Investigating the Role of Rho Kinase in Cardiomyocytes during Heart Development

Kathleen Christie* 120039014 BSc Biomedical Genetics
Kate Bailey, Dr Helen Phillips
k.a.christie@ncl.ac.uk

Introduction

Congenital heart anomalies are one of the most common birth defects, affecting 1 in 145 births. Adult cardiovascular disease accounts for approximately 1/3 of all deaths. It is thought that some non-lethal defects that occur in embryonic development can result in cardiovascular disease later in life. In light of these facts, it is vital that we study the heart and gain a better understanding of why these defects can occur.

Heart development is a complex process. My project focussed on the development of the muscle layer of the heart, known as the myocardium. The myocardium begins as a thin layer of muscle cells (cardiomyocytes). The myocardium thickens during development as the cardiomyocytes proliferate (Zhang *et al.,* 2013). In the mouse the heart is fully formed by embryonic E15.5 day as shown by a transverse section through the heart in Figure 1. If the myocardium does not thicken properly it will not be able to efficiently pump blood around the body.

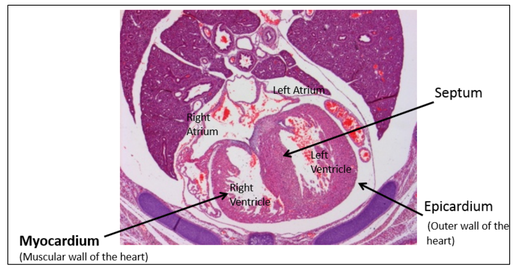

Figure 1: Haematoxylin and eosin paraffin stained section to show the structure of the heart.

This project entailed understanding the function of the gene, Rho Kinase (ROCK) specifically within the heart during embryonic development. ROCK has roles in many cellular functions which have been shown in Figure 2 and is known to be expressed within the developing heart (Amano *et al.,* 2010). It has been shown that Rho Kinase is upregulated in cardiomyopathy (Shi *et al.,* 2011), so it is vital that we understand its role in heart development.

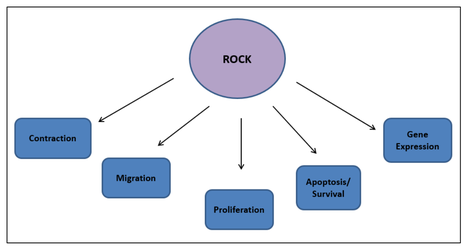

Figure 2: Cellular functions of Rho Kinase (ROCK)

Aims

To determine the phenotype of a mutant mouse in which ROCK had been downregulated specifically in the cardiomyocytes. This was achieved using the *TnT-Cre* (Jiao *et al.,* 2003) mouse, which is expressed only in the myocardium, crossed with the transgenic *ROCKDN* mouse (Kobayashi et al 2004).

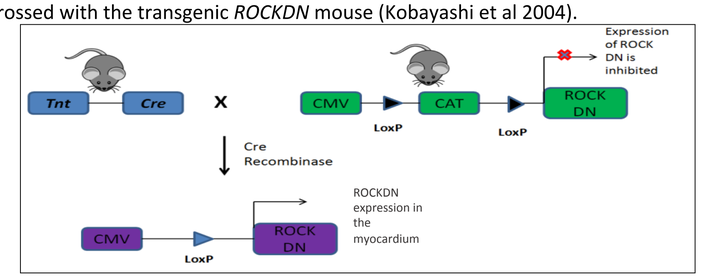

Figure 3: A mouse expressing *Cre* under control of a gene specific promoter (for e.g. *Tnt*; specific for cardiomyocytes) is crossed with a *ROCKDN* transgenic mouse. In the mutant mouse embryos which express *Cre* and *ROCKDN*, the *ROCKDN* construct is activated by the removal of the CAT box. The *ROCKDN* protein binds to and inactivates the endogenous ROCK protein present in the cardiomyocytes.

Methods

The *ROCKDN* and *Tnt-Cre* mice were mated and embryos were collected at different developmental ages from embryonic day (E)10.5 to E15.5.

For each embryo:

- DNA was extracted followed by PCR using specific primers – this enabled the genotype of each embryo to be determined
- Embryos were processed and embedded in paraffin wax
- Transverse sections (Figure 4) were collected onto slides

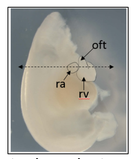

Figure 4: A mouse embryo collected to study heart development. The line indicates where the embryo is transversely sectioned. Oft = out flow tract, ra= right atrium, rv= right ventricle.

