UNIVERSITY OF
BIRMINGHAM

Project 1: Investigating the Possible Interactions Between the Meis1 and Onecut1 Proteins with Adjacent LincRNAs

and

Project 2: Investigating Hypoxia Inducible Factor Transcriptional Activity in the Liver

by

Nicholas Ross Frampton

A combined research thesis submitted to the University of Birmingham as part of the requirement for the degree of MASTER OF RESEARCH in Molecular and Cellular Biology.

College of Life and Environmental Sciences
School of Biosciences
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September 2013

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Abstract

LincRNAs are molecules transcribed from regions with no protein coding function, which may have regulatory functions specifically over nearby proteins. These ncRNAs are often expressed during early development and may exert their regulatory function in a number of ways; either as decoys, guides, scaffolds or enhancers. Linc_meis1 and Linc_Onecut1 are LincRNAs located adjacent to two well-studied proteins Meis1 and Onecut1, these are the focus of the project and were studied using a range of techniques in an effort to elucidate their expression patterns and potential regulatory function. These particular LincRNAs are suspected of having Cis-regulatory function over their neighbouring proteins, this assumption was made because the proteins and LincRNAs appear to be co-expressed. Analysis using whole mount in situ hybridisations attempted to visualise the native expression patterns of each LincRNA within a zebrafish model, however so far this has not been successful. Anti sense oligonucleotide morpholino knockdowns were utilised to try and determine the importance of each LincRNA for adjacent protein expression and study any generated phenotypes. So far the data has been inconsistent and is therefore still continuing. A better understanding of these RNA molecules may help towards an improved understanding of developmental disorders and future development of treatments.

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LIST OF ABBREVIATIONS

BCIP	5-Bromo-4-chloro-3-indolyl phosphate
DNA	Deoxyribose Nucleic Acid
Dpf	Days post fertilisation
EDTA	Ethylenediaminetetraacetic acid
HM	Hybridisation Mix
Hpf	Hours post fertilisation
LincRNA	Large Intergenic Non-coding RNA
LncRNA	Long Non-coding RNA
M-MLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
miRNA	Micro RNA
NBT	Nitro Blue Tetrazolium Chloride
ncRNA	Non-coding RNA
Nt	Nucleotides
PBS	Phosphate Buffered Saline
PBT	Phosphate Buffered Saline with Tween
PCR	Polymerase Chain Reaction
RNA	Ribose Nucleic Acid
SSC	Saline Sodium Citrate buffer
WISH	Whole-mount In Situ Hybridisation

1 Introduction

1.1 Non-coding RNAs

Until recently the most well studied and important sequences of the genome were considered to be the protein coding genes. It is now apparent that these make up only 2% of the human genome, the other 98% consisting of non-protein coding transcripts (*Esteller, 2011*). Huge interest has emerged over the possible functional roles for these other transcripts. Over years it has become increasingly obvious that this non-protein coding portion of the genome has important roles in both normal development and physiology as well as in disease (*Esteller, 2011*). Non-coding RNAs (ncRNAs) have become popular for study in research due their potential functional relevance; discovery of these transcripts and realisation that they might have a number of different regulatory roles led to a frenzy of research attempting to identify the functions of these transcripts and therefore divide them into classes (*Esteller, 2011*. *Rinn & Chang, 2012*). A non-coding RNA is an RNA molecule that is not translated into protein. NcRNAs belong to several groups and are involved in a number of different cellular processes. This allows some separation into classes (*Guttman et al, 2011*).

MicroRNAs (miRNAs) are the class of ncRNAs that are most widely studied, epigenetic and genetic defects within this class and their mechanisms of action have been attributed to many diseases (*Esteller, 2011*). These are encoded in a huge range of tissues and have multiple functions; of these functions it is important to note their ability to target mRNAs to different locations and regulate

gene expression levels (*Esteller, 2011*). A good example is the miR-200 family of miRNAs, this group of miRNAs are highly expressed in epithelial cells and it has been suggested they contribute to cancer metastasis by inhibiting epithelial-mesenchymal transition (EMT). This process is a crucial step in eventual metastasis and involved in the spread of cancers (*Esteller, 2011*). Generally this class of ncRNA acts through post-transcriptional regulation of mRNA. As an example of how widespread these non-protein coding transcripts can be, miR-200 family members have been found in association with bladder, breast, ovarian, pancreatic, prostate, stomach and lung cancers (*Esteller, 2011*).

MiRNAs are however just one class of ncRNAs that have emerged, other classes such as long non-coding RNAs (LncRNAs) and large intergenic RNAs (LincRNAs) also appear to play important roles in normal development and in disease. Evidence suggests that ncRNAs can be involved in a range of different disorders, such as neurological, cardiovascular, autoimmune, imprinting and monogenic disorders (*Rinn & Chang, 2011. Wapinski & Chang, 2011*).

Many ncRNAs are conserved over all or most cellular life. These are considered to be remnants from LUCA (last universal common ancestor) or the RNA world (*Mercer et al, 2009. Wang & Chang, 2011*). Unlike protein-coding genes it is not as necessary for the transcripts of ncRNAs to maintain sequence conservation, rather the locations of ncRNA transcripts are highly conserved coupled with short sections of highly conserved sequences (*Rinn & Chang, 2012*).

NcRNAs are associated with the propagation and progression of many human disorders, it is therefore important to develop an understanding of how ncRNAs

are involved in pathological effects and the mechanisms by which they act. There are a number of different genomic, epigenomic and bioinformatics approaches used to help achieve this (*Rinn & Chang, 2012*). The ENCODE project (an Encyclopaedia of DNA elements) is a collaboration of multiple research groups which aims to identify all the functional elements within the genome (*Rinn & Chang, 2012*). Other methods aimed at studying ncRNA functions are based on and utilise second-generation sequencing (RNA seq); these methods provide more detailed observations about the whole human RNA transcriptome (*Rinn & Chang, 2012*). These methods however can run into complications when trying to interpret ncRNA complex secondary structures; additional algorithms have been designed to complement second-generation sequencing (RNAfold, RNAalifold, EvoFold etc.) (*Rinn & Chang, 2012*). Even with these additional algorithms there have only been a few ncRNAs identified with this method. The issues with identification are based in a lack of complete understanding of the ncRNAs functional motifs and domains, their low expression levels and a general need for better characterisation of regulatory regions (*Rinn & Chang, 2012*).

1.2 Long Non-coding RNAs

Long non-coding RNAs (LncRNAs) are a class of ncRNA that appear to resemble mRNA, however they do not behave as templates for protein synthesis (*Rinn & Chang, 2012*). Rather these function as RNA genes that coordinate and manage genetic regulatory outputs. Evidence suggests that they are at least partially involved in a number of different cellular processes such as; imprinting (an epigenetic process involving DNA methylation and histone

modifications which results in silencing of an imprinted gene and expression of a certain gene from only the non-imprinted allele inherited from the mother), pluripotency, cell cycle regulation, diseases and many more (*Rinn & Chang, 2012*. *Guttman et al., 2011. Hung et al., 2011*). LncRNAs have been implicated in post transcriptional gene regulation through control of processes such as protein synthesis, RNA maturation, RNA transport and gene silencing through regulation of chromatin structure (*Khalil et al, 2009*).

It has been suggested that LncRNAs are involved in a number of different diseases; both developmental and cancerous (*Calin et al, 2007. Scaruffi, 2011*). Their potential role as regulators of protein function means that the scope of their effect could be enormous. The use of DNA microarrays in studying ncRNAs has indicated that there are at least as many LncRNA transcripts as those encoding proteins (*Rinn & Chang, 2012*). So far these ncRNAs have been characterised by their lack of protein coding function and their size. Typically these ncRNAs are considered to be transcripts longer than 200 nucleotides in length (*Rinn & Chang, 2012*). However a number of different roles for these RNA transcripts have been put forward (*Mercer et al, 2009*). LncRNAs can act through a range of different mechanisms, which have been called “Archetypes” (*Wang & Chang, 2011*).

Firstly LncRNAs may act as decoys; in this instance the ncRNA serves by titrating away DNA binding proteins such as transcription factors to prevent activation or repression of its target gene (Figure 1A) (*Rinn & Chang, 2012*). A good example of the effect is seen with PANDA, an LncRNA that associates

with the NF-YA transcription factor and titrates it away thereby preventing its transactivation of p53 mediated apoptosis (*Rinn & Chang, 2012*).

LncRNAs may also behave as a scaffold; this involves the compilation of two or more proteins into a complex or within spatial proximity of one another (Figure 1B) (*Tsai et al, 2010. Rinn & Chang, 2012*). This mechanism is seen with the LncRNA HOTAIR (encoded in the HOXC cluster), which binds and brings together the polycomb repressive complex 2 (PRC2) and the LSD1-CoREST complex; this combination of proteins orchestrates H3K27 methylation and H3K4me2 demethylation. Amalgamation of these two processes ensures gene silencing (*Gupta et al, 2010. Yang et al, 2011*. *Rinn & Chang, 2012*).

The third “archetype” is when LncRNAs act as guides; they recruit proteins (such as chromatin modifying enzymes) to DNA, which may occur either through a direct interaction with the protein or by RNA interaction with a DNA binding protein (Figure 1C) (*Rinn & Chang, 2012*). LncRNAs involved in imprinting are often called guides, in that they result in targeted gene silencing in an allele specific manner (*Rinn & Chang, 2012*). HOTAIR can also be considered an example for this mechanism as well; it guides PRC2 to the correct location at multiple sites throughout the genome in development and cancer related gene expression (*Gupta et al, 2010. Yang* et al, 2011). These LncRNAs utilise two basic molecular functions to achieve their regulatory function, the binding of protein partners and localisation specific points within the genome (*Rinn & Chang, 2012*).

The final LncRNA mechanism is an enhancer-like function. LncRNAs are able to exert guidance through chromosome looping (Figure 1D) (*Rinn & Chang,*

2012). A great example of this mechanism is the HOTTIP LncRNA, which is encoded on the 5' end of a HOXA gene cluster and through chromosomal looping is brought into spatial proximity of multiple HOXA genes where it maintains H3K4me3 and gene activation (*Rinn & Chang, 2012*).

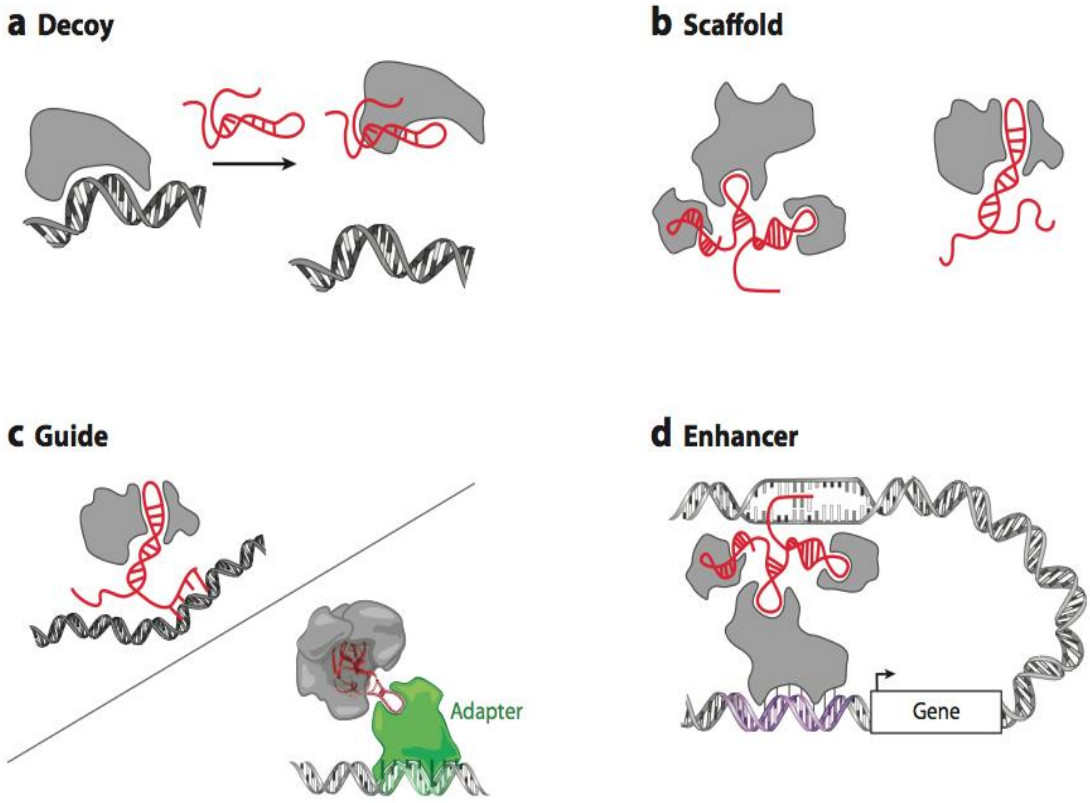


Figure 1. Roles of LncRNAs (Taken from *Rinn & Chang, 2012*) A) Decoys. B) Scaffold. C) Guides. D) Enhancers.

In order to study LncRNAs experimentally it is important to examine their native expression patterns and identify specific cell types or cellular processes associated with the candidate LncRNA. LncRNAs show cell type specific expression and respond to diverse stimuli. A method called "guilt by association" has been used to help develop a global understanding of LncRNAs and protein-coding genes that are co-expressed (*Rinn & Chang, 2012*). While not necessarily always the case, this tight co-expression often suggests that the

two genes are co-regulated. This means the adjacent LncRNA may in some way control the expression of the protein via one of the mechanisms mentioned above (*Rinn & Chang, 2012*). The ability of LncRNAs to regulate associated protein function might result in diseases such as cancer. Evidence suggests that altered expression patterns for LncRNAs in human cancers are seen in relation to those controlled through tumour suppressor pathways (*Calin et al, 2007. Esteller, 2011. Wapinski & Chang, 2011*). A recent study has identified that numerous LncRNAs are either up or down regulated within different cancers (*Tsai et al, 2011. Yang et al, 2011*). An example given in this study shows that HOTAIR (encoded in the HOXC cluster) was highly indicative of breast cancer metastasis. High levels of HOTAIR expression is associated with a poor prognosis and death; this observation has been found in colon and liver cancers as well which suggests that elevated HOTAIR LncRNA might be an oncogenic trait (*Yang et al, 2011. Rinn & Chang, 2012*). Other examples of LncRNAs, which have been shown to affect the expression of adjacent proteins include HOTTIP, a LncRNA located on the HOXA locus and is involved in regulating a number of HOXA associated genes. In addition it binds to WDR5 protein, which forms part of a complex involved in methylation and activation of the HOXA locus (*Wang et al, 2011*). H19 is another LncRNA, which is implicated in a number of cancers but has no known protein coding function (*Brannan et al, 1990*). Another good example of a LncRNA is Xist, which has been shown to regulate the process of X inactivation in early development of mammalian females (*Chow et al, 2005*).

1.3 LincRNAs

LincRNAs are a subgroup of LncRNAs that originate from intergenic regions (the spaces between protein coding genes) that have separate transcriptional units from the protein-coding genes, which they may regulate (*Ulitsky et al, 2011. Rinn & Chang, 2012*). These LincRNAs typically have highly conserved promoter regions which are involved in the recruitment and binding of transcription factors to adjacent proteins (*Ulitsky et al, 2011*). For LincRNAs the majority of sequence conservation is found over the introns and intergenic regions (*Ulitsky et al, 2011*). However syntenic LincRNAs found in different species can maintain similar functionality despite a difference in sequence (*Ulitsky et al, 2011*). The literature suggests that at least a third of the known LincRNAs interact with and utilise chromatin modification complexes to regulate a range of cellular processes (Khalil et al, 2009. *Rinn & Chang, 2012*).

A study that utilised numerous annotation sources and combined them with RNAseq was able to show in excess of 8,000 LincRNAs within the human genome (*Rinn & Chang, 2012*). This helped to identify a number of characteristics common in LincRNAs. Such ncRNAs have a propensity for location near developmental regulators and appear to maintain tissue-specific expression patterns (*Rinn & Chang, 2012*). Thousands of orthologous LincRNAs have been found between mice and humans and the transcript is often found in “gene deserts” which are megabase-sized genomic segments which lack any protein-coding genes associated with vertebrate genomes (*Rinn & Chang, 2012*).

