount of times, and siRNA 3 was chosen for further studies as it was the most effective on average and had been tested the most (6 samples as compared to 4 each for siRNAs 1 and 2).

It is feasible that further optimisation of the transfection procedure could increase knockdown efficiency even further. Transfections were performed at both higher and lower siRNA concentrations, but results were inconclusive, did not appear to increase efficiency and were eventually abandoned because of time constraints. It is also possible that these are the maximum efficiencies of the tested siRNA sequences, and that a differently designed siRNA would be needed to reach the 14% expression level that would correspond to a tinamou cardiomyocyte. In some cases, siRNA machinery has been reported to be limiting for highly expressed transcripts, although this would be rare (McManus & Sharp 2002).

**Figure 1** – Transfections duplicates performed at a cell density of 150,000 cells/well reduced ERK expression to approximately half. These results were the first time a knockdown effect could be detected and experiments were continued at this level of confluency.

**Figure 2** – ERK expression levels in transfected cardiomyocytes. All three siRNAs produce significant knockdown effects (p=0.0007, 0.03 and 0.002 respectively). 6 samples used for control and siRNA 3, 4 samples for siRNA 1 and siRNA 2. Data from Figure 1 has been included in this graph.
4.3 Effects on proliferation

The effects of ERK knockdown on proliferation were tested after two different protocols: a shorter one where only proliferation was assayed, and a longer one where MTS and qPCR assays were performed on cells from the same transfection. In the first one, cells were transfected directly in a 96-well plate, leaving approximately 72 h between transfection and the MTS proliferation assay. Cells were then treated with two different concentrations of IGF-1 and FBS respectively, and while FBS treatment increased proliferation no differences could be found between knockdown and control cells.

In the second setup, cells were transfected in 6-well plates at the previously established cell density of 150,000 cells/well, trypsinated and then re-seeded in a 96-well plate. In this case a full week had passed when cells were finally subjected to the MTS assay. During re-seeding (24 h after transfection) all the cells were counted, and had reached similar cell numbers, indicating no immediate toxic effects on the cells or innate differences in viability between the samples. Cell counts were approximately double that which had been seeded, but this proliferation is expected to have occurred during the 48 h adherence window when serum was present and would thus not reflect any transfection-related effect. After adherence, cells were subjected to 100 ng/ml IGF-1 or 10% FBS, both equal to the higher concentrations used in the first proliferation protocol.

Absorbance measurements from the MTS assay were first normalised to untreated cells from the same original sample to correct for possible differences in cell numbers during re-seeding. Results were then compared to those of untreated, control-transfected cells. As shown in Figure 3, IGF-1 induced an average increase in proliferation of 20.3% in control-transfected cells. In knockdown cells the effect was reduced to a mean of 10.1%, a decrease that is statistically significant at p=0.009. It is interesting to note that the decrease in IGF-1 effect shown here is approximately proportional to the achieved ERK knockdown at the transcriptional level.

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FBS has a stronger pro-proliferative effect (see Figure 4). In control-transfected cells, FBS increases proliferation by an average of 64.3%. Partial ERK knockdown reduces this to 49.2%, also a statistically significant effect at p=0.006. In terms of percentage points (both IGF-1 and FBS results are normalised to the same untreated control group of cells), this decrease is somewhat larger than in the IGF-1 experiment. This is expected, as part of the pro-hyperplastic effect of serum is expected to be the result of IGF-1 contained in the serum.
5 Discussion

The results of this project indicate that even a partial knockdown of ERK in embryonic cardiomyocytes reduces the cells’ ability to respond to proliferative stimuli. This agrees with previous studies that point out ERK itself as the rate-limiting step of the ERK cascade, as an excess of ERK would have been able to counteract the effects of the knockdown. A similar argument was made by Glennon et al. (1996) after seeing 50% protein activity reduction at 90% protein-level knockdown. While FBS has a stronger stimulatory effect than IGF-1, the ERK knockdown in these experiments decreases the effects of both similarly (by 10% and 15% respectively). This corresponds to half the effect of IGF-1 and about a quarter of the effect of FBS. As IGF-1 is known to be present in FBS, some of the proliferative effects of serum can be expected to be due to this Kajstura et al. (1994). From the rather large differences in magnitude in the responses it would seem, however, that other constituents of the serum also contribute, and that the effects of some of these are also inhibited by the ERK knockdown. While it would still be interesting to test the effects of a near-complete interference of ERK expression, especially as it would more closely resemble the situation in the small-hearted tinamous, these results offer a proof of concept that proliferative stimulation by IGF-1 and FBS are at least partly dependent on the ERK cascade for their respective effects.

Previously, ERK has mostly been implicated in hypertrophic growth. This is not surprising as that is the type of cardiac growth typically relevant in clinical settings. However, several studies indicate that ERK may be equally important in antenatal and non-mammalian cardiomyocytes where hyperplasia is the main mode of heart growth, and that ERK is a major mediator of all cardiac growth Reiss et al. 1996, Li et al. 1997, Sundgren et al. 2003a. For the embryonic chicken cardiomyocytes used in these experiments, and probably for most avian cardiomyocytes, hyperplasia is the main mode of growth available and the type of growth expected to be induced by increased load on the heart, whether due to an increase in body mass during developmental growth or strenuous activity Li et al. 1997. Because of the bottleneck nature of the ERK pathway in this process, the low ERK expression levels in tinamous hearts will make these cardiomyocytes less sensitive to proliferative stimulation. Even when growth or other forms of physiological stress challenge the tinamous heart and trigger the release of growth factors, the low ERK levels can be expected to severely limit the cardiac growth response. This naturally imposes a major constraint on the activity and lifestyle of the tinamous.

