# Investigating Differential Gene Expression in Cardiac Developmental Defects which lead to Adult Cardiomyopathy

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## 1. Introduction

Cardiovascular disease in adults causes more than a quarter of all deaths in the UK. Non-lethal defects which can occur during embryonic heart development may result in cardiovascular disease later in life.

Inherited hypertrophic cardiomyopathy (HCM) is a disease which causes the muscle layer of the heart (myocardium) to become thickened, leading to heart failure. This project focused on a hypothesis that Rho Kinase (ROCK) has a role in HCM development. ROCK is expressed throughout the heart, has a role in heart development, and is involved in fundamental cellular functions.

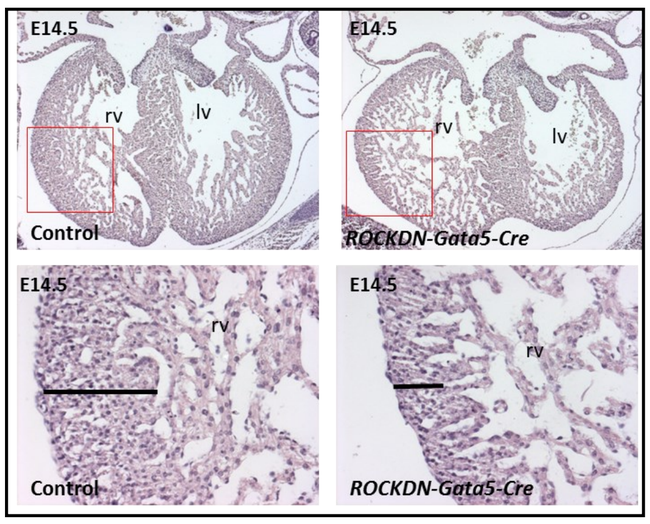


**Figure 1: *ROCKDN* mutants have a thinner myocardium compared to controls**

Using transgenic mouse lines (*Gata5-Cre* and *Tnt-Cre*), ROCK can be downregulated within the myocardium and epicardium (the outer layer of the heart). *ROCKDN* mutants have a thinner myocardium layer (**Figure 1**)and a non-lethal developmental heart defect subsequently developing HCM in adulthood.

A previous microarray experiment using RNA from embryonic day (E)10.5 hearts, identified genes whose expression level were significantly different in either *ROCKDN;Gata5-Cre, ROCKDN;Tnt-Cre* or both mutants compared to control embryos. The expression pattern of 8 genes in the developing heart was studied to determine whether they could contribute to the development of HCM (**Table 1**).

| Gene name | Up or down regulated | Protein information | Location |
|---|---|---|---|
| **Ano3** | Upregulated in *ROCKDN:Tnt-Cre* | A potassium channel regulator. | In the cell membranes, particularly in neural regions. |
| **Itm2a** | Downregulated in *ROCKDN:Gata5-Cre*. | Involved in cellular differentiation. | In the cell membranes of the neonatal calvaria, paws, tail and skin. |
| **Nbbp** | Upregulated in *ROCKDN:Tnt-Cre* | Cardiac hormone which plays a role in cardiovascular homeostasis and cell growth in cardiac muscle. | In the nucleus, perinuclear cytoplasm of the ventricles, some expression in the atrium. |
| **Ptn** | Downregulated in *ROCKDN:Gata5-Cre* | Important in anti-apoptotic signalling and regulation of cell proliferation. | In the membrane, ER and cytoplasm of the neural epithelium. |
| **Slit3** | Downregulated in *ROCKDN;Gata5-Cre* | Acts as molecular guidance cue in cellular migration. | Expressed in the ventral neural tube, floor plate and motor columns. |
| **Tfc21** | Downregulated in *ROCKDN:Gata5-Cre* slightly upregulated in *ROCKDN:Tnt-Cre* | Involved in the organogenesis of the spleen and heart and in cardiac and coronary artery development. | In the nucleus of cells that surround the epithelium of the gastrointestinal, genitourinary, respiratory systems and in the septum of the heart and in epicardial precursor cells. |
| **Tns3** | Downregulated in *ROCKDN:Gata5-Cre* | Involved in actin remodelling. May have a role in cell migration and bone development. | In the cytoplasm of the lung, liver, spleen, stomach, aorta, trachea, and perichondrium. |
| **Trp53inp1** | Upregulated in *ROCKDN:Tnt-Cre* | An apoptotic stimulator which can also bring about cell cycle arrest. | In the cytoplasm and the nucleus throughout the organism. |

**Table 1: information on each gene in situ hybridisation analysis was done on. Information from Uniprot.**

## 2. Aims

To investigate the expression pattern of genes identified from a microarray study of wild type and Rho kinase mutant embryos within the developing heart. This was achieved by using two genetic mouse lines (**Figure 2**).