The complexity of LincRNAs makes them difficult to study effectively and hard to develop inhibitors that will allow study of the regulatory effects of these ncRNAs. Techniques such as SELEX (systematic evolution of ligands by exponential enrichment) have been used to identify sequences of RNA that will bind to the LincRNAs (*Rinn & Chang, 2012*).

Targeting splice sites or conserved regions with antisense reagents may result in developmental defects allowing effective study of their regulatory roles, this form of study may also elucidate the cell types within which the LincRNA resides and exerts its influence and help to demonstrate the importance of LincRNAs during embryonic development (*Corey & Abrams, 2001*. *Guttman et al, 2011*).

1.4 Meis1

The Meis1 protein is a member of a homeodomain subfamily of cofactors that regulate the activity of Hox proteins (*Azcoitia et al, 2005*). It has been shown that overexpression of Hox proteins leads to an increased population of haemopoietic stem cells (HSC) (*Azcoitia et al, 2005*). Hox proteins are involved in hematopoietic system pathologies or in normal hematopoietic development and as such the Meis1 protein can be considered a regulator or at least involved in the regulation of haematopoiesis. Interactions between Meis1 (and other Meis family members) and Hox proteins results in an increased DNA affinity and target specificity; these traits are necessary if correct transcriptional regulation of Hox targets is to be achieved. Meis1 proteins role in haematopoiesis has largely been suggested because of its localisation; this protein can be found within adult bone marrow and the HSC compartment of

fetal livers (*Azcoitia et al, 2005*). During the early stages of embryogenesis, Meis1 expression can be seen in paraxial, intermediate, and lateral plate mesoderm (*Azcoitia et al, 2005*). During organogenesis, Meis1 is involved in the development of a number of organs and embryonic structures, such as early eye formation and limb development (*Azcoitia et al, 2005*). Overexpression of this protein has been shown to contribute to the development of leukaemias in cooperation with a number of Hox proteins (*Azcoitia et al, 2005*). Meis1 may also have a role in the development of HSCs, it was shown using Meis1 deficient mice (which died mid-gestation) that the protein is involved in the genesis of the first HSC because when lacking Meis1 haematopoiesis fails to occur (*Azcoitia et al, 2005*). The Meis1 protein may also be involved in later development and differentiation of vascular networks.

1.5 Onecut1

The Onecut1 protein (or Hepatocyte Nuclear Factor 6 –HNF6) belongs to a family of transcription factors called ONECUT. This protein is involved in regulating the hepatocyte specific transcription, which is necessary for normal adult liver function and the development of the hepatic biliary tree and hepatocytes from hepatoblasts (*Clotman et al, 2002*). Onecut1 protein is also known to be expressed in the pancreatic duct epithelium during development; when inhibited, normal duct growth was prevented (*Zhang et al, 2009*). Further study into this protein revealed that it regulates a network of genes responsible for cilium development and hereditary polycystic disease (*Pierreux et al, 2006. Zhang et al, 2009*). A more recent paper shows that this protein has roles in the activation of pro-endocrine transcription factor Ngn3 and that early Onecut1

inactivation resulted in pancreatic defects (*Jacquemin et al, 2000. Zhang et al, 2009*). Onecut1 has both early and late roles in the developing pancreas. A more recent discovery is this proteins (and other ONECUT family members) involvement in the development of the nervous system; expression of Onecut1 has been observed in spinal motor neurons during chick development (*Francius & Clotman, 2010*). Expression of the protein is conserved in spinal motor neurons and is involved in differentiation during the later stages of motor neuron development (*Francius & Clotman, 2010*). This family of transcription factors typically has a C-terminal DNA binding motif, which is comprised of a single Cut-domain and a homeodomain (*Clotman et al, 2002*).

1.6 Meis1 and Onecut1 LincRNAs

These LincRNAs are located in the intergenic regions adjacent to the proteins Meis1 and Onecut1; they were chosen for study because they appear to be co-expressed with the adjacent proteins and therefore may be co-regulated (work done by Swaraj Basu). So far neither of these LincRNAs has been studied before and that means there is a lack of functional data for each. Also, while both the associated proteins have been studied quite extensively and therefore have known sites of tissue specific expression, the same cannot be said for the LincRNAs. Based upon the co-expression and potential regulation a theory has been developed; suggesting that they function as Cis-regulatory elements (a region of DNA or RNA that regulates the expression of genes located on the same molecule of DNA) which are typically located upstream from the coding sequence which they control or interact with. The exact mechanism by which these two LincRNAs may act upon their nearby protein counterparts is

unknown; they may act through any of the mechanisms described in section 1.2 or perhaps through another mechanisms which remains un-described.

Different stages of development will be used to demonstrate the tissue specificity of the LincRNAs and show that they are only expressed at certain stages, when required. The Meis1 protein and LincRNA are separated by 1155 base pairs; the Onecut1 protein and LincRNA are separated by 332 base pairs. Figure 2 below shows a snapshot of the genome browser displaying the distance between each LincRNA and the adjacent protein.

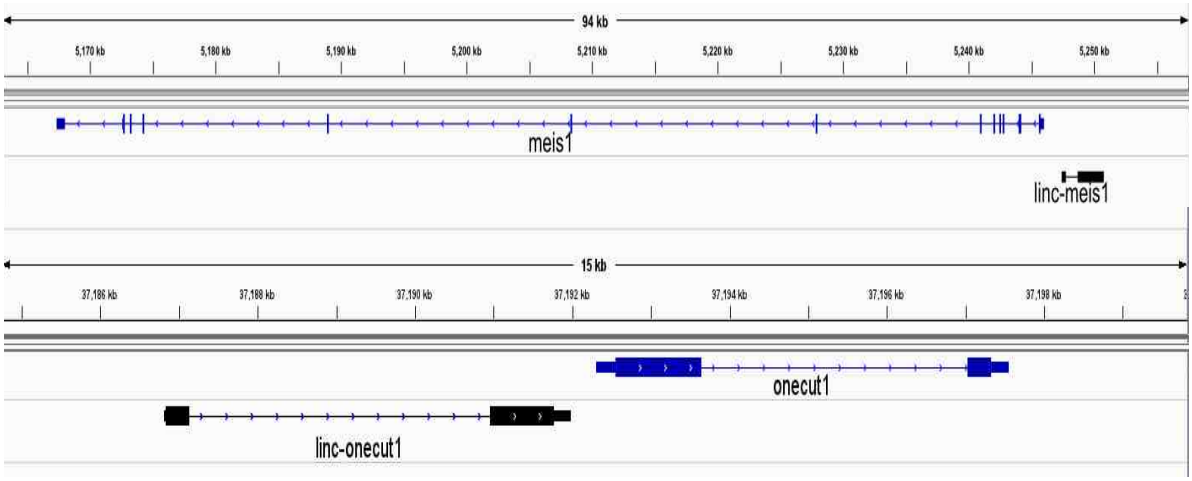


Figure 2. Snapshot from the Genome Browser displaying distance between LincRNAs and adjacent proteins.

Figures 3 and 4 demonstrate the co-expression seen between each LincRNA and its adjacent protein. These graphs show expression patterns across early development within zebrafish models; they were generated using RNAseq data.

Morpholinos will be used to knock down the LincRNA and then examine changes in morphology. Both of the proteins have been previously studied and therefore phenotypes are available to demonstrate the effect of knockdowns. This can be used to compare with LincRNA knockdowns, if the same effect is

observed it would suggest a regulatory function. Following this experiment it would be interesting to know if LincRNA knockdown causes overexpression or a lack of expression of the adjacent proteins. This could indicate whether it is positively or negatively regulating the protein. This LincRNAs were picked for study based upon previous work by Swaraj Basu; he determined that Meis1/ Onecut1 and their LincRNAs are syntenic in mammals and fish. Also the CAGE data showed that they share a similar expression profile in zebrafish; with similar peaks visible at sites of promoters/ over the length of each transcript.

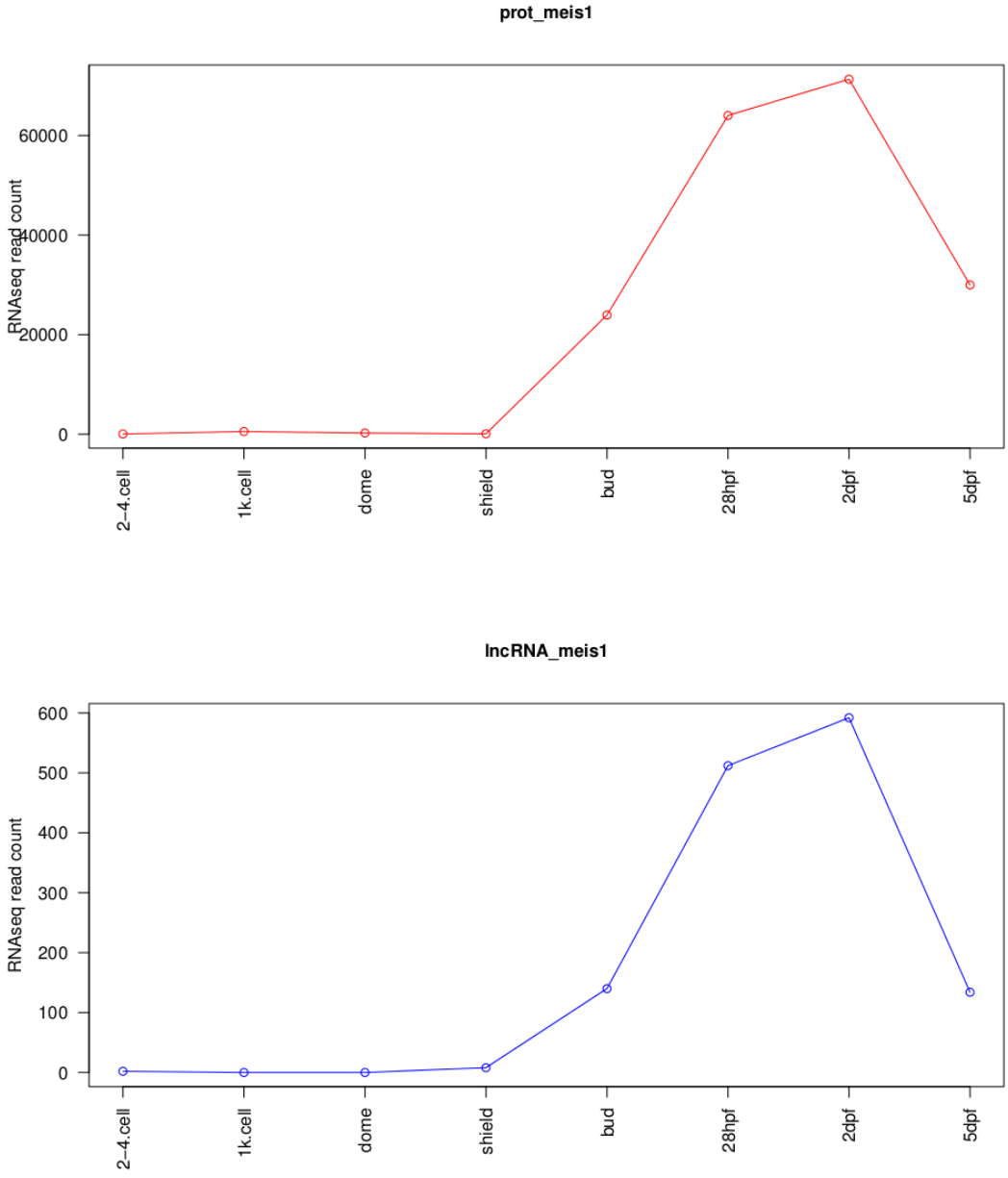


Figure 3: RNAseq expression pattern across early developmental stages in zebrafish for the meis1 gene and its upstream LincRNA (Taken from a presentation by Swaraj Basu).

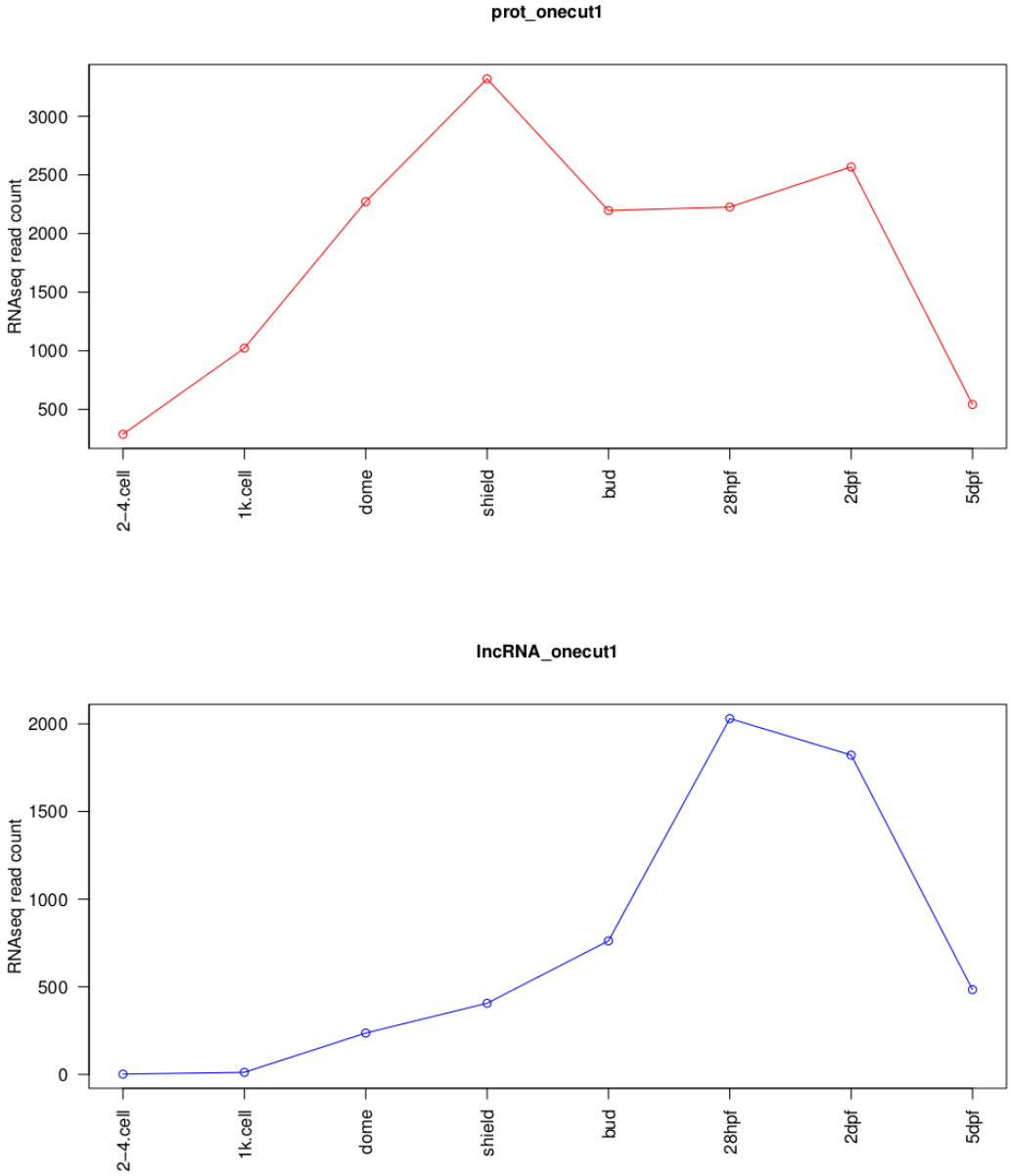


Figure 4: RNAseq expression pattern across early developmental stages in zebrafish for the onecut1 gene and its upstream LincRNA (Taken from a presentation by Swaraj Basu).

1.7 Zebrafish Model

Zebrafish are a vertebrate model system that is regularly used in scientific research because it has many advantages and shares 70% of human protein

coding genes and 84% of disease associated genes (*D'Costa & Shepherd, 2009*). Firstly, the genome has been fully sequenced. In combination with well-understood and easily observable behaviours these models provide a great platform to study gene expression and alteration. In addition, Zebrafish lay large numbers of transparent embryos in a controlled and predictable manner. These enable the study of embryonic development because they grow in well-defined embryonic stages over short periods of time. The development from fertilisation to hatching takes 48 hours. The Zebrafish can then develop to sexual maturity over the following 3 months and live up to 5 years. Also, they maintain a near constant size during early development, which enables the use of simple staining techniques such as Whole-Mount In Situ Hybridisations. Genetic studies commonly use Zebrafish models where knocked down gene expression is achieved using either small-interfering RNAs or antisense Morpholino technologies (*White et al, 2007; D'Costa & Shepherd, 2009; Bradford et al, 2011*).