When it comes to further developing the protocol, it is interesting to note that no effects of the
knockdown were observed in the proliferation assay performed 3 days after transfection. Glennon et al. (1996) achieved protein-level depletion after 48 h and maximal effect 72 h after ERK knockdown in rat cardiomyocytes. This time span agrees well with what is generally recommended for protein-level assays by RNAi suppliers. Assuming that no other variable inadvertently affected these two 96-well plates, it is possible that avian ERK has a slower turnover rate than its mammalian counterparts. This would make it necessary to leave more time between transfection and the MTS assay. It is also possible that the time in starvation is affecting ERK levels in control cells, effectively making them indistinguishable from knockdown cells. If so, it is the recovery time in FBS-supplemented medium that needs monitoring. Because of differences in length between transfection periods, Glennon’s cells had had 64 h of recovery time in serum-supplemented media when measured while the cells used in my experiments had only had 48 h to recover. Reproducing these experiments allowing for more or less time in between procedures should effectively clear up this uncertainty in the future. Since no protein-level assay was performed in this study, adding one to future experiments should also help resolve these questions.

The discrepancy in estimated efficiency of transfection between qPCR measurements and fluorescence microscopy results is another question that may need more investigation. Although the amount of available of cell surface area could conceivably affect lipofection efficiency, both the results of others and the confocal imaging indicates that transfections are taking place even in near-confluent cells. Several other factors may of course affect the qPCR results. Because the full protocol from cell seeding to qPCR assay takes around a week it is difficult to double-check every step along the way, and the risk of introducing undesirable variables is comparably high. From this perspective the fluorescence microscopy may give a better estimate of the transfection efficiency itself, although this is not very useful without any quantitation of knockdown. RNA quality was tested just before cDNA synthesis in an early run of the experiment and found to be excellent. The most probable source of qPCR problems is therefore remaining ethanol contamination that affects either cDNA synthesis, qPCR or both.

6 Acknowledgements

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7 Bibliography


8 Appendix 1: mRNA sequence of *G. gallus* MAPK1 (ERK)

The sequences targeted by siRNA in these experiments are written in red. NCBI accession number is NM_204150.1.

ATGGCGCAGGCGCTGCTAGGGCCGATGTTGCTCGGGGCAAGGCAGGATGTGCGGGGCCAAGTGGACGTTGCTACACCAATCTCCTACGACGAGGGGGCGTACGGCATGGTGTTCTGCCTACGATAACGTCAACAAAGTTCGAGTTGCTATAAAGAAAATCAGTCCTTTTGAGCATCAGACGTACTGCCAGAGAACTCTGAGAGAGATAAAGATCTTACTGCGCTTCAGGACCTCAAACCTTGAAATTTCG

ACGTACTGCACAGAGACCTGAGAGAGATAAAGATCTTACTGCGCTTCAGGACCTCAAACCTTGAAATTTCG

AAATGATATTTATCAAGAGCTCACAAGTTGAAACAAATGAAAAGGATAGCTATACATTTGCGAAAGCCTTTATTG

AACAGACTCTTTACAAAGCTCTTTAAGACTCACAACCTCAGCAACGCCACATTTTGTATTTTTATTTACCAG

ATTCTGAGAGCTTAAATATACATCCATACGCCAATAGCTGCTACTGCGACCTCAAACCTTGAAATTTCG

TGCTTAATACACTTGAGTCTCAAGATATTGTGACTTTGGACTGGCTGCGTTGCCAGATCCGCAACCATGA

TCACACAGGATTTGCTCACAGAGTATGTGGCACAAGTTGGTAGACAGGCTCTGAAAAATCATGCTGAAATTCT

AAGGGTACACCACGACATCTGGACTCGAGCTCTATTCTGAGGATAGCTCTCAGACATTACCAGGACTTTCCCTG

CCATTTTACGAGAAAACACTACCTTCAGCGTCATACCATTTTTGTAACATTTTTGATCCCATACCCCTTCCA

AGAAGATTGGAATTTGATATTAAATTTTTAAGGCAAAGAATTTTTGTTCTCTCCCTCCCAACACAAATAAAG

GTACCATGGAACAGGCTGTTTCCCAATGCTGACCCCAAAGCCTTGATCTGTTGGATAAAATGTTGACCT

TTAATCTTACAAAGGGAATTGGAATGCTGGAGGAGCTTTTAGCCCATACCATATCTGGAAAGATGATACGC

AAAGTGATGAGCCTGTAGCTGAAGCACCCTTCAAGTTTGATATGGAATTGGATGACTTGCCGAAGGAAAAG

CTGAAAGAAGCTGATTTTGGAGGAAACTAGCATTTCCAGCCAGGATATCGATCTTAA