Validation of potential downstream targets of Pax9

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Introduction

Congenital heart disease is a major cause of childhood death, and occurs in up to 9 in 1000 births in the UK. It has been found that knocking out of Pax9 in mice causes complex congenital defects in the aortic arch arteries. Mouse models are used as they have genetic similarities to humans and can be manipulated genetically to produce similar disease states to those found in humans. Pax9 is a transcription factor which is expressed in the pharyngeal endoderm during development and becomes restricted to the pharyngeal pouches at E9. To increase understanding of how the pharyngeal endoderm controls cardiovascular development, we identified differentially expressed genes in Pax9 null pharyngeal arches using RNAseq and selected 10 genes for validation with qPCR.

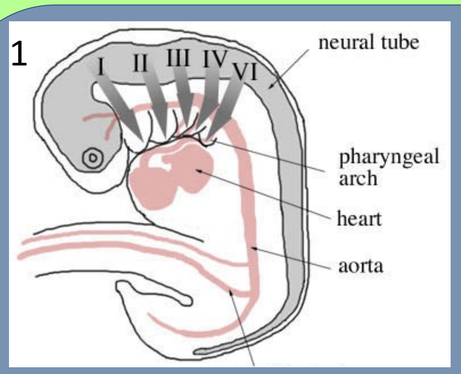

Figure 1: Shows the location of the pharyngeal arches, heart and aorta in the developing embryo (Gilbert, 1994)

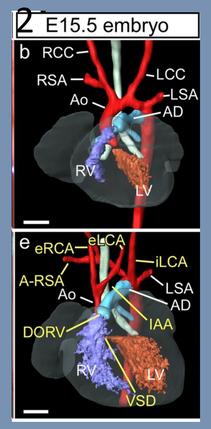

Figure 2: Images comparing wild type and Pax9 null cardiovascular structures at E15.5. The Pax9 null shows ventricular septal defect, double-outlet right ventricle, hypoplastic aorta, interruption of the aortic arch, incorrect formation of the right subclavian artery and absent common carotid arteries.

Aims

- To measure the expression levels of our 10 genes of interest in wild type and Pax9 null mice
- To determine if there is a statistically significant change in expression in any of the genes of interest, and whether this could contribute to disease.

Methods

Stage matched E9.5 embryos (embryos that have been fertilized 9 days before extraction), were collected from the wild type and Pax9 nulls. E9.5 is a critical stage in the development of the aortic arch arteries.

The pharyngeal arches were removed and snap-frozen. The mice were genotyped by carrying out PCR to identify the mutant and wild type embryos.

PCR is a process which is used to scale up DNA samples. It uses primers and nucleotides to produce copies of a DNA sample when rotated through cycles of different temperatures.. qPCR is a similar process in which the amounts of DNA present are measured to give a numerical value using fluorescent dye to label the DNA during the cycling process.

RNA was extracted from the snap-frozen aortic arches using and was tested for concentration and quality by use of a spectrophotometer.

Reverse transcription was then carried out on 500ng of the extracted RNA to produce cDNA copies of the transcript.

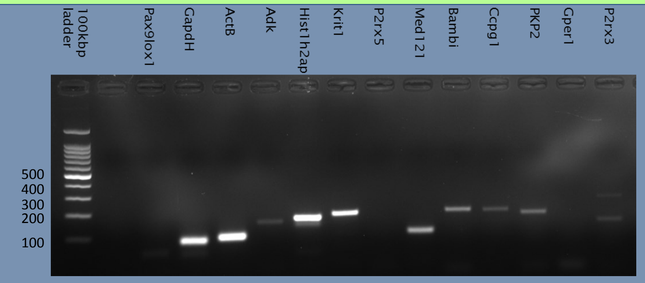

Figure 3: Image of the agarose gel electrophoresis used to see if the genes and their specific primers were working and suitable for qPCR

The primers were tested using PCR in order to examine if a noticeable level of cDNA was present, that the primers were functional and that the product was of the correct size.

The primers which were found to be effective were used in the qPCR. The genes of interest were tested along with housekeeping genes ActB and B2M in order to investigate the change in expression between the Pax9 null and the wild type. The qPCR tested four mutant and four wild type cDNA samples.

The results were normalised using the expression levels of the housekeeping gene ActB. The data was processed and the statistical significance of the differences between the mutant and the wild type were calculated.

Results

We started with 10 genes of interest, five which were up-reglated in the RNAseq and five which were down-regulated.

Up-regulated genes: Hist1h2ap, Bambi, PKP2, Krit1, Adk

Down-regulated gene: P2rx5, Gper1, P2rx3, MED121, Ccpg1

The primers for Adk, P2rx5, Gper1 and P2rx3 did not pass the initial quality control in the PCR examination and so were not tested in the qPCR.

Hist1h2ap was found to have increased expression as suggested by the RNAseq. RNAseq suggested increased expression of PKP2, Bambi and Krit1, but these were found to be reduced. MED121 and Ccpg1 were both predicted to show reduced expression which was shown in the qPCR.

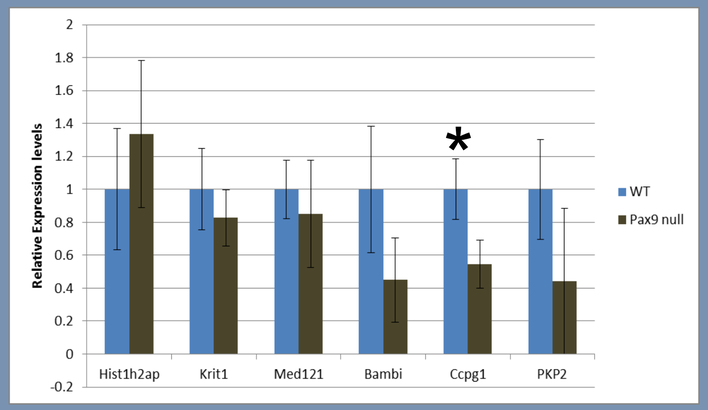

Figure 4: Graphical representation of the qPCR analysis. Only Ccpg1 levels were significantly changed. (*P<0.05).

The experiment produced a single statistically significant result – the decrease in Ccpg1 expression in the mutant. This had a significance value of 0.0491.

Discussion

Ccpg1 expression is reduced to 54.7% of that seen in the wild type. Ccpg1 is a scaffold protein which has a role in the assembly of Rho protein signalling complexes and interacts with Dbs.

Dbs has in vitro guanine exchange activity which is specific to RhoA and Cdc42. When Ccpg1 is expressed with Dbs, it binds the pleckstrin homology of Dbs and inhibits its guanine exchange activity with RhoA specifically and also inhibits transcriptional activation by Dbs.

Ccpg1 expression can affect the expression of other genes and acts to regulate the cell cycle and division processes. When expression is reduced in Pax9 nulls, this could be disturbing the regulatory activity and changes in cell division may contribute to the disease phenotype.

Conclusion

The knockout of Pax9 produces a reduction in the expression of Ccpg1. Ccpg1 is involved in the activation of a number of pathways and can affect expression of other genes and cell division processes. The changes to Ccpg1 expression may contribute to disease, but further study of Ccpg1 and it's role in cardiac development would be required.

Acknowlegements

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