1.8 Methodology Crucial to the Project

1.8.1 PCR

Polymerase chain reaction, a biochemical technology used in molecular research to amplify single or multiple strands of DNA by large orders of magnitude. Millions of copies of the DNA strand of interest can be generated in a relatively short time (*Ochman et al, 1988*). This technique has a number of

different applications such as DNA cloning for use in sequencing, functional analysis of genes and diagnosis of some hereditary diseases (*Cold Spring Harbor Laboratory, 2003*). The process involves varying temperatures over a number of cycles; the repeated heating and cooling of DNA allows for replication and enzymatic activity. Short DNA sequences (primers) that are complimentary to stretch of DNA being amplified are used in combination with DNA polymerase to enable the selective replication. A few cycles into the process the DNA so far generated is then used as a template resulting in exponential amplification through a chain reaction (*Cold Spring Harbor Laboratory, 2003*). These cycles can be broken down into a few steps; first comes initialisation where the reaction mixture is heated to a high temperature to activate the DNA polymerase. Following this a short cycle where DNA is heated to a high temperature to allow unwinding of the DNA double helix occurs, this is called denaturation (*Chantler, 2004*). The annealing step is at a lower temperature and allows the primers to bind to their complementary sections of DNA. Finally there's the elongation step in which the polymerase synthesises a new stretch of DNA; the temperature for this step is dependent on the type of polymerase being used (*Chantler, 2004*). This process is then coupled with agarose gel electrophoresis to check whether the anticipated DNA fragment was successfully made (when compared against a DNA ladder of known size). This technique will be useful in generating the probe and can be used to confirm expression of genes of interest (when coupled with agarose gel electrophoresis).

1.8.2 Whole-Mount In Situ Hybridisation

This process uses a labeled complementary RNA strand as a probe, which hybridises at a target location on a specific sequence of DNA or within a specific tissue; this type of in situ in particular relies on small tissues and hybridisation can occur over the entire tissue (*Darnell, 2010*). The process means researchers can measure and detect the localisation of specific RNAs. During this experiment the sample tissues are usually fixed in place, this increases the access for the probe during hybridisation (*Darnell, 2010*). The probe hybridises to the target location at a high temperature, following which the excess probe is washed away. The probe had been previously labeled with antigen-labeled bases (*Darnell, 2010*); which means that immunohistochemistry could be used to detect expression and allow quantification and localisation (this works for both native expression and over expression studies, and can be used to confirm successful knockdown experiments in theory). The study of expression patterns is important because it gives an indication of functionality. This pattern can be either within a specific cell type or a specific cellular process (*Darnell, 2010*). With meis1 and onecut1 there is previous data regarding the proteins, which each LincRNA is adjacent to, and therefore a good starting point for understanding their potential role. This process of using gene expression analyses to study adjacent protein-coding genes and pathways that significantly correlate with the LincRNAs is often called “guilt by association” studies. It would be useful to combine WISH with morpholino knockdowns to determine if the antisense oligos successfully silenced expression of the target.

1.8.3 Morpholino Knockdowns

A morpholino is a molecule used in research that can alter gene expression; they bind to specific sequences of DNA and prevent other molecules from accessing the same stretch or prevent transcription of certain genes (*Corey & Abrams, 2001*). This technique can be utilised in research to study functional aspects of proteins and ncRNAs by knocking down said functions (*Corey & Abrams, 2001*). The mechanism has been called “steric blocking”. These molecules are often used in developmental research to study the roles of specific transcripts; this is achieved by injecting embryos at a very early stage with a solution containing these synthetic oligos (*Corey & Abrams, 2001. Langenau & Zon, 2005*). Binding to their complementary sequence should prevent proper development of the protein or transcript and have adverse effects on the embryos development.

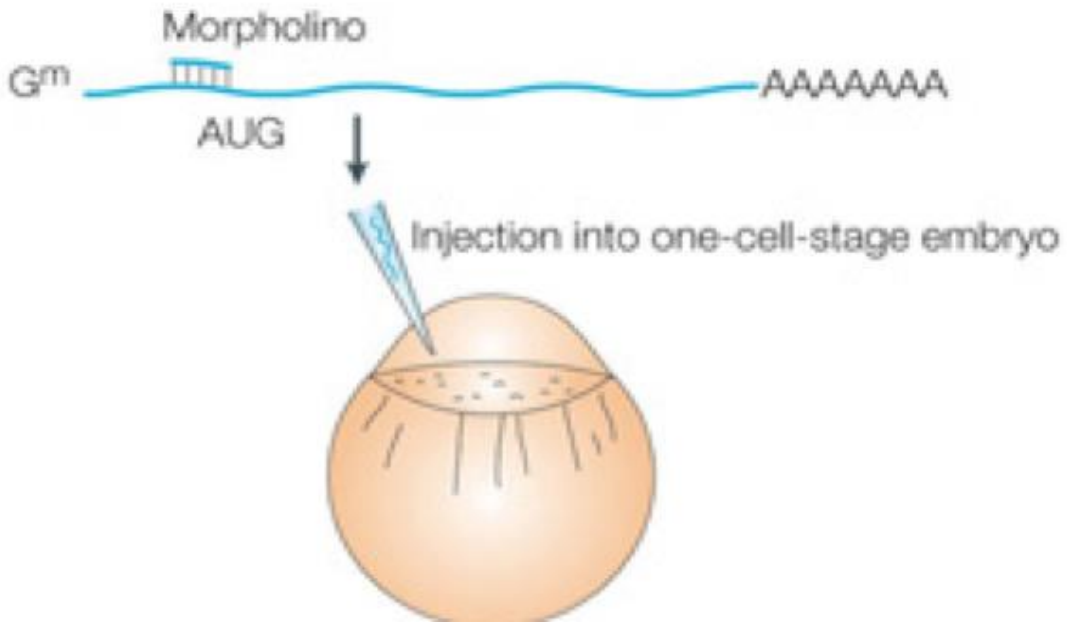


Figure 5: Morpholino methodology (taken and modified from Langenau and Zon, 2005).

This image simply outlines how morpholinos are used in research.

Gene knockdown studies enable the establishment of the importance of each LincRNA in the developmental process. Following successful identification of the LincRNAs for Meis1 and Onecut1 using WISH, it would be useful to knock them down and determine whether expression of the associated proteins still occurs.

1.9 Aims and Objectives

The aim of this project was to study the LincRNAs adjacent to Meis1 and Onecut1 proteins and their potential regulatory role during early development. If possible the experiments were meant to help elucidate the functions of each LincRNA and determine their expression patterns. The main objectives of the project were to develop an anti-sense probe using PCR from total genomic DNA for each of the LincRNAs. Following probe design, Whole-mount in situ hybridisations would be utilised to study where the LincRNAs were being expressed and if possible to what level. And after that morpholino knockdowns would reveal any regulatory function of the LincRNA by development of new phenotypes in embryos following injection. If possible, identification of the nature of said LincRNA functions as either positive or negative regulators of proteins could be achieved using loss of function analyses and over expression studies.

2 Materials and Methods

2.1 Buffers and Solutions

Buffer	*Ingredients*
5x Buffer	20mM Tris-HCl (pH 7.5 at 25°C), 0.2M NaCl, 0.1mM EDTA, 1mM DTT, 0.01% Nonidet® P-40 and 50% glycerol
Elution Buffer	10 mM Tris-HCl; pH 8.5
Hybridisation Mix	5x SSC, 0.1% Tween, pH 6 with Citric Acid (Monohydrate), 50 µg/ml heparin, 500 µg/ml torula RNA and 50% Formamide
NTMT staining buffer	100 mM NaCl, 100 mM Tris-HCl, 50 mM MgCl2, 0.1% Tween 20; pH 9.5
NTMT staining solution	100 mM NaCl, 100 mM Tris-HCl, 50 mM MgCl2, 0.1% Tween 20, 1.88 mg/ml NBT, 0.94 mg/ml BCIP; pH 9.5
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4
PBS EDTA	PBS pH 5.5/2mM EDTA
PBT	0.1M PBS, 0.5% Tween 20, pH 7.4
PCR Buffer	1.5 mM MgCl2
PFA	4g Paraformaldehyde in 100 ml PBS
PTU	0.003% 1-phenyl-2-thiourea
RNA-Loading Buffer	47.5% Formamide, 0.01% SDS, 0.01% bromophenol blue, 0.005% Xylene Cyanol, 0.5mM EDTA
SSC	3M NaCl, 0.3M sodium citrate (for 20X concentration)
TE Buffer	10 mM Tris-HCl, 1 mM EDTA, pH 8.0

2.2 Plasmids

Vector	*Insert*
pCS2+	Linc_Meis1 Exon 1/ Linc_OC1

2.3 Antibodies

Number	*Antibody*	*Type of Antibody*	*Brand*
1	In Situ Hybridization	Goat Monoclonal Anti-Digoxygenin	Roche

2.4 Zebrafish Husbandry

Wild type Tubingen zebrafish strains were used for all the experiments. These were kept at 28^{o}C in an incubator within the laboratory. When required, a number of breeding tanks (roughly 10) were set up in the fish facility the evening before embryo collection. Dividers and separators are used to keep the male (1 per tank) and females (2 per tank) apart until the next morning. Upon removal of the dividers the fish will lay and embryos can be collected. This are transferred to petri dishes with standard embryo media for transportation to the lab. If developmental stages beyond 24 hours of development were required, PTU was added to the embryo media to suppress pigmentation.

2.5 Morpholino Injections

The antisense Morpholino oligonucleotides that we used to knockdown the LncRNAs were purchased from GENE TOOLS, LLC in the US. The Morpholinos targeted specific locations for each LncRNA; for linc_onecut1 a conserved transcript initiation site was chosen, and for linc_meis1 a splice junction site. The Morpholinos stock solutions were combined with nuclease free water and phenol red. Then using glass micropipettes and a microinjector setup, 4nl of each Morpholino solution was injected into embryos at the one to two cell stage of development.

Antisense MO sequences (5'→3').	
Morpholino linc_onecut1	GCTGAAAGGGAATCAGTATGCAAAT
5 mispair oligo for *linc_onecut1*	GCTcAAAcGGAATgAcTATGgAAAT
Morpholino linc_meis1	AGCGACTTAGAGAGAGAAAGATTCA
5 mispair oligo for *linc_meis1*	AGCcAgTTAcAGAGAcAAAcATTCA

2.6 Isolation of total RNA

Total RNA was isolated using the TRIZOL® reagent made by Invitrogen in the US. 1ml of TRIZOL® reagent for every 50-100 mg of tissue sample was used to homogenise the samples; these were taken from 24hpf. The samples were then incubated for 5 min at room temperature. Chloroform was added to each of the tissue samples 0.2ml for every 1ml of TRIZOL®. The samples were then shaken vigorously for 15 seconds by hand; following this the tissue samples were left to incubate at room temperature for 3 minutes. The phases were separated by centrifugation (12000xg; 15 minutes; at room temperature). The upper aqueous phase formed after centrifugation was transferred to a fresh tube Eppendorf tube and precipitated with 0.5 ml of isopropyl alcohol per every 1 ml of TRIZOL®. Further centrifugation (12000xg, 10 minutes, at 4°C) was used to collect the RNA which was subsequently washed with 1 ml of 70% ethanol for every 1 ml TRIZOL®. After this wash step, the RNA pellet was dried for 10 minutes by upending the Eppendorf tube and allowing to air dry. This pellet was then solubilised in RNase-free water, the concentration calculated using a nanodrop and then stored at -80°C.

2.7 Isolation of genomic DNA from embryos

Embryos were collected and allowed to develop to 3dpf; these were then used to isolate genomic DNA. Upon reaching the right developmental stage the embryos were washed in Methanol (which dehydrates them), this was followed by drying for 15 minutes at 70°C in an incubator. Following this, the embryos were treated for 4 hours with an enzyme called Proteinase K (0.5 mg/ml), which

was diluted to its working concentration using TE buffer (see section 2.1). The embryos were incubated with the enzyme at 55°C. After this digestion the enzyme was inactivated by a shorter incubation at 75°C. Subsequently the mixture of digested embryos and enzyme was diluted using nuclease free water at a ratio of 1:2.5 and stored at -20°C.

2.8 Agarose gel electrophoresis of DNA and RNA

Agarose gel electrophoresis was used to separate DNA and RNA fragments. Due to differences in molecular weight, this method allows isolation of specific bands of nucleotides, which travel at different rates through the gel. The DNA and RNA fragments were run on a 1% agarose gel which contained ethidium bromide, this allowed visualisation of both DNA and RNA fragments. RNA was loaded into the wells with an RNA-loading buffer.

2.9 Isolation of DNA from agarose gels

A 1% agarose gel with ethidium bromide was used to visualise PCR products and DNA fragments; this could then be placed under a UV-light. This allowed easy visualisation of the DNA bands, which were then carefully cut out using a scalpel. Following this extraction from the gel the DNA had to be separated from the remaining agarose gel; this was achieved using the NucleoSpin® Gel and PCR Clean-up kit from Macherey Nagel in Germany. The section of cut gel was incubated at 50°C with the kits provided NT1 binding buffer (200 µl of buffer for every 100 mg of gel) until the gel had fully melted. The resultant sample was transferred to a filter column and spun in a centrifuge (11000xg, for 1 minute, at room temperature). After centrifugation the column was washed twice with 750 µl of NT3 washing buffer for 30 seconds each time with a spin in the centrifuge

after each wash (11000xg). The DNA that was now bound to the column and had most impurities washed away was eluted with 50 µl of elution buffer (see section 2.1) and stored at -20°C.

2.10 Reverse Transcription

An M-MLV Reverse Transcriptase and RNase H Minus kit from Promega in the US was used to induce production of cDNA from RNA. This reaction used 100 ng of total RNA (isolated in section 2.6), which was incubated with 5.0 µl of 5x Buffer at room temperature, 5.0 µl of dNTP Mix (10 mM concentration of each dNTP), 1 µM Oligo-dT primer and 2.0 µl of M-MLV Reverse Transcriptase, Rnase H Minus (M-MLV RT). The total reaction volume was 20 µl, nuclease free water was used to make up the final volume. The reaction mixture was then incubated for 1 hour at 37°C. Following incubation the mixture was kept at -20°C.

2.11 Polymerase Chain Reaction

When performing PCR each of the reaction mixtures contained: 100 ng of template DNA, 200 µM dNTPs, 1 µM forward primer, 1 µM reverse primer, 2 units AmpliTaq® DNA-Polymerase from Roche in Switzerland and PCR Buffer (section 2.1) were incubated together in a thermocycler for 30 cycles with alternating temperatures. During the first cycle the reaction mix was denatured at 95°C for 5 min. After initial denaturation the samples were denatured at 95°C for 30 sec, primers were allowed to anneal at 55°C for 30 sec and DNA synthesis was carried out at 72°C for 45 sec; this occurred 30 times. This was followed by a final elongation step at 72°C for 10 min. The reaction mix was then cooled down to 4°C and further processed.

PCR primers (5'-3')	
linc_meis1 Exon1 FP	GCTATAAGAGGCTTTAAACAAAAAC
linc_meis1 Exon1 RP	CGACAAACGCGTGCGTGTTTATC
linc_meis1 Long FP	GCTATAAGAGGCTTTAAACAAAAAC
linc_meis1 Long RP	TGTCAAAAGGGAAAGCTGACAAA
linc_meis1 Short FP	TTACTATCAAATCCCCAAAGC
linc_meis1 Short RP	TTTGTGTGTTGATTTAAAATTTATTC
linc_onecut1 FP	TAATTTGCATACTGATTCCCTTTCAGC
linc_onecut1 RP	GTATTTAAACATTTTCTTTATTTTCAGAAAATC

2.12 Enzymatic digestion of DNA

3 µl of pCS2+ plasmid DNA was incubated at 37°C for 2 hours in a 50 µl reaction. 1 µl of restriction digestion enzyme and 5 µl of corresponding buffer purchased from New England Biolabs in the US. The total volume of this digest was adjusted using nuclease free water.