- Histological analysis was performed – Haematoxylin and Eosin staining (Figure 5A) to look at the morphology of the heart and Immunohistochemistry (Figure 5B) using the α smooth muscle actin (α sma) antibody to specifically label the cardiomyocytes

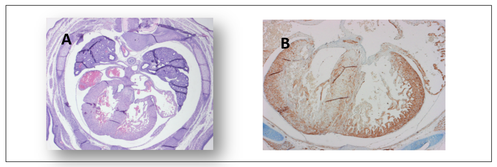

Figure 5: **A –** A Haematoxylin and Eosin paraffin stained section. **B –** A section stained by immunohistochemistry using an α sma antibody.

- Microscopy – allowed visualisation of structural differences in the hearts

Results

At E10.5, the myocardium is thin (arrow in Figure 6) and the chambers of the heart have not septated. By E12.5, the myocardium has begun to thicken and the septum is developing (asterisk in Figure 6ii). At these early stages of embryonic development it was not easy to differentiate between mutants and controls (Figure 6i and ii). However, at E14.5, there were obvious differences between the mutant and control heart structures. The septum and myocardium of the right and left ventricles of the E14.5 mutant embryo is much thinner than that of the control (Figure 6iii). The heart is also dilated and full of blood, which is a sign that the embryo is dying.

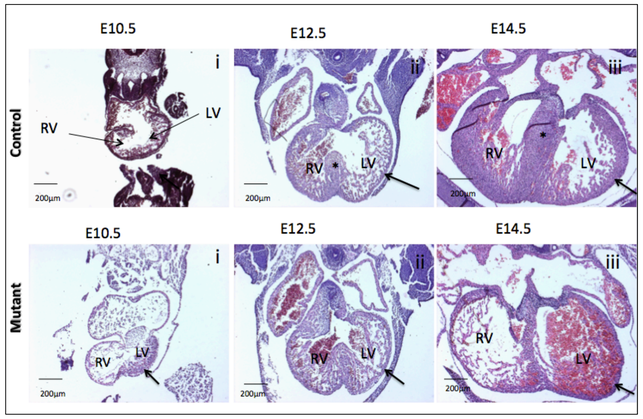

Figure 6: Haematoxylin and Eosin stained paraffin sections showing the contrast in the thickness of the myocardium between the mutants and controls across different developmental ages. The arrows indicate the myocardium in i) E10.5, ii) E12.5 and iii) E14.5. The asterisk indicates where the septum is in the E12.5 and E14.5 hearts. RV = right ventricle and LV = left ventricle.

Statistical analysis was performed by measuring the thickness of the myocardium for both the left and right ventricle on 3 sections for each embryo, then an average was calculated and a t test was used to determine the significance (Figure 7).

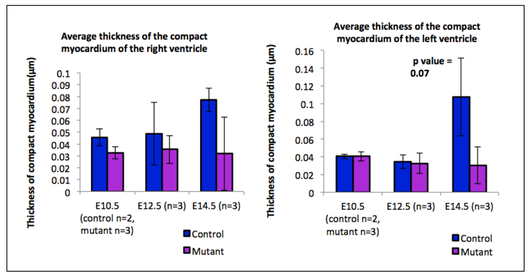

Figure 7: Bar chart showing the difference in average thickness of the compact myocardium in the i) left ventricle and ii) right ventricle between controls and mutants across different developmental ages. I performed a t test, it showed there is no significant difference between the thickness of the myocardium at E10.5 and E12.5 however the decrease in thickness at E14.5 in the left ventricle of the mutant embryo is close to significance (p=0.07).

Conclusions and Further Study

- These findings suggest ROCK plays an important role in the cardiomyocytes during the maturation of the myocardium.
- Downregulating ROCK in the cardiomyocytes results in a thin compact myocardium.
- This occurs at some point between E12.5 and E14.5.
- ROCK signaling is essential for the expansion of the myocardium and is an integral part of heart development. ROCK is known to be involved in controlling cell proliferation and cell death (Zhao and Rivkees, 2003).
- Future work could include looking at the proliferating cells using phospho-Histone H3 antibody and cell death could also be investigated using the caspase 3 antibody. Additionally, embryos could be collected later in development to determine if the mutants recover and survive or if the phenotype is too severe.

Acknowledgements

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