FAYE

A clinical study was conducted at the University of Sheffield in which a group of patients presented at hospital with complaints of chest pains. All these patients were found to have atherosclerosis, a hardening of the arteries caused by fatty deposits, however only half the patients had suffered a heart attack. The other half of the patients then acted as a control group.

Over a 90 day period, gene expression patterns of both groups were analysed using AFFymetrix gene chips. This was done by extracting messenger RNA from blood samples, labelling the RNA fluorescently and hybridising it to the gene chip. After scanning, the two data sets were compared. Genes showing increased or decreased expression, relative to the control, were highlighted and statistically analysed by Dr Marta Milo. Although 82 genes were found to have significant differences in expression,

To decide which of these genes to study At Tapton we used ensembl, an open source genetic database to identify zebrafish orthologs by performing sequence alignments. We chose orthologs which had a high percentage identity to humans which means they are more likely to perform the same function

We have decided to investigate CDKL1 as this was the most differentially expressed, persisting for 30 days.

CDKL 1(cyclin dependent Kinase 1) has been classed as Cyclin dependent “like” as it contains the conserved domain for binding cyclin “KKIARLE” as well as a kinase domain. Its structure is also shown to be similar to human CDK2. It is a novel gene as no function has been assigned to it however the cyclin dependent kinase family of proteins are very important in controlling the cell cycle.

**This means that anything we find out about this gene is genuinely real science and we will be part of the scientific process!!!!!**

**Nicola**

Why zebrafish?

There are several reasons why we use zebrafish as our model organism to investigate heart disease.

\* Well firstly, zebrafish are transparent organisms, so the development of the blood vessels and the staining of the hypochord and notochord are easily visible down a microscope.

\* Secondly, zebrafish embryos develop rapidly so all of their major organs and systems, including the cardiovascular system, have developed within 36 hours post fertilisation. This means the development of the dorsal aorta, which is the main blood vessel, is easier to observe.

\* Some experiments used when studying the cardiovascular system involve restricting blood flow. It is therefore essential that the effects of blood flow are separate from the effects of a lack of oxygen, which is called hypoxia. In mammals, a lack of oxygen causes ischaemia, and results in death. Zebrafish embryos, however, are small enough to survive in hypoxic conditions, as oxygen diffusion can occur across their outer membrane?

\* Also, zebrafish embryos are still attached to the yolk sack, meaning that they are not free feeding. This makes ethics approval easier to obtain. Also, zebrafish breeding programmes are cheaper than those in mammals for example, and the specimens take up less laboratory space.

\* We are closely supported by Dr Tim Chico’s lab at The University of Sheffield. They provide us with fixed embryos and the zebrafish mutants.

\* Finally, recent studies suggest that zebrafish develop collateral blood vessels (arteriogenesis) to bypass an arterial block.

**Eloise**

In-situ hybridisation is a technique used to identify where a gene is being expressed, using the fact that if it is be being expressed then mRNA will be transcribed in that location.

(Mention that this is a method that they have learnt about or will learn about in their A levels)

A DNA template for CDKL1 was PCR amplified, a probe synthesised and a chemical called digoxygenin attached to the probe, before applying it to the fixed zebra fish embryos. The probe hybridised to the mRNA where CDKL1 was being expressed. We then washed the embryos with a monoclonal antibody, complementary to DIG, which had an enzyme attached. Finally we washed the embryos with a colourless substrate to the enzyme which went blue where CDKL1 was expressed.

Our results show CDKL1 expression in day 1 and 2 post fertilisation embryos, but not day 3. Expression was localised to the hypochord and the notochord. The hypochord signals the development of blood vessels in adjacent tissue and is reported to be involved in signalling for the development of the dorsal aorta. Furthermore, this expression regresses anterior to posterior- the same direction that the dorsal aorta develops.

We also looked at CDKl1 expression in VHL mutant hypoxic embryos with increased blood vessel formation and observed that it was upregulated, which again suggests a link between CDKL1 and blood vessel development.

**This data is quite remarkable as we had a list of 80 genes to investigate and with our first screen we have identified a gene that is linked to Human CVD and have evidence for its role in the development of the cardiovasculature of the Zebrafish**

**Khadija**

This figure was taken from a paper *(Hsu et al 2008)* that performed a preliminary investigation into the role of CDKL1 in Zebrafish. Reverse genetics was used to determine CDKL1’s function; this means CDKL1 was inhibited or removed and phenotypic consequences were observed. Expression was removed using a technique called morphilino knockdown. This involves adding a short section of RNA, which is complementary to the target gene mRNA found in the cell. This prevents the attachment of the mRNA to the ribosome and hence the protein cannot be translated as there is no information being received from the mRNA.