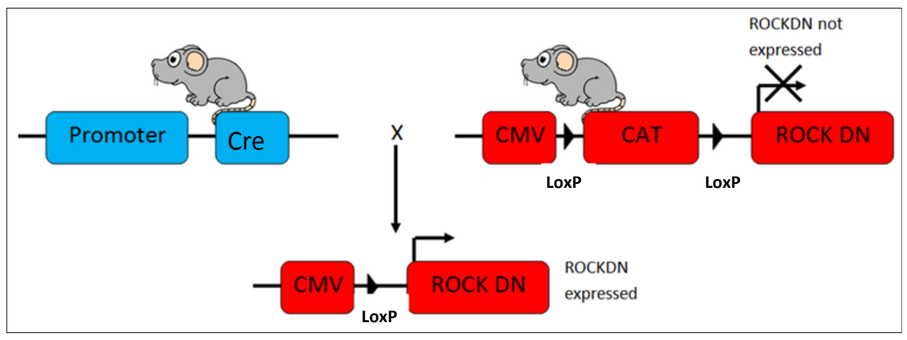


**Figure 2: Transgenic mouse model to downregulate ROCK. The Cre recombinase is downstream of a specific promoter; *Gata5* specific to the epicardium and myocardium or *Tnt* specific to the myocardium. In the presence of *Cre*, the CAT box is removed allowing for the expression of *ROCKDN* which binds and inactivates endogenous ROCK protein.**

## 3. Methods

Primers were designed and tested by PCR for the genes of interest. DIG–labelled probes were made using the PCR products of working primers

Mice were mated and embryos collected at embryonic day E10.5. Embryos were processed and embedded in paraffin wax. Transverse sections were collected onto slides.

*In situ* hybridisation was performed on the slides using the DIG-labelled probes made. Microscopy allowed for the visualisation of where the genes were expressed.

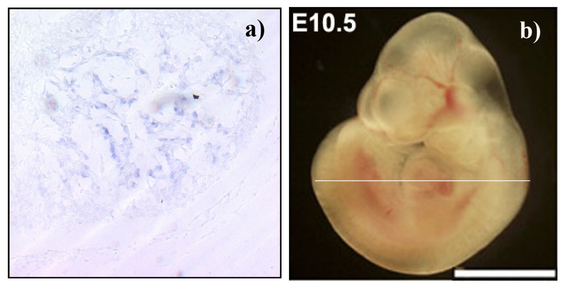


**Figure 3: a) An example of staining from *In situ* hybridisation within the myocardium of wild type mice using the control probe *Bmp10.* b) an image of a mouse embryo at E10.5 demonstrating the orientation of sectioning, (white line) used in this project (Embryology.med.unsw.edu.au, 2015).**

## 4. Results

The expression pattern in control embryos was examined by *in situ* hybridisation to determine where within the heart each gene of interest was expressed. Each gene was done in triplicate.

A negative control, with no probe included, was used to establish whether non-specific background staining was present.

| Gene name | Location of expression within hearts | Subcellular location |
|---|---|---|
| ***Ano3*** | Epicardium and myocardium | Cytoplasm |
| ***Itm2a*** | Myocardium, and to a lesser extent the epicardium | Cytoplasm |
| ***Nbbp*** | Trabeculae myocardium | Nucleus |
| ***Ptn*** | Epicardium | Cytoplasm |
| ***Slit3*** | Epicardium and myocardium | Nucleus |
| ***Tfc21*** | Epicardium and myocardium | Nucleus |
| ***Tns3*** | Epicardium | Cytoplasm |
| ***Trp53inp1*** | Myocardium, and to a lesser extent the epicardium | Cytoplasm |