2.13 Ligation of PCR-product with the pCS2+ plasmid vector

The PCR reaction mix was column purified for each LincRNA. In the case of *linc_meis1* exon1 the band was cut from a gel and purified to reduce the number of breakdown products. The PCR products were then ligated with a pCS2+ vector, which had been previously digested using StuI enzyme. This ligation was achieved using an In-Fusion® HD Cloning Kit from Clonetech in the US. According to the manufacturers instructions: 4 µl of the PCR product were incubated with 2 µl of 5X In-Fusion HD Enzyme Premix and 2 µl of Vector. Using nuclease free water the volume was increased to a 10 µl total volume. This reaction mixture was then incubated at 50°C for 15 minutes. Half of the ligation reaction was used for transformation into competent E. coli DH5α cells.

2.14 Transformation of bacteria with DNA

Competent E. coli DH5α cells were transformed with vector or DNA-ligation reactions; the vector only acted as a control. 50 µl aliquots of DH5α cells were thawed on ice and mixed with either 5 µl of plasmid DNA or DNA-ligation reaction. The mixtures were then incubated on ice for 30 minutes. Following this incubation the cells were heat-shocked for 45 sec at 42°C. The resultant cell suspension was mixed with 500 µl of Luria-Bertani (LB) medium and incubated at 37°C for 1 hour. After incubation the DH5α cells (which now contain either the control plasmid vector DNA or DNA-ligation reaction samples) were plated onto LB-ampicillin agar plates (Ampicillin at 0.1 mg/ml concentration) and incubated overnight at 37°C. The formation of colonies indicated that the DNA-ligation reaction had been successful. Formation of colonies on the control plates was not desirable.

2.15 Isolation of plasmid DNA from bacteria

10 Eppendorf tubes each containing 1 ml of LB medium were inoculated with a single colony of E. coli DH5α picked from a LB-ampicillin agar plate (these were labeled 1-10). These LB cultures were grown overnight at 37°C with vigorous shaking. After allowing sufficient growth overnight, the cells were collected by spinning in a centrifuge (14000xg; for 5 minutes; at room temperature). Using a QIAprep® Miniprep kit from Qiagen in the US, plasmid DNA was isolated. The manufacturers instructions are as follows: The pelleted bacterial cells were resuspended in 250 µl of Buffer P1 and transferred to a fresh microcentrifuge tube. 250 µl of Buffer P2 was added to the centrifuge tube and mixed thoroughly by inverting 4–6 times. Following this, 350 µl of Buffer N3 was added

and again the tubes were inverted immediately to thoroughly mix the solution. After addition of each of the buffers the solution was centrifuged for 10 minutes at 13,000 rpm (~17,900xg). Following centrifugation the supernatant was transferred to a QIAprep spin column by pipetting, this spin column was then centrifuged for 30–60 seconds. The flow-through was discarded and the spin column was washed by addition of 0.5 ml of PB buffer followed by a 30 second spin, and then washed with 0.7 ml of PE buffer and spun again. After these washes the spin column was placed into a fresh 1.5 ml microcentrifuge tube. DNA was eluted using 50 µl of Buffer EB; this was left to soak into the membrane within the column for 1 minute followed by a 1 minute spin in the centrifuge.

2.16 In-vitro transcription of digoxigenin-labeled RNA

Digoxigenin (DIG)-labeled probes for in situ hybridisation (ISH) were generated using the DIG labeling kit made by Roche in Switzerland. 2 µg of linearised template plasmid-DNA (EcoRV digested) was incubated with 4µl 5X transcription buffer, 2µl NTP-DIG-RNA, 2µl T7 Polymerase and 1.5 µl RNase inhibitor. The total volume was increased to 20 µl with nuclease free water and the mixture was incubated for 2 hours at 37°C. In order to purify the RNA, any remaining template DNA had to be removed by adding 20 U DNase I and incubating the solution at 37°C for 15 min. The RNA was purified with GE Illustra™ MicroSpin™ G-25 Columns as per the manufacturers instructions: The resin in the column was resuspended by vortexing. The cap was loosened by one-quarter turn and the bottom closure twisted off. The column was placed in the supplied collection tube followed by a 1 minute centrifugation (735 ×g). It

was placed into a fresh DNase-free 1.5 ml microcentrifuge tube and the Dig labeled RNA was added to the top-centre of now prepared resin. The column was then centrifuged for 2 minutes at 735×g then the eluate collected, the concentration measured using a nanodrop and stored at -20 °C.

2.17 Whole-mount *In Situ* Hybridisation

2.17.1 Embryo Fixation

Zebrafish embryos were collected and allowed to develop to stages at which the LncRNAs of interest are reportedly expressed. These were then dechorionated and fixed overnight in 4% PFA in PBS. Following fixation, PBS was used to wash the embryos and 100% methanol was added to them. This dehydrated the embryos and meant they could be stored at -20°C.

2.17.2 Day 1 of *In Situs*

Using serial dilutions the embryos were rehydrated. A range of methanol solutions in PBT were used to wash the embryos in Eppendorf tubes (75%, 50% and 25% methanol). Following rehydration the embryos were washed 4 times in PBT for 5 minutes each at room temperature. This was followed by a digest with proteinase K (10 µg/ml) in PBT (15 min for embryos over 24 hpf and 30 min for 32-72 hpf embryos) at room temperature (this step, subsequent fixation and washes are not necessary in embryos younger than 24 hpf). The digest was stopped by washing 2 times with PBT for 5 minutes at room temperature. Following digestion with proteinase K the embryos were fixed in 4% PFA in PBS for 20 minutes at room temperature and washed 4 times for 5 minutes with PBT at room temperature. Hybridisation buffer (Hyb Mix or HM)

was added to each of the embryos, which were then left to sink to the bottom. Fresh hybridisation buffer was added and the embryos were left to incubate at 68°C for 2 hours. After the stipulated time the embryos were incubated overnight at 68°C with the DIG-labeled RNA probe (1 µg/ml concentration) in fresh hybridisation buffer.

2.17.3 Day 2 of *In Situs*

Following the overnight hybridisation with a probe (the probe was carefully recovered and stored at -20°C for re-use) the embryos were washed with a number of different solutions: 50% HM/50% 2XSSC at 68°C for 5 minutes, 2XSSC at 68°C for 15 minutes, 0.2XSSC at 68°C for 30 minutes, twice; 50% 0.2XSSC/50% PBT at room temperature for 10 minutes and finally with PBT at room temperature for 10 minutes. Fresh PBT/2% goat (or sheep) serum/2mg:ml BSA was prepared and the embryos were incubated in this blocking solution at room temperature for several hours. The embryos were then incubated in antibody solution overnight at 4°C (PBT/2% goat serum/2mg:ml BSA/1:2500 anti-DIG antibody).

2.17.4 Day 3 of *In Situs*

Unbound anti-DIG antibody was washed off the following day by washing the embryos multiple times at room temperature in PBT (1 X 1 min, 3 X 5 min, 4 X 15 min). Following the washes, embryos were then incubated 2 times for 5 minutes each in NTMT staining buffer (section 2.1) at room temperature and then in NTMT staining solution (section 2.1). The staining reaction was stopped by removing the staining solution and washing the embryos for 3 minutes in

stop-solution (PBS EDTA, section 2.1). Embryos were fixed with 4% PFA for 20 minutes at room temperature or longer (it was possible to store the embryos at this stage at 4°C). For imaging the embryos were given 3 quick washes with methanol followed by serial dilutions of methanol/PBT (75% MeOH, 50% MeOH, 25% MeOH, PBT). The final step before imaging was a series of washes in serial dilutions of glycerol/PBT (25% glycerol, 50% glycerol, 75% glycerol, 100% glycerol).

3 Results

3.1 Amplification and cloning of the LncRNAs

Using RT-PCR, a 1.2KB cDNA fragment representing linc_onecut1 was amplified and cloned; the genetic material (total DNA) used as a template was taken from 24 HPF embryos. For linc_meis1, the process was more complex due to the presence of two different possible transcripts (one was 2 KB, the other 2.4 KB). This was determined using RNAseq and CAGE data and therefore two different sets of primers were designed. No RT-PCR product was detected for the *linc_meis1* transcripts. This may be caused by a low expression level in the RNA sample (Figure 6). The exons (Exon1 400 bp, Exon2 2 KB) of *linc_meis1* long transcript were PCR amplified from zebrafish genomic DNA (Figure 7). After successful amplification of both exons, PCR and ligation reactions were used to attempt to fuse each fragment together and into the PCS2+ plasmid. This was ultimately unsuccessful, yielding no positive colonies. Consequently the 400 bp fragment of linc_meis1 was amplified alone and cloned with a PCS2+ plasmid. Anti-sense RNA probes were prepared with T7 RNA polymerase, for the full-length linc_onecut1 transcript and the 400 bp fragment of linc_meis1 (Figure 8).

Figure 6. Agarose gel: PCR amplification of linc_meis1 and linc_onecut1 from 24 HPF whole embryo RNA. Meis1 LS : lincMeis1 Long primer specific, Meis1 SS: lincMeis1 Short primer specific, Onecut1 S: lincOnecut1 primer specific, Meis1 LO : lincMeis1 Long primer reverse with Oligo dt, Meis1 SO: lincMeis1 Short primer reverse with Oligo dt, Onecut1 SO: lncOnecut1 primer reverse with Oligo dt.

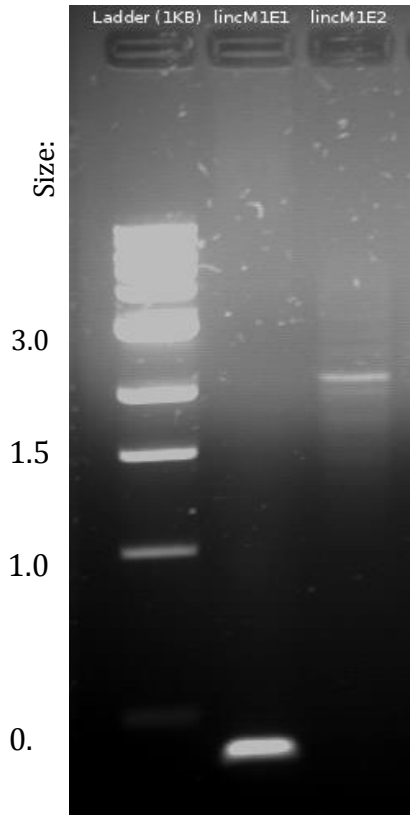


Figure 7. Agarose gel: PCR amplification of exons of linc_meis1 from genomic DNA. LincM1E1: 400 bp first exon. LincM1E2: 2 KB second exon.

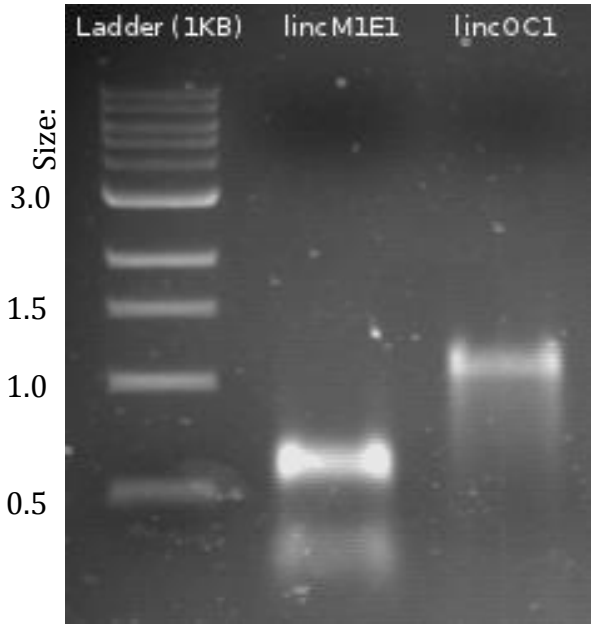


Figure 8. Agarose gel: RNA probe prepared for ISH. LincM1E1: probe from 1st exon of linc_meis1, lincOC1: probe from full-length linc_onecut1 transcript.

3.2 Whole-mount in situ hybridisation

So far, attempted detection of expression patterns has been unsuccessful with LncRNAs simply showing non-specific background staining; the reason remains unclear. The hybridisation protocol was repeated using a low temperature (4 °C) slow staining which again failed to show a specific binding of the probes (Figure 9, 10, 11). Specific staining was visible for the probe against the *neurogenin1* coding gene (positive control) in the diencephalon, tegmentum and hindbrain at 24 hours post fertilisation in zebrafish embryos (Figure 11). Due to the non-specific background staining seen in each of the attempts using a standard in situ protocol, a number of small alterations were made upon repeats. More washes with PBT on day 3, no dehydration after embryo fixation and slow staining. An image was taken for each of the different methods and can be seen below (Figure 12). Discussion about the different protocols and lack of positive results can be found in section 4.

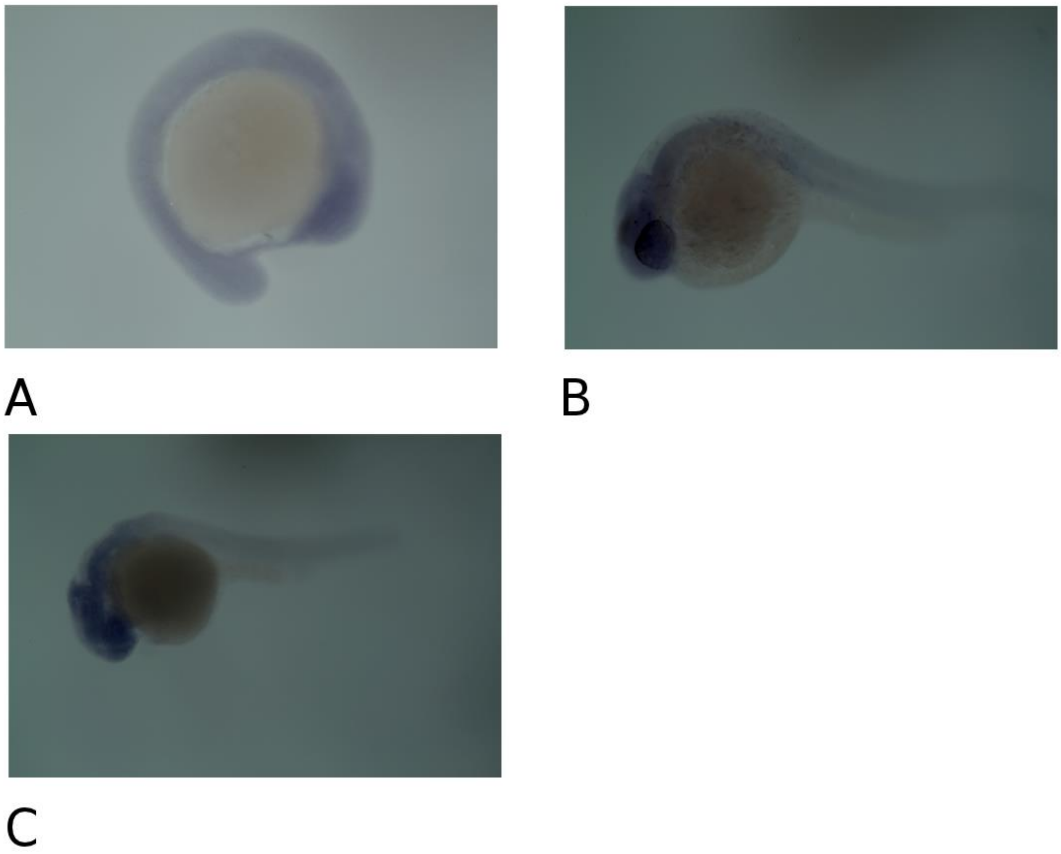
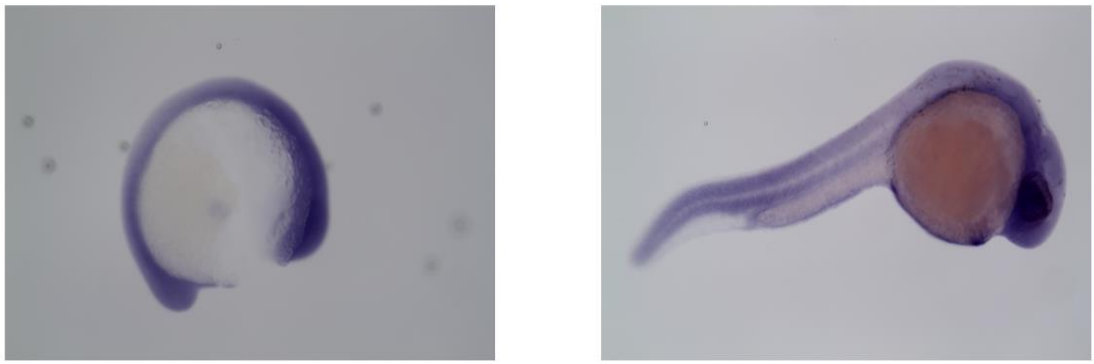
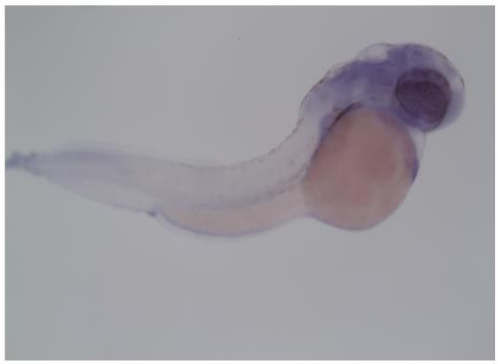


Figure 9. ISH: Whole mount in situ hybridisation of zebrafish embryos with the *linc_onecut1* antisense probe across early developmental stages **A)** 15 somites **B)** Prim 5 **C)** Prim 15.