This study was looking at the possible link to neuronal development by studying neurogenin and sonic hedgehog. We used the results of the effects of CDKL1 on shh to support our hypothesis. It was found that when CDKL1 is lost through morphilino knockdown, the levels of sonic hedgehog mRNA fell. As a control in all morphilino experiments, the mRNA that was inhibited is added back. On adding back CDKL1 mRNA, the levels on shh mRNA are restored.

Interestingly Sonic hedgehog signalling is involved in blood vessel development which involves signalling through the Vascular endothelial growth Factor VEGF and NOTCH.

These data demonstrate a possible role for CDKL1 to supersedes shh in signalling pathway. Because when CDKL1 is inhibited there are lower levels of shh would be present too.

**THIS IS EXCITING because not only do we now have a Novel gene linked to blood vessel formation but have a potential mechanism to investigate.**

**James**…………..

Negative feedback hypothesis

Before embarking on expensive time consuming experiments we decided to use GEO……

**Our hypothesis now seemingly is supported by other studies.**

**Sabba**

Now we had to confirm these results experimentally.

Essentially we were to repeat the GEO experiments but in zebrafish as oppose to just endothelial cells as in the graphs that James has just shared with you.

Our first candidate was VEGF…. Well actually we did inhibit Sonic Hedgehog but it turns out we had not done this correctly, so we are not showing this data.

VEGF signalling can be inhibited with AV951- our hypothesis would suggest that this drug should inhibit VEGF and thus via a negative feedback mechanism increase the presence of CDKL1.

Here are our results, the top panel is the control experiment and the lower is the drug treatment. Embryos were exposed to the drug treatment for 6hrs from 24 hours post fertilisation so the fish here are actually 30 hours old (?) I may come back to that!

I should also mention that these experiments were carried out in the Chico lab by one of our students on a summer placementas we do not have the home office licencse for this type of experiment…we only perform the Insitus on fixed embryos subsequent to any treatments.

It does not look like we have the expected effects as we had predicted. However I when performing these comparative In Situs the idea is that you stop the staining reaction once we see the staining. In these experiments we may have stopped the staining too soon as you can see we stopped staining once the Notochord appeared……perhaps if we had left it longer for the Hyperchord to stain then we would have seen a difference.

Alasdair,

Have you got all that?

So I am going to talk to you about our experiments with NOTCH, and its going to get a bit complicated.

NOTCH signalling enables cells to orientate themselves when growing across a surface. If we are considering Endothelial cells that line a blood vessel then these cells are one cell thick. The cells in development will grow across the vessel but when they contact each other they inhibit their growth which results in a single cellular layer …this is called Contact inhibition.

NOTCH is a membrane spanning protein with extracellular domains and intracellular domains. In this figure the signal sending cell has Delta another membrane spanning protein this will Interact with the NOTCH protein of the signal receiving cell… this results in the intracellular domain of NOTCH being cleaved which is now untethered from the membrane and can translocate to the nucleus to initiate gene expression which would change cellular behaviour such as the cessation of cell division.

We can inhibit NOTCH with the Drug DAPT, which is a gamma secretase inhibitor….gamma secretase cleaves the intracellular domain.

The results……….as before no real difference!!!

IONA

There is one other way we used to ask the same question. As oppose to inhibiting NOTCH signalling with a drug we did it genetically by looking for CDKL1 staining in a mutant zebrafish called MINDBOMB.

Mindbomb is a gene that codes for a Ubiquitin ligase… this basically targets proteins for degredation and recycling in the cell. Mindbomb is important in NOTCH signalling as it functions in the signal sending cell.

In this figure you can see that the internalisation (endocytosis) of the interacting Delta and NOTCH proteins is initiated by their ubiquitination by Mindbomb….this process is essential for the NOTCH signalling to proceed.

In Mindbomb mutants this recycling of the NOTCH extracellular domain does not occur and therefore the intracellular domain of OTCH can not be released to the nucleus so there is no effect of the cellular contact.

Our results……

In the mutant CDKL1 staining is reduced. Well this I suppose is better than no difference even though it refutes our hypothesis.

**We still think this is a promising result as CDKL1 was highlighted in a human trial, Mindbomb is important in veascular development in zebrafish and yet again CDKL1 is linked.**

**I think this has just shown us that science is very complicated- the cross talk between multiple signalling pathways is very difficult to dissect and everything that we attempt to change hasmultiple effects.**

**Our Future Work! Next slide**

**Aknowledgements.**