**Table 2: The location of staining for each gene within the wild type mouse heart**

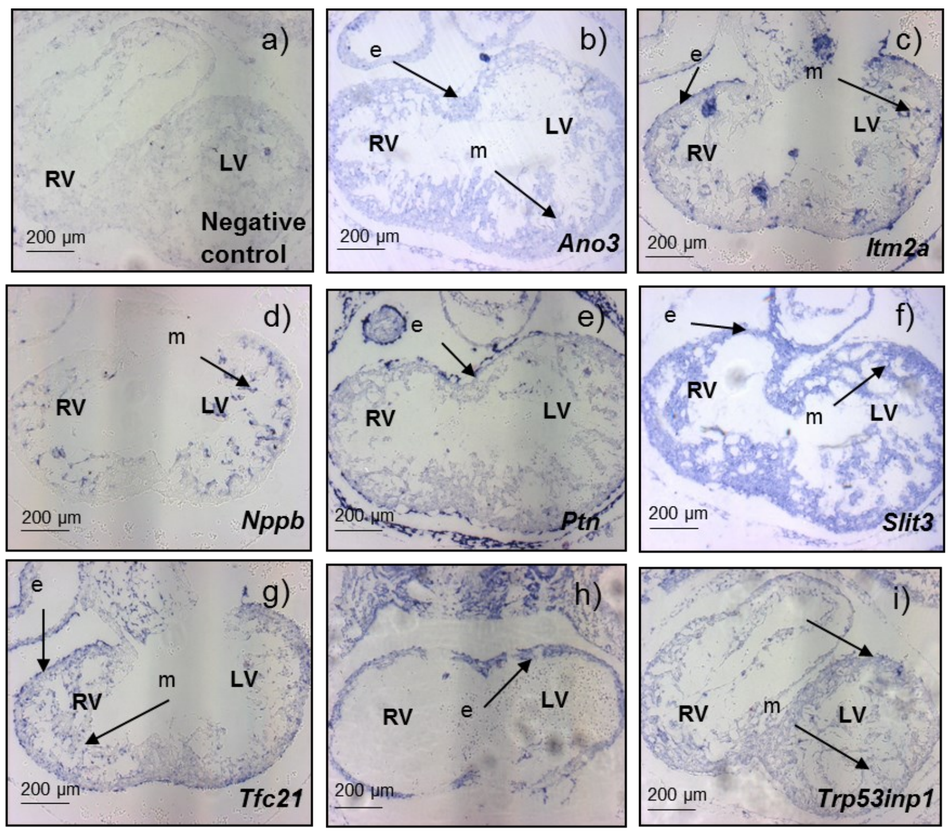


**Figure 4: a) A negative control in which no probe was included, was used to establish the level of any non-specific background staining. b) *Ano3* is expressed in the epicardium and myocardium of the heart at E10.5. c) *Itm2a* is expressed in the epicardium and, to a lesser extent, myocardium of the heart at E10.5. d) *Nppb* is expressed in nucleus the myocardium of the heart at E10.5. e) *Ptn* is expressed in the epicardium of the heart at E10.5. f) *Slit3* is expressed in the nucleus of the epicardium and myocardium of the heart at E10.5. g) *Tfc21* is expressed in the nucleus of the epicardium and myocardium of the heart at E10.5. h) *Tns3* is expressed in the epicardium of the heart at E10.5. i) *Trp53inp1* is expressed in the myocardium and to a lesser extend the epicardium of the heart at E10.5. Images were taken at magnification x10.**

**e = epicardium; m = myocardium; LV = left ventricle; RV = right ventricle.**

## 5. Conclusions

Each gene tested was found to be expressed in the developing heart of control embryos suggesting the results from the microarray were not false positives.

All of the genes studied, except *Itm2a*, have functions which may lead to the phenotype observed in *ROCKDN* mutants (thinner myocardium and longer trabeculae).

- The proteins encoded by the genes: *Ano3, Slit3* and *Tns3* have functions related to cell proliferation.
- The protein encoded by *Nppb* has roles in cardiac muscle development and ventricular remodelling.
- Proteins encoded by the genes *Ptn* and *Trp53inp1* have functions that control apoptosis.
- The protein encoded by *Tfc21* has a function related to organogenesis, which allows for the growth of organs.

The functions of the above genes and the results from the microarray could explain the phenotype observed in *ROCKDN* mutants.

## 6. Future work

Future work could include looking at the expression of the genes, which were expressed convincingly in control embryos (all apart from *Ano3* and *Slit3*) in *ROCKDN* mutant embryos to confirm whether expression level is altered as suggested by the microarray.

## Acknowledgments and references

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