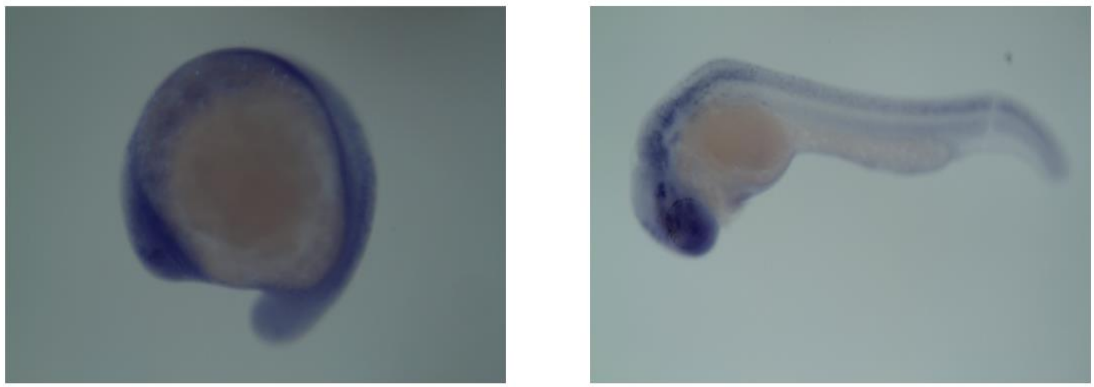


A B



C

Figure 10. ISH: Whole mount in situ hybridisation of zebrafish embryos with the *linc_meis1* exon1 antisense probe across early developmental stages **A)** 15 somites **B)** Prim 5 **C)** Prim 15.



A B

Figure 11. **ISH:** Whole mount in situ hybridisation of zebrafish embryos with the neurogenin1 antisense probe across early developmental stages **A)** 15 somites **B)** Prim 5.

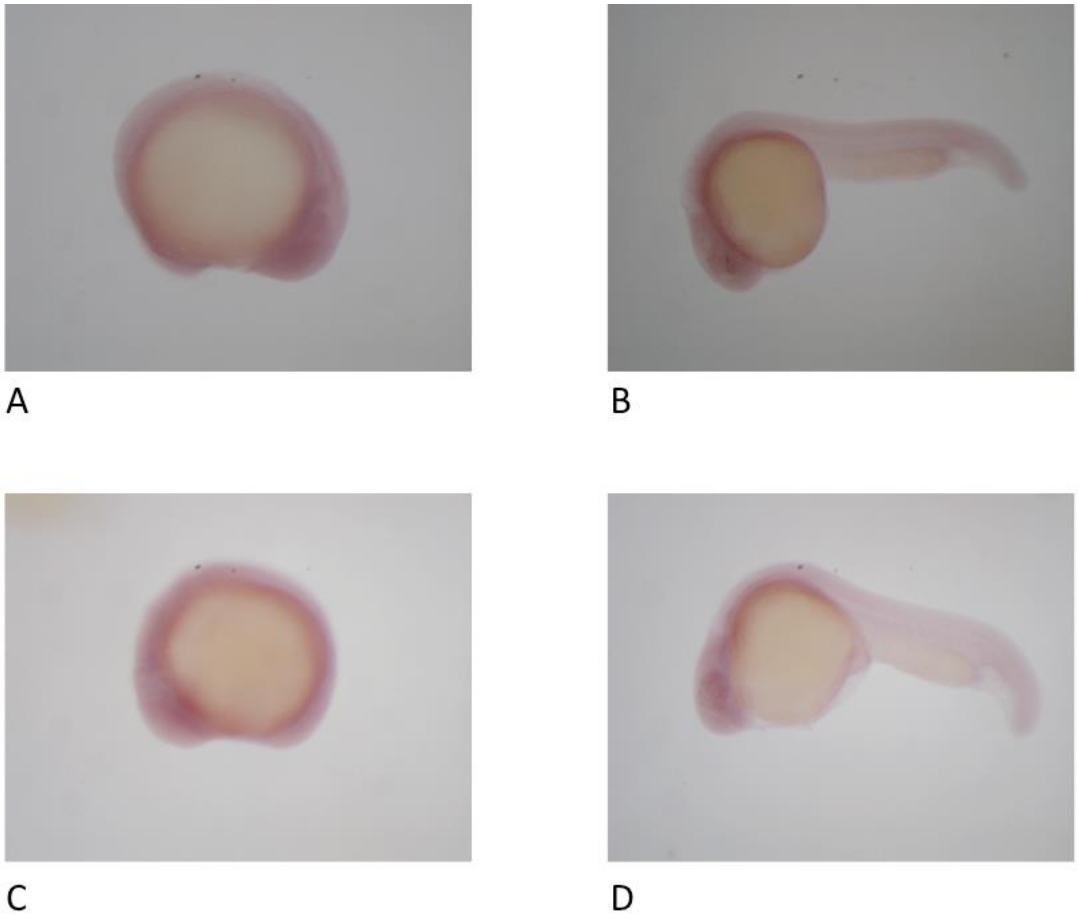


Figure 12. **Images of repeats showing non-specific background staining**. **A)** 15 somites, linc_OC1 probe, slow staining/ extra washes **B)** 24 hpf, linc_OC1 probe, slow staining/ extra washes **C)** 15 somites, linc_meis1 probe, slow staining/ extra washes **D)** 24 hpf, linc_meis1 probe, slow staining/ extra washes.

3.3 Antisense morpholino oligonucleotide based knock-down

In an attempt to determine the optimum concentration initial injections were performed using concentrations of 50, 100 and 150 μM for both linc_onecut1 and linc_meis1 antisense morpholinos. Observable effects were seen at different concentrations for each morpholino; however despite the difference neither has yielded consistent results. At 50 μM concentration there were no signs of toxicity, non-specific cell death, or lethality. For each dose, approximately 100 embryos were injected and then scored at 24hpf and 48hpf for normal and abnormal phenotypes. 5-base mispairing morpholinos in which

five of the 25 residues are altered to prevent binding to the target were used as controls. This checks that the morpholino itself is not mistargeting other transcripts or causes toxicity. The mispairing morpholinos did not cause any gross phenotypes or developmental delay with any concentration. A smaller head phenotype was observed with the linc_onecut1 morpholinos at 150 μM concentration at 48hpf (Figure 13). A greater number of abnormal fish were observed when injected with the linc_onecut1 morpholino by comparison to the 5mm morpholino (Figure 15A, 15B) The linc_meis1 morpholino injected fish showed non-specific cell death and necrosis at 24 hpf and increased mortality at 48 hpf. Following these results the concentrations were reduced to 100 μM for each morpholino; this however resulted in a lack of specific phenotype for the linc_onecut1 morpholino. The fish for linc_meis1 morpholino showed a delay in growth and cell-death in the notochord (Figure 14). The linc_meis1 morpholino and its corresponding 5mm control show a better resolve between the number of normal and morphant embryos at this concentration (Figure 15C, 15D). It was observed that a high percentage of the linc_meis1 morpholino injected fish showed almost no movement at 48hpf at all tested concentrations. The embryos were considered dead when no visible heartbeat was observed and therefore not counted. Figure 16 shows the percentage survival of embryos after injection with morpholinos at two different concentrations. Clearly the higher concentrations resulted in the death of more embryos even in the 5mm controls. A desirable concentration has yet to be established.

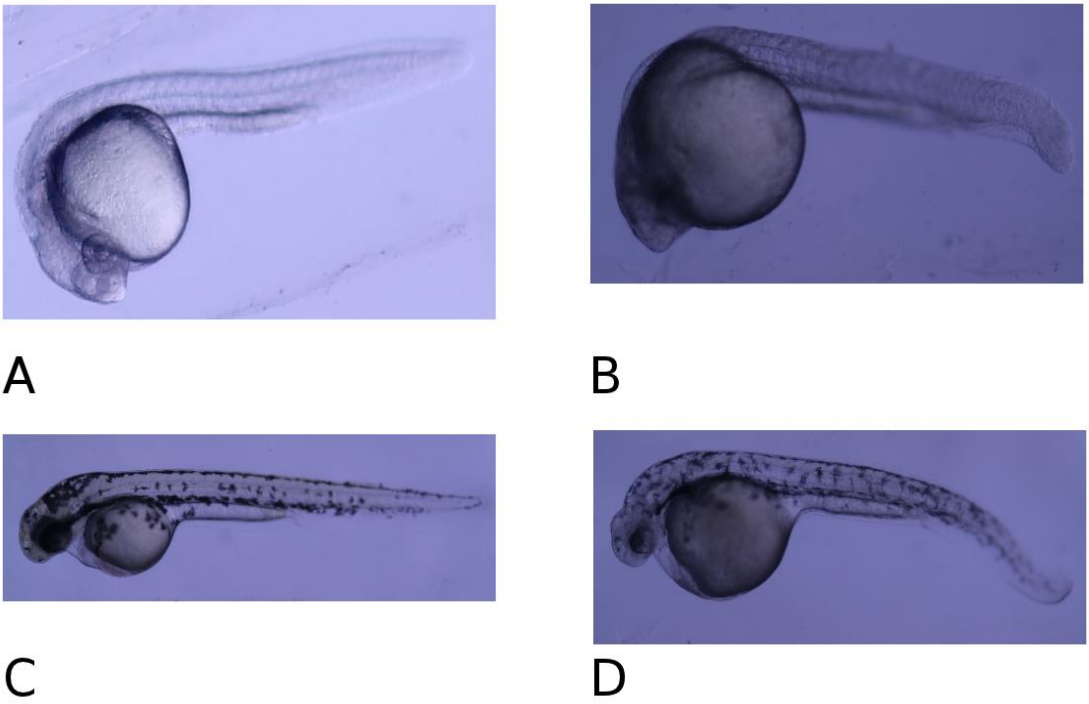


Figure 13. **Morpholino injections at 150 µM:** *linc_onecut1* antisense oligonucleotide and its 5 base pair mis-match **A)** 5mm phenotype at 1dpf **B)** MO phenotype at 1dpf **C)** 5mm phenotype at 2dpf **D)** MO phenotype at 2dpf.

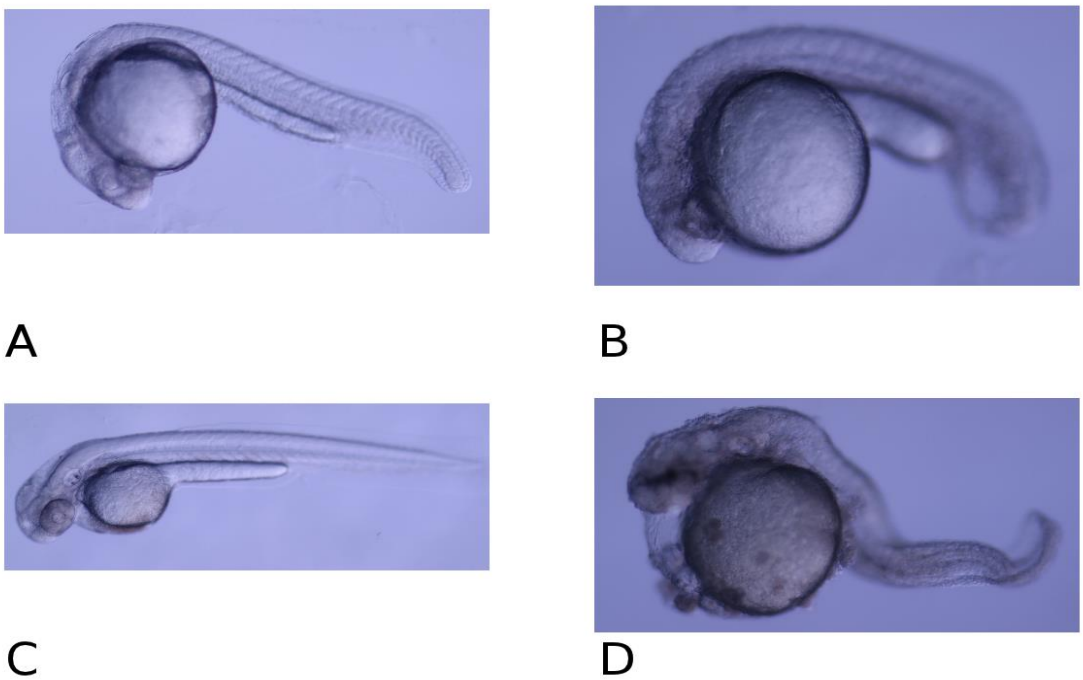


Figure 14. **Morpholino injections at 100 µM:** *linc_meis1* antisense oligonucleotide and its 5 base pair mis-match **A)** 5mm phenotype at 1dpf **B)** MO phenotype at 1dpf **C)** 5mm phenotype at 2dpf **D)** MO phenotype at 2dpf.

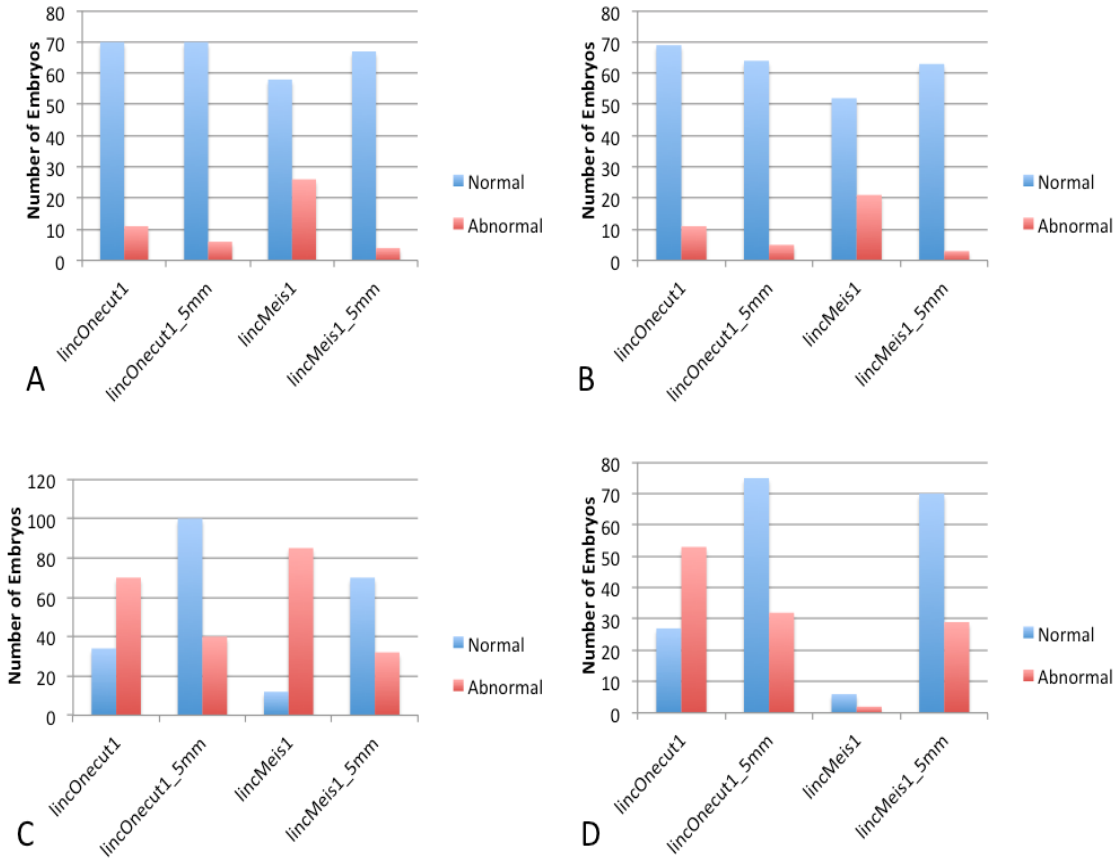


Figure 15. **Morpholino injection statistics: A)** 24 HPF, MO concentration of 100 µM **B)** 48 HPF, MO concentration of 100 µM **C)** 24 HPF, MO concentration of 150 µM **D)** 48 HPF, MO concentration of 150 µM.

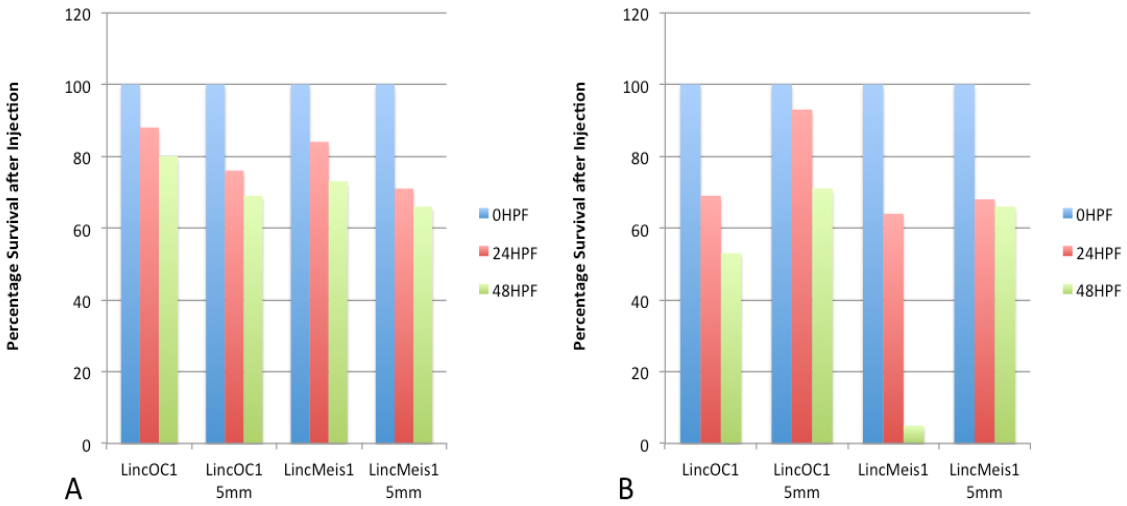


Figure 16. Morpholino injection statistics: A) MO concentration of 100 µM **B)** MO concentration of 150 µM.

4 Discussion

The purpose of the investigation was to study the LincRNAs adjacent to two previously well-studied proteins called Meis1 and Onecut1. This project involved the use of a number of different experiments, which combined aimed to elucidate the expression patterns of the LincRNAs and perhaps gain an insight into their functionality and mechanisms of actions. PCR was used in order to develop an anti-sense RNA probe from total RNA of 24hpf zebrafish for each of the LincRNAs. Anti-sense RNA probes were prepared with T7 RNA polymerase, for the full-length linc_onecut1 transcript and from a 400 bp fragment of linc_meis1 corresponding to the first exon. A probe for each of the LincRNAs was successfully prepared, along with a control probe (Neurogenin) with a known expression pattern to prove the probes were correctly made (Figures 8, 11). These probes were then used in whole-mount in situ hybridisations (WISH). This method is regularly used to visualise the expression patterns of proteins and RNA in zebrafish models. Other vertebrate models such as mice may be used, however the rapid and external development coupled with transparent embryos enables researchers to study developmental diseases quickly and effectively. The in situ hybridisations carried out over the course of this project have so far been unable to show a native expression pattern for either of the LincRNAs. An example of the Neurogenin probe in zebrafish has been provided to show successful application of the WISH protocol (Figure 11). So far the results have simply shown non-specific background staining in the zebrafish models that appears pink all over the fish

(Figures 9, 10); a clear expression pattern would appear a dark purple or blue colour in a specific location or tissue. The protocol was repeated a number of times, with various changes made to the process to try and develop a set of steps sensitive enough to show the expression of these lowly expressed LincRNAs. Figure 12 has been added to show a lack of difference between sets of results. At the same time we ran anti-sense oligonucleotide morpholino knockdowns; this type of “Loss of function” experiment was meant to prevent the expression of the LincRNAs within zebrafish models. Injections performed at an early stage mean that the synthetic molecule binds to its complementary strand within the genome and prevents transcription of the target. A morpholino was designed for each LincRNA; the purpose was to study the phenotype developed and perhaps illuminate their functions, potential regulatory roles and importance to survival. Our results were not consistent with this experiment; the effective dosage was difficult to ascertain, with a low dosage showing little or no change in phenotype (as compared to a 5mm control) and high dosages often causing rapid death in the embryos as well as stunted growth. A phenotype for linc_onecut1 was observed with some fish, a smaller head at 48hpf. This result was not consistent in all of the fish or in every repeat (performed with a range of morpholino concentrations). This suggests that it may have been an artifact of morpholino injection. Likewise for the linc_meis1 morpholino injected fish, cell death was seen in the notochord for some embryos, but for most the cell death was non-specific. This again would suggest there might be an artifact of morpholino injection involved in the resulting phenotype. None of the results throughout this experiment resulted in significant successes; however there is

still much work that needs to be done and a number of extra controls should probably be performed to ensure that the techniques were being performed correctly.

Each of the experimental techniques used during this project have strengths and weaknesses that are important to discuss. PCR is an essential technique in research because it is a simple procedure that is quick to run and requires very little starting material in order to generate millions of copies of the desired stretch of DNA; this technique is regularly utilised to screen for expression of desired fragments of DNA when coupled to agarose gel electrophoresis (*Chantler, 2004*). There are however some drawbacks to the technique; in order to amplify the gene of interest prior and detailed knowledge of the DNA sequence are required in order to design primers. Also in order to ensure a successful result, the process must be optimised by varying the concentrations of chemicals involved and the temperatures of each cycle; this is largely dependent on the primers (*Ochman, 1988. Chantler, 2004*). The PCR process is limited to a certain size, the maximum regularly achieved is 5kb, however the larger the stretch of DNA the less efficient the process. PCR is a highly sensitive technique and is therefore susceptible to contamination from non-template DNA present in the lab (*Slish, 2013*). This can lead to false results and therefore when preparing for PCR the area of work is kept as sterile as possible. The polymerases used in PCR often lack 3' to 5' exonuclease activity and therefore lack the ability to correct misincorporated nucleotides resulting in a higher error rate (*Slish, 2013*). Taq polymerase is a good example of a DNA

polymerase regularly used in PCR that lacks this activity. Some recombinant polymerases have been developed in order to increase PCR activity.

Whole-mount in situ hybridisation was the second technique utilised throughout this project and is a standard for detecting RNA presence within embryos. This experiment is important because a lot of knowledge can be gained from studying gene expression patterns; often genes with developmental regulatory function are expressed in a localised manner (*Darnell, 2010. Rinn & Chang, 2012*). This type of screening can be used in zebrafish models to identify differentially expressed developmental regulators. Anti-sense RNA probes are commonly used and are made from transcribed cDNA copy of the target. The problem with this is that the correct stretch of cDNA can be hard to come by. Digoxygenin (DIG) labeled nucleotides are inserted into the anti-sense RNA every 10-15 nucleotides and enable detection via specific antibodies that bind to the DIG molecule (*Darnell, 2010*). While this is an effective method, there are a few problems. RNA probes are easily degraded by RNases and generally are not very effective if shorter than 300bp in size (*Darnell, 2010*).

The final method used during this project is anti-sense oligonucleotide morpholino knockdowns. This approach is enables study of gene function *in vivo*, however the results are sometimes considered to be ambiguous (*Corey & Abrams, 2001*). As seen in this project, high levels of cell death were seen in morpholino injected embryos, but this may simply be due to the process of injection. For this reason a number of controls are required. In this project we used 5 mis-match morpholinos as controls. These injected embryos usually displayed a wild type phenotype, however we did see cell death on occasion,

which shows that simply injecting with a morpholino can alter the normal growth of an embryo. The process on the whole is great for study of early embryo development, but non-antisense effects may be seen and cause false positives when unintended interactions occur between the morpholino and proteins or DNA nearby; also the morpholino may bind to other “non-target” sections of nucleotides with similar sequences (*Corey & Abrams, 2001*).

Based on the results so far it is unclear what role these LincRNAs play in regulating the proteins encoded next to each, both the in situ hybridisations and the morpholino knockdowns provided inconclusive data. The work is still in progress within the lab and still has potential to yield some significant results when successful protocols can be developed. The in situ hybridisations could not tell us the expression specificity of the long non-coding RNAs. Initial morpholino based targeted knock down of the non-coding transcripts yielded visible phenotypes, however so far these are not consistent and may be artifacts caused by injections. There are a number of things that could be done to further work given enough time. To ensure that the right targets are being studied, it would be a good idea to check for meis1 and onecut1 expression using Northern blots (while RT-PCR is more sensitive, Northern blots are more specific and therefore is less likely to get false positive results). If possible it would also be good to perform a series of morpholino injections with rescue experiments, this could confirm whether the cell death and stunted growth observed were simply due to injections or actually an effect of the morpholinos. As a control a p53 knockdown morpholino (which has been shown to work previously) could be injected simultaneously with the LincRNA morpholinos; if

the cell death is prevented then the cell death being observed is a normal p53 mediated apoptosis and not an effect of LincRNA knockdown. With repeated experiments it would hopefully be possible to develop an in situ protocol sensitive enough to capture the expression dynamics of the candidate genes. If this doesn't work however, it might be worth performing an over expression study of the LincRNAs because their expression is so low. Coupled with ISH this could at least allow study of the localisation (this however would not be valid alone and would require the native expression pattern at some point). Failing that, another probe may have to be designed for each LincRNA if study into their regulatory function is to continue.

These LncRNAs may have regulatory functions over adjacent proteins and as such are important to study. Successful study and interpretation of their roles may help towards the better understanding of developmental disorders and future development of treatments.

5 Acknowledgments

I would like to thank everyone in the Müller lab for their help and supervision throughout the time spent in the laboratory; in particular I would like to thank Emma and Yavor for their help throughout the experiments and Swaraj Basu, for his good advice and help through out my project. Finally I'd like to thank Ferenc Müller for the opportunity to work in his lab and learn a range of new techniques.

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Investigating hypoxia Inducible Factor transcriptional activity in in the Liver

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A thesis submitted to the University of Birmingham for the degree of MASTER OF RESEARCH

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September 2013

Abstract

Hypoxia inducible factors (HIFs) are heterodimeric transcription factors that regulate oxygen homeostasis and stress responses, are involved in causing or responding to liver disease. The aim of this investigation was to study HIF2α stabilisation and activity within hepatocytes after exposure to hypoxia and the pro-inflammatory cytokine TNFα. The kinetics of HIF stabilisation were determined using a hypoxia responsive element (HRE) luciferase reporter gene assay. Exposure to low oxygen stabilised HIFs and stimulated HRE transcriptional activity and luciferase reporter gene expression in Huh-7.5 and HepG2 cells. HRE transcriptional activity differed under two different hypoxic conditions (1% versus 3% oxygen; $P<0.05$, unpaired t-test). Hypoxic responses were compared in DMSO-differentiated and naïve Huh-7.5 cells with no significant difference, but a change in activity followed chronic hypoxic exposure. Comparing the effects of TNFα on both cell lines indicated no significant activation beyond hypoxia alone when plotted relative to normoxic controls. However, data obtained with lipopolysaccharide-stimulated macrophage conditioned media indicated an increase in activity under hypoxia, perhaps suggesting the presence of additional cytokines or factors that regulate activity. These kinetic data have not been shown before and contribute to the understanding of HIF regulation and identification of potential therapeutic targets for liver disease.

Table of Contents

List of Abbreviations

AkT	Protein Kinase B
DAPI	4’6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular Signal-regulated Kinases
FBS	Fetal Bovine Serum
FIH-1	Factor Inhibiting HIF 1
HCC	Hepatocellular Carcinoma
HCL	Hydrochloric Acid
HCV	Hepatitis C Virus
HGF	Hepatocyte Growth Factor
HIFs	Hypoxia Inducible Factors
HRE	Hypoxia Responsive Elements
IκB	Inhibitor of κB
IPAS	Inhibitory PAS domain protein
LB	Laemmli Buffer
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
NEAA	Non-Essential Amino Acids
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ODD	Oxygen-dependent Degradation Domain
PAS	Per/Arnt/Sim
PBS	Phosphate Buffered Saline
PHDs	Prolyl Hydroxylases
PHH	Primary Human Hepatocytes
PI3-K	Phosphoinositide 3-kinase
RIPA	Radioimmunoprecipitation Assay Buffer

RLU	Relative Light Units
RNA	Ribonucleic Acids
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate, Polyacrylamide Gel Electrophoresis
STAT3	Signal Transducer and Activator of Transcription 3
TBST	Tris-Buffered Saline with Tween 20
TEMED	Tetramethylethylenediamine
TLR	Toll-like Receptors
TNFα	Tumour Necrosis Factors alpha

7 Introduction

7.1 The Liver

The liver, which is the largest organ in the body, is located on the right side of the abdomen below the diaphragm. A nutrient rich blood supply circulates through the liver via the hepatic portal artery, traverses the entire organ through sinusoidal capillaries before leaving via the hepatic portal vein. The liver is highly specialised with diverse functions including a major role in metabolism, toxin removal, and the synthesis and storage of carbohydrates. Consequently, it is comprised of numerous cell types (*van Leeuwen et al, 1994*). The hepatocytes, also known as parenchymal cells, comprise 80% of the liver mass and perform the majority of liver functions. The remaining 20% of cells, non-parenchymal cells, includes endothelial, Kupffer, and stellate cells, which have several functions, including initiating a response to hepatic injury (*Kolios et al, 2006*). A cartoon depiction of the liver architecture is in Figure 1.

Under normal conditions in an adult healthy liver, hepatocytes do not divide. However, under toxic conditions or in a disease state the hepatocytes are able to proliferate, a process known as regeneration. This occurs when the liver is damaged, but additionally, when occurring in an uncontrolled manner, is involved in the development of cancers. It is well known that the liver is able to replace up to two thirds of its mass after a liver transplantation; it will grow back until the point the organism requires and then proliferation stops (*Taub, 2004*).

7.2 Oxygen Range

Anatomically the liver is divided into three zones, each of which has a different protein and carbohydrate profile due in part to variation in oxygen levels; zone 1 is located near the portal triad, zone 2 is the centrio-lobular region, and zone 3

adjoins the hepatic vein (Figure 1) (*Adams & Eksteen, 2006*). The oxygen concentration ranges from 11% to 3% from the portal triad to the hepatic vein, respectively. An oxygen level of 3% is considered a hypoxic environment, therefore part of a healthy liver is likely to exist under a hypoxic state (*Jungermann & Kietzmann, 2000*).

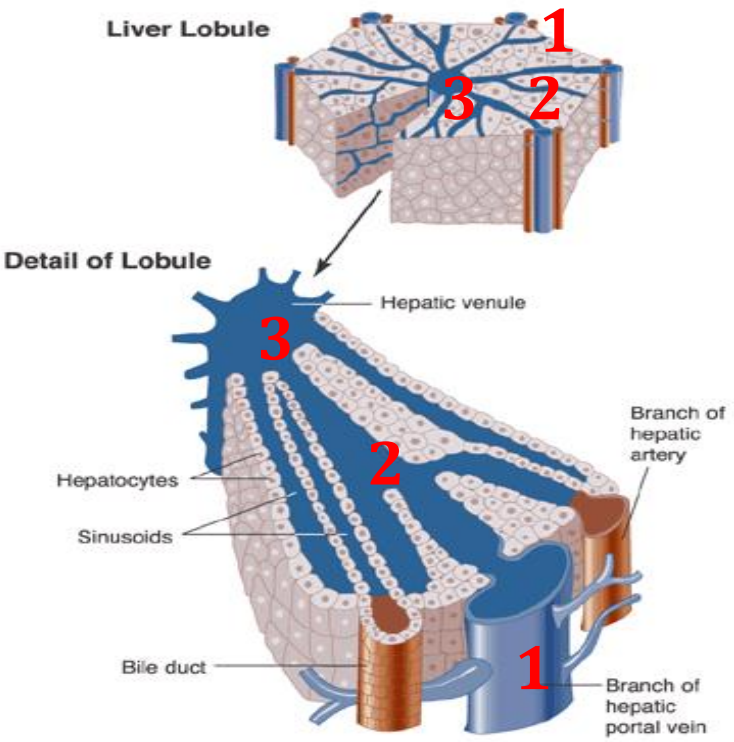


Figure 1: A cartoon depicting different zones within a liver lobule (*Adams & Eksteen, 2006).*
Figure 1 represents the anatomy of a section of liver. The numbers indicate zones of the liver. From zone 1 to 3 the gradient of oxygenation ranges from 11% to 3%.

Several liver diseases are associated with hypoxia and the tightly regulated host responses to hypoxia. For example, ischemia-reperfusion, obstructive sleep apnea, alcoholic liver disease, hepatitis C virus (HCV) infection, and hepatocellular carcinoma are all known to contribute to the development of, or persist within, a hypoxic environment. All these diseases are associated with

activation of hypoxia inducible factor (HIF) responses (*Nath & Szabo, 2012*). When hepatic parenchymal and non-parenchymal cells are exposed to hypoxic stress the varying oxygen concentrations induce a variety of effects and can determine whether a hepatocyte is capable of responding to the hypoxic stress and to what extent. Low oxygen conditions result in increased metabolic demand, tissue ischemia, as well as other conditions. These can *all* contribute to liver injury while under hypoxic stress (*Broughan et al, 2008*).

7.3 Hypoxia and Diseases of the Liver

Hypoxia can have a deep impact on the tissues of an aerobic organism. The liver has a normal homeostatic response to hypoxic conditions, which may become altered while in a diseased state. Normal adaptation to hypoxia results in the activation of stress response genes, which usually have beneficial effects, improving cell survival or eliminating aberrant cells. Stress responses include angiogenesis, erythropoiesis, glycolysis, proliferation, and apoptosis, activities regulated through the action of an evolutionarily-conserved family of transcription factors called hypoxia inducible factors (HIFs) (*Corpechot et al, 2002; Maegdefrau et al, 2009*). A number of diseases associated with the liver up regulate HIFs beyond normal levels (*Nath & Szabo, 2012*). Of most importance to the research in this project are the effects of HIFs in viral hepatitis and hepatocellular carcinoma (HCC).

7.3.1 Viral Hepatitis

There are at least three liver tropic viruses that are responsible for liver pathogenesis, hepatitis B, C, and E. Hepatitis B is the major cause of liver cancer in China and has been linked to hypoxia and liver damage through the

association of hepatitis B virus protein X (HBx) with HIF1α expression (*Xie et al, 2008*). The transfection of this protein into HepG2 cells has been shown to result in HIF1α accumulation. HBx protein has also been shown to increase levels of metastasis associated protein 1 (MTA1) and histone deacetylase 1 (HDAC1), both of which are involved in HIF1 stabilisation and directly interact with the transcription factor (*Han et al, 2007*).

Hepatitis E virus is associated with liver disease through the development of hepatitis and liver injury. Hepatitis E virus open reading frame 3 protein (ORF3) is a viral protein that is thought to be important for infection. This protein has been shown to up regulate a number of enzymes involved in the glycolytic pathway. In addition, there is an associated up regulation of HIF1α expression and stabilisation through phosphorylation of the CBP/p300 co-activator in an ERK-dependent manner. This correlates with HIF1α up regulation and increased AkT phosphorylation (*Moin et al, 2009*).

Chronic HCV infections are a common cause of chronic liver disease and cirrhosis. A persistent infection is enabled by the rapid replication of virus particles combined with continuous cell-to-cell spread and the lack of a vigorous host immune response (*Chen & Morgan, 2006*). There is a strong association between chronic HCV infection and the development of cirrhosis and hepatocellular carcinoma (HCC) (*Shepard et al, 2005*). There are a number of mechanisms by which HCV might interact with HIF1α. Stabilisation may be mediated by oxidative stress and calcium signalling caused by the expression of HCV genes; it has been shown that the NFκB, STAT3, PI3-K/ AkT and p42/44 mitogen-activated protein kinase pathways are all involved in this stabilisation. It

has also been shown that stabilisation of HIF1α through these pathways leads to stimulation of VEGF and angiogenesis as well as numerous other stress responses, including glycolytic enzyme up regulation (*Nasimuzzanan et al, 2007; Ripoli et al, 2010*). An association between HCV core protein and HIF1α has been observed in hepatoma cells. The core proteins induced an up regulation in VEGF expression and HIF1α DNA binding by EMSA. Transient infection with HCV or the presences of HCV subgenomic replicons are both linked to HIF1α stabilisation (*Nath & Szabo, 2012*).

7.3.2 Hepatocellular Carcinoma

There is a strong evidence to suggest the involvement of HIFs in the development of HCC. HIF associated genes such as the glucose transporter GLUT1 and growth factor VEGF have been shown to be up regulated in individuals with HCC and these have been implicated in malignant transformations. As with viral hepatitis, multiple pathways appear to be involved in the development of HCC. PI3K, ERK and NFκB are all associated with HIF stabilisation and involved in disease progression (*Sun et al, 2009; Daskalow et al, 2010; Fu et al, 2011*).

Interestingly, it has been shown that both HIF1α and HIF2α isoforms are up regulated in HCC. Up regulated HIF2α is found in 69.5% of HCC cases and correlates with tumour size, capsule infiltration, portal vein invasion, and necrosis. HIF2α up regulation is also associated with VEGF expression; however, there is evidence showing a decreased chance of survival. HIF2α was not shown in non-cancerous tissue, suggesting that its expression may be a

feature of tumours and their formation (*Bangoura et al, 2004; Bangoura et al, 2007*).

A better understanding of HIF regulation, activity and stabilisation could contribute towards future therapies for liver disease by providing targets to regulate HIF activity.

7.3.3 Hypoxia Inducible Factors

Hypoxia inducible factors (HIFs) are a family of transcription factors involved in the host adaptation to low oxygen environments. They are master regulators of the homeostatic response to changing oxygen concentrations and are found within nearly all cells and tissues. Stabilisation occurs under low oxygen and indicates the transcription factors are no longer being actively degraded. There are three currently known isoforms of HIF: 1α, 2α, and 3α, and the latter has multiple splice variants. HIF1α is expressed ubiquitously within all cells, whereas HIF2α and 3α appear to be more tissue-specific; their expression has been demonstrated within liver parenchymal cells. These subunits bind to a common HIFB subunit, also called ARNT, which is continuously expressed and not dependent on local oxygen concentration. Hundreds of genes are reportedly regulated through the action of hypoxia and HIFs; these genes are activated in response to stress (*Bertout et al, 2008; Kaelin & Ratcliffe, 2008*).

A number of genes reported to be HIF targets may only be responsive to HIFs within a small range of tissue types, with activation dependent on interaction with other cooperative molecules. There is evidence to suggest that different isoforms of HIF alpha subunit may be differentially regulated by multiple

mechanisms and that each subunit is capable of stimulating expression of distinct genes within the same environment, or activating the same genes with different observed effects (*Majmundar et al, 2010*).

7.3.4 HIF1α

HIF1α, the alpha subunit of a heterodimer called HIF1, is involved in glucose metabolism, regulation of lipid metabolism, liver injury and tumour-associated angiogenesis, metastasis and inflammation. It is responsible for converting metabolism from oxygen-dependent ATP production to glycolysis under low oxygen conditions through the promotion of glycolytic enzyme activity and LDHA expression. Combined, these actions provide additional NAD^+ required for glycolysis under hypoxia. Also, HIF1α promotes the expression of its target gene PDK1, which prevents the conversion of pyruvate into acetyl-CoA and reduces the oxygen consumption within cells. HIF1α has also demonstrated activity in converting glycolytic intermediates into RNA or DNA through the pentose phosphate pathway. These metabolic changes are part of an important role in facilitating cell survival and growth under low oxygen conditions (*Simon, 2006; Gordon et al, 2007*).

HIF1α is involved in the angiogenic response to low oxygen. The dimer binds to promoter regions within the target gene VEGF, enhancing expression of this growth factor. This effect has been observed in a number of injury models such as myocardial infarction, skin wound healing, and hepatic injury (Heinl-Green et al, 2005; *Mace et al, 2007; Sano et al, 2007; Botusan et al, 2008; Liu et al, 2008*). It is noteworthy that it is also responsible for stimulating angiogenesis

within tumours in a similar manner. However, the surrounding environment is filled with different cell types such as fibroblasts, which might respond differently to the hypoxic stress and contribute differently to the stress response (*Jain, 2005; Du et al, 2008*). Whether these responses require HIF stabilisation under hypoxic or normoxic conditions is currently unclear (*Bertout et al, 2008; Kaelin, 2008*). It has been suggested that HIF1α has a role in the regulation of cancer metabolism in certain tissues. Mutations that cause inactivation of the enzymes fumerate hydratase (FH) and succinate dehydrogenase (SDH), both involved in the TCA cycle, result in increased levels of HIF1α stabilisation within tumours through increased ROS activity inhibiting the prolyl hydroxylases (PHDs) after accumulation of fumerate and succinate (*King et al, 2006*).

HIF1α also has a role in cancer metastasis and invasion. The expression of HIF1α can contribute to the loss of E-cadherin. The mechanism of action differs between types of cancers; however, the evidence is clear that HIF1α expression is involved in epithelial to mesenchymal transition (EMT). In head and neck squamous cancers this transcription factor directly regulates TWIST1 transcription, which is responsible for tumour cell invasiveness and metastasis. HIF1α can also produce a similar response in prostate cancer through up regulation of SNAIL1 via VEGF (*Kaelin, 2008; Yang et al, 2008; Mak et al, 2010*).

HIF1α is also involved in inflammation. An inflammatory response to hypoxic stress is linked to the NFκB pathway within macrophages and neutrophils. NFκB activation alone is insufficient to stabilise HIF1α; rather, the additional regulation through exposure to low oxygen is required. This suggests the

possibility of graduated HIF stabilisation within hypoxic stress environments (*Rius et al, 2008*).

HIF1α, which represents a potential target for therapy in a large number of liver associated diseases, have been studied in macrophages, renal cells and hepatocytes, among others, under both hypoxia and normoxia. The McKeating lab is interested in HIFs because it has been shown that HCV virus is capable of stabilising the HIF1α protein under normoxic conditions (*Nasimuzzamann et al, 2007; Ripoli et al, 2010*), suggesting that this transcription factor has beneficial effects on the viral lifecycle.

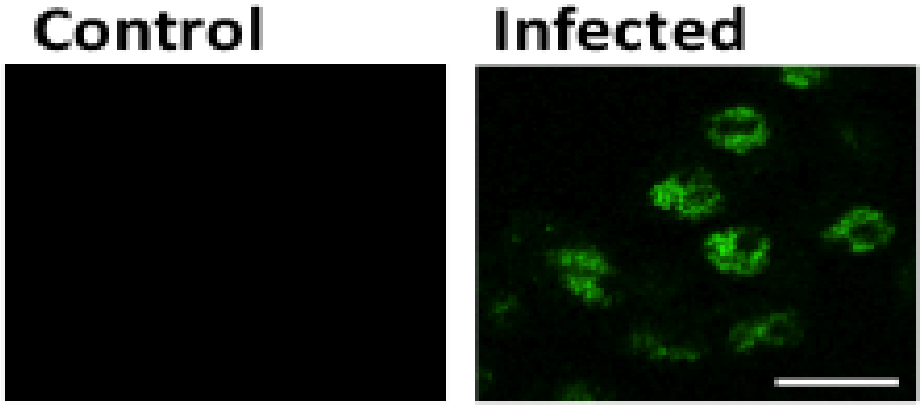


Figure 2: HCV stabilises HIF1α (*Wilson et al, 2012*)
Figure 2 presents a confocal image displaying the stabilisation of HIF1α in cells infected with HCV using GFP.

7.3.5 HIF2α

Much like HIF1α, HIF2α is important for regulating stress response genes in an oxygen-dependent manner. HIF2α has only recently been investigated and while there are currently no studies showing the stabilisation or activity of HIF2α within the liver, it appears the target genes activated by HIF1α and HIF2α overlap, suggesting they have similar roles or perhaps that they complement

one another (*Kim et al, 2006; Keith et al, 2012*). In contrast, other studies suggest that they oppose one another, having opposite effects on several different genes. However, there is evidence to suggest that the gene silencing of one isotype results in the over expression of the other (*Pan et al, 2004; Fang et al, 2009; Majmundar et al, 2012*). Could this be a compensation mechanism? When considering potential therapeutic targets for cancers, HIF1α is probably not the end of the story.

Similar to HIF1α, HIF2α is a subunit part of the HIF2 heterodimer involved in the regulation of stress response genes and has diverse roles in regulating stress response pathways, including glucose and lipid metabolism, angiogenesis, inflammation, and redox homeostasis. HIF2α has also been implicated in tumour associated angiogenesis and tumorigenesis (*Majmundar et al, 2012*).

Both HIF1α and HIF2α are able to alter the expression of cytochrome C oxidase in order to increase the efficiency of the electron transport chain. However, HIF2α has some unique roles in the regulation of redox homeostasis. It is responsible for up regulation of an anti-oxidant enzyme called SOD2, which regulates aberrant ROS accumulation. The loss of HIF2α activity contributes towards the accumulation of ROS and ultimately leads to associated activation of p53 and tumour cell death (*Scortegagna et al, 2003; Gordan et al, 2007*).

HIF2α has been associated with lipid catabolism, although little is known about its involvement at present. Similarly, HIF2α has a role in angiogenesis, but in comparison with HIF1α it has not been extensively studied its role is yet to be elucidated. However, it has been shown that it can stimulate the production of

functional blood vessels in endothelial cells, which has implications in tumour progression (*Beroukhim et al, 2009; Morris et al, 2009; Dalgliesh et al, 2010*).

Unlike HIF1α, the presence of HIF2α within a tumour microenvironment is indicative of a poor prognosis, suggesting opposite roles within the development of tumours. In renal carcinoma cells the selective inhibition of one or both of the HIF isoforms showed that HIF2α is essential for tumour progression whereas HIF1α is not (*Leek et al, 2002; Kawanaka et al, 2008*) possibly related to their differential interaction with the transcription factor MYC. MYC is responsible for controlling the G1/S stage of the cell cycle and promoting proliferation through induction of most glycolytic enzymes and enhanced protein synthesis. HIF1α has been shown as an antagonist of MYC whereas HIF2α promotes this activity (*Gordon et al, 2007a; Mylonis et al, 2006; To et al, 2006; Kalousi et al, 2010*). Other observations suggest that the aberrant activity of HIF2α may be more oncogenic than HIF1α.

HIF2α is also activated through the action of pro-inflammatory cytokines and the NFκB pathway. However, the responsible cytokines differ between HIF2α and HIF1α. This differential activity warrants further investigation because there is evidence suggesting a correlation between tumour associated macrophages (TAMs) in breast cancers and poor prognosis. It has also been shown that the deletion of HIF2α in mice with HCC prevented migration of TAMs into the tumour environments. HIF2α has roles in the stimulated migration or chemotaxis of macrophages (*Leek et al, 2002; Murdoch et al, 2004; Kawanaka et al, 2008*).

7.3.6 HIF3α

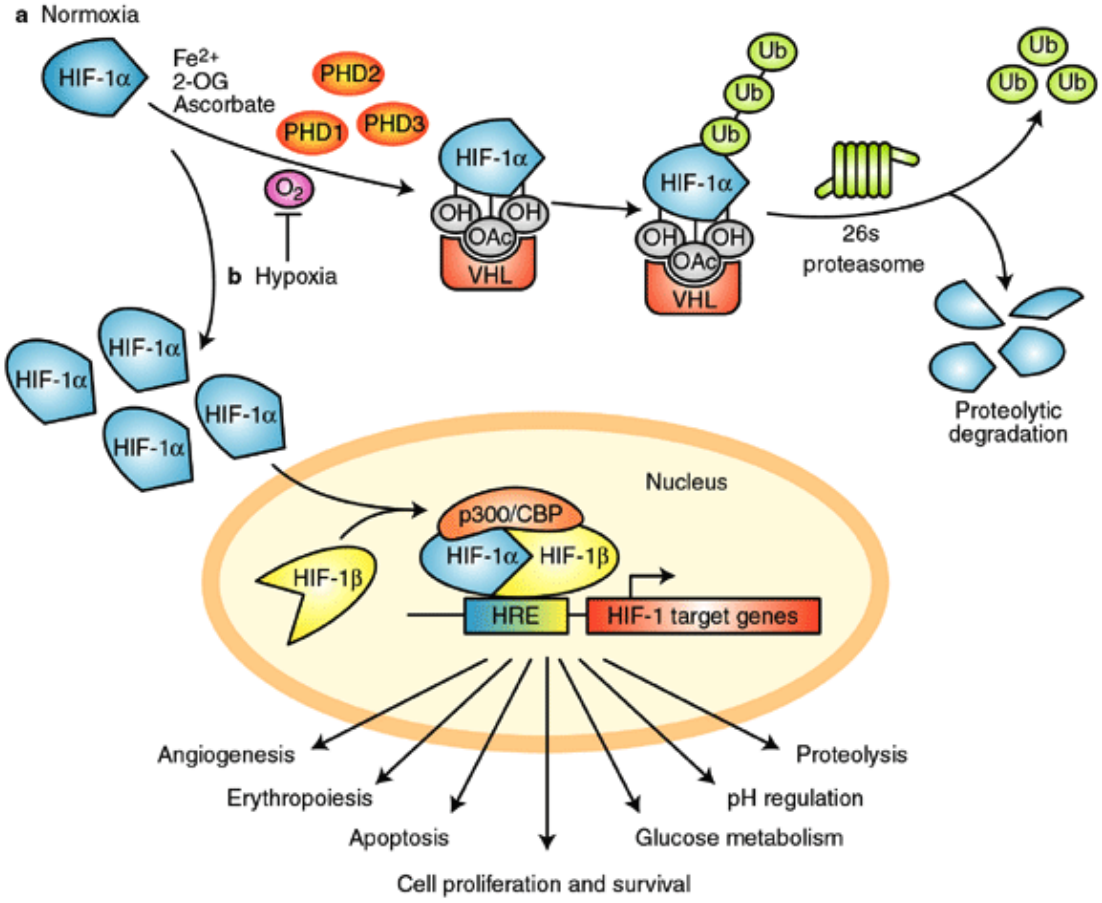
The third isoform of HIFs is the least studied within the family. However, a recent paper has observed that the protein undergoes polyubiquitination and proteosomal degradation in the same way as the other HIFs. Unlike the other two isotypes, HIF3α has a number of different splice variants. So far at least 7 variants have been identified, which can be separated by size. It has been suggested that the long variants are located within the nucleus while under hypoxia, whereas the short transcripts can be found in the cytoplasm. The interaction of HIF3α variants with other HIFs inhibited nuclear translocation. None of the HIF3α variants are capable of efficient hypoxia responsive element (HRE) activation. However, it was shown that the down regulation of HIF3α caused a reduction in some HIF target gene expression (*Augstein & Poitz, 2011. Heikkilä et al, 2011*), perhaps indicating that HIF3α is not limited to a negative regulatory role over HIFs, but rather is responsible for multiple functions in controlling the host cell response to hypoxia.

HIF3α lacks a transactivating domain, much like an identified negative regulator of HIF-1 called IPAS (inhibitory PAS domain protein). IPAS prevents the interaction and dimerisation of HIF1α with HIF1β by binding to the HIF1α subunit, which prevents the binding to HRE and subsequent activation of any pathways. HIF3α might therefore be a negative regulator of HIF pathways (*Augstein & Poitz, 2011. Heikkilä et al, 2011)*. Previous experiments studying this isoform were conducted in Chinese hamster ovary (ChoK1) cells and most studies have shown that HIF3α is expressed abundantly within the kidneys and lung epithelial cells. While expression of HIF3 α has been shown in

hepatocytes, there are few studies showing HIF3 α activity in liver cells (*Augstein & Poitz, 2011. Heikkilä et al, 2011)*.

7.3.7 HIF Stabilisation

Under normoxia HIFs are rapidly degraded, which is important in the regulation of activation of certain stress response genes. Figure 3shows the pathway that results in proteosomal degradation under normoxia for HIF1α. The other HIF isotypes (2α/3α) may be regulated in the same or similar manner. Under normoxia, the PHDs hydroxylate HIF1α at two conserved proline residues found within the oxygen-dependent degradation domains (ODD) (*Carroll & Ashcroft, 2005*). Hydroxylation of these domains results in the recruitment of the Von Hippel Lindau protein (VHL) E3 ligase complex, which results in the polyubiquitination of HIF1α and subsequent proteosomal degradation (*Carroll & Ashcroft, 2005*). At the same time, factor inhibiting HIF (FIH-1), an asparaginyl hydroxylase, interacts with the HIF1α, modifying the protein. This prevents the interaction of HIFs with co-activator p300/CBP (*Lando et al, 2002*). The combination of these two activities results in transcriptional inactivation under normoxic conditions.



HIF-1α regulation by proline hydroxylation

Expert Reviews in Molecular Medicine 2005 Published by Cambridge University Press

Figure 3: HIF1α regulation under hypoxia or normoxia (*Carroll & Ashcroft, 2006*)

Under hypoxic conditions the activities of these two hydroxylases is inhibited, which results in the stabilisation of HIF1α, following which, the protein begins to accumulate then translocates to the nucleus. After translocation to the nucleus the protein dimerises with its HIFβ counterpart. This heterodimer then binds to co-activators p300/CBP and this dimer binds to the HREs found within the regulatory regions of target genes, stimulating a wide range of stress responses (*Carroll & Ashcroft, 2005*).

Mitochondria appear to be involved in oxygen sensing, PHD inhibition, and HIF stabilisation. Under "moderate" hypoxia (approximately 1.5% oxygen),

mitochondria release reactive oxygen species that inhibit the activity of PHDs (*Kaelin et al, 2005; Klimova & Chandel, 2008*). It has been suggested that the radicals may originate from electron transport chain complex III directly and that mitochondria might be involved in HIF regulation (*Klimova & Chandel, 2008*). This effect may be limited to moderate hypoxic conditions, below which HIFs may stabilise in the absence of mitochondria. This pathway may be similar to or a part of the pathways described in the section below.

Environmental oxygen concentration can determine which HIF becomes stable and begins to accumulate. Evidence suggests that at oxygen concentrations of 0-2%, HIF1α is stabilized, and at 2-5%, HIF2α is stabilised (*Nilsson et al, 2005; Holmquist-Mengelbier et al, 2006; Li et al, 2009*). However, studies have shown that different factors such as pro-inflammatory cytokines, growth factors and bacterial products can contribute to HIF stabilisation even under normoxia (*Imtiyaz & Simon, 2010*) and the majority of pathways involved in stabilisation mechanisms are currently not fully understood. Cytokines such as TNFα and IL-1β, which act through the NFκB pathway, have been shown to induce the accumulation of the HIF1α isoform and increase transcriptional activity (*Imtiyaz & Simon, 2010*). Others, such as TGF-β, act through inhibition of PHD2 expression. These cytokines have roles in inflammation and inducing HIF activity, which suggests that HIFs may have a role in inflammation. HIFs may also be stabilised through stimulation with bacteria and bacterial products such as lipopolysaccharide (LPS). This activity may be linked to a number of pathways, including NFκB, ROS, PHD,s and mitogen activated protein kinases (MAPKs) (*Imtiyaz & Simon, 2010*).

The pathway most frequently described in HIF stabilisation after oxygen regulation is the NFκB pathway. NFκB (nuclear factor κB) is a transcription factor found within most cell types and involved in pathways stimulated by stress responses, cytokines, ROS, and bacterial or viral antigens. This pathway is predominantly regulated through the activity of IκB (inhibitor of κB) proteins, which sequester NFκB dimers within the cytoplasm. These proteins inhibit activity by masking the nuclear localisation signal (NLS) of NFκB proteins. IκB proteins activity is inhibited through the activity of enzymes IKK (IκB kinases), which utilise phosphorylation of IκB to target the protein for degradation (*van Uden et al, 2008*). This activation of NFκB has been reported to contribute to basal levels of HIF1α mRNA and proteins through mediation of the subunit expression and stabilisation as well as promoter activity (*Görlach & Bonello, 2008*). Various factors such as TNFα, bacterial products such as LPS, and reactive oxygen species have been shown to stabilise HIF1α via the NFκB pathway by phosphorylating IκB; NFκB binds to a conserved region within the promoter of the HIF1α gene (*Imtiyaz & Simon, 2010*). In turn, activation of this pathway and subsequent HIF1α stabilisation regulate the production of numerous cytokines and multiple stress response pathways.

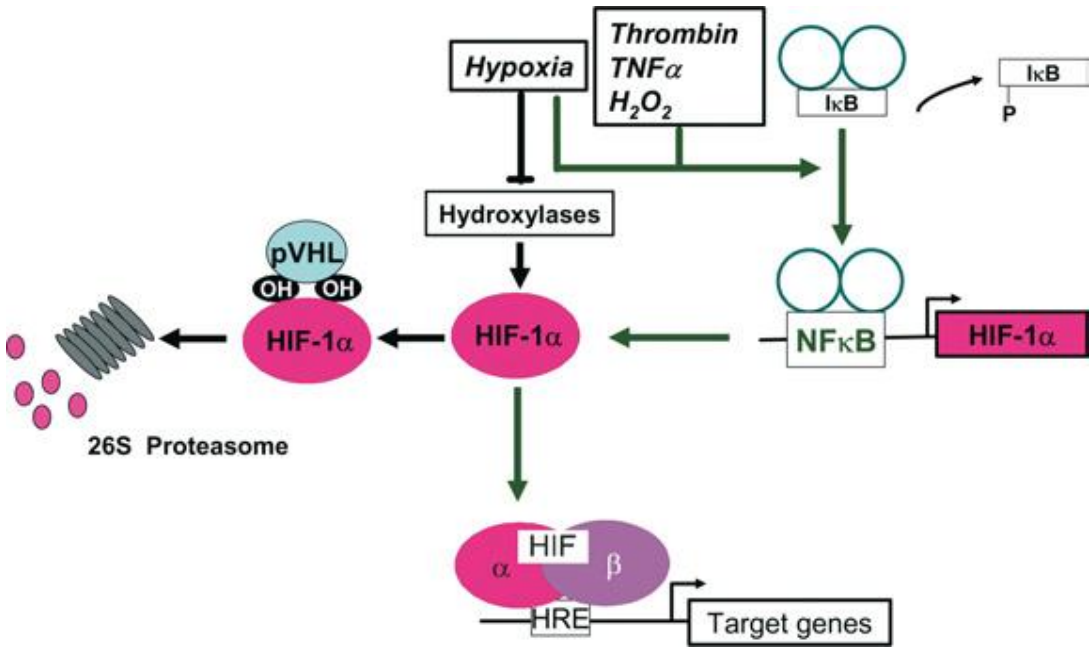


Figure 4: Cartoon depicting HIF1α stabilization through NFκB activation (*Görlach & Bonello, 2008*).
Figure 4 represents two pathways involved in HIF1α stabilisation. It illustrates how both hypoxia and cooperative factors thrombin, TNFα, and H_2O_2 stabilise the transcription factor and result in subsequent stress response activation.

The activation of Toll-like receptors (TLR) by exposure to pathogens and their products (such as LPS) is also associated with disease pathogenesis and HIF stabilisation (*Imtiyaz & Simon, 2010*). A number of previous reports indicate that HIF protein accumulation is increased by PHD2 and PHD3 inhibition in a TLR4 dependent manner under normoxia (Uesugi et al, 2001; *Scharte et al, 2006; Peyssonnaux et al, 2007; Jantsch et al, 2011*). There is evidence to suggest that HIF also binds directly to TLR4 and up regulates TLR4 expression while under hypoxia, which in turn increases HIF stabilisation and subsequent cytokine/chemokine production. This interaction suggests the presence of a positive feedback loop resulting in increased HIF responses under hypoxia and during infection (*Kim et al, 2009*).

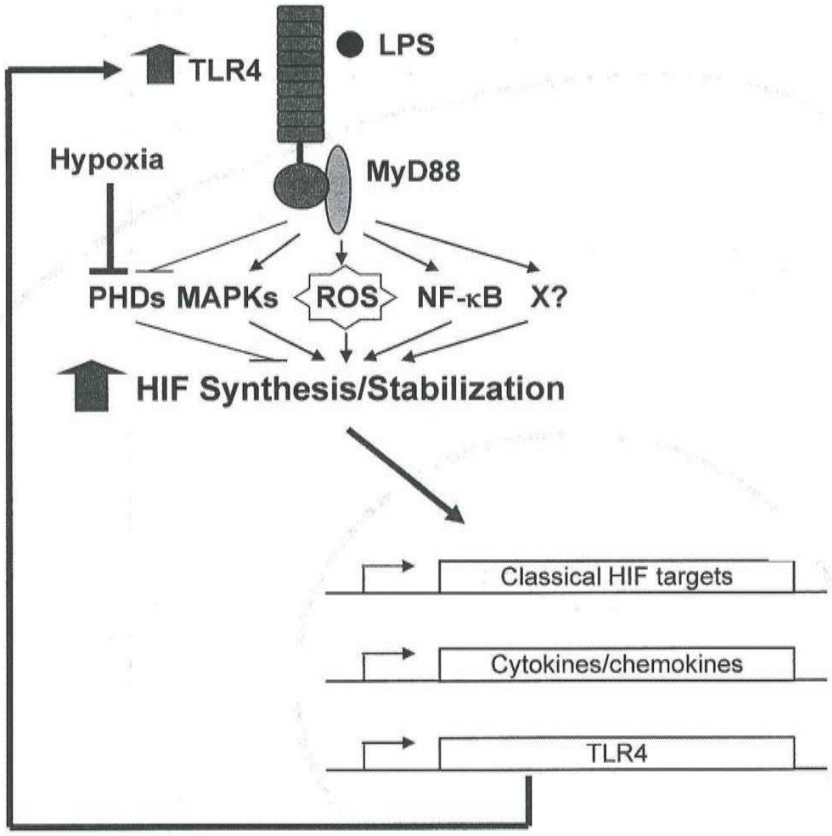


Figure 5: Cartoon depicting HIF stabilisation through TLR4 signalling (*Imtiyaz & Simon, 2010*)
Figure 5 represents the TLR4 pathway of HIF stabilisation and the various factors associated with receptor activation.
TLR (toll-like receptor), MyD88 (myeloid differentiation primary response gene 88), LPS (lipopolysaccharide), PHDs (prolyl hydroxylases), MAPKs (mitogen activated protein kinases), ROS (reactive oxygen species), NFκB (nuclear factor κB).

7.4 Aims and Objectives

The main aim of the project was to study the role of HIFs in HCV biology and liver injury. This project is part of a larger whole in which HCV utilisation of HIFs as part of the viral lifecycle is being studied. The work within this thesis is not a complete project, but rather part of an ongoing study.

The objectives were to study the kinetics of HIF stabilisation and transcriptional activity in hepatoma cells at varying oxygen concentrations and to investigate

the role of TNFα in stabilising HIF-transcriptional activity in hepatoma cells. In a broader view, it is of interest to investigate the role of HIF2α within the liver and adaptation to stress, whether HIF1α and HIF2α regulate the same genes in hepatocytes and are they regulated in the same way, and what benefits might this confer on the virus lifecycle or tumour microenvironments.

8 Materials and Methods

8.1 Plasmids

Vector	Insert
pGEX-4T	HRE Luciferase
pCDNA	NFκB Luciferase

8.2 Cell Lines

Name	Tissue	Growth Media	Source
Huh-7.5	Human Hepatoma	DMEM	Jhaveri Laboratory at Duke University
HepG2-CD81	Human Hepatoblastoma	DMEM	Prepared in the McKeating Laboratory

8.3 Tissue Culture

Huh7.5 cells and HepG2 cells were obtained from Charles Rice (Rockefellar University, New York and the American Tissue Culture Collection. Cells were maintained in Dulbecco's modified Eagles medium (DMEM) (Gibco, USA) supplemented with 10% foetal bovine serum (FBS), 1% v/v penicillin-streptomycin (pen/strep) and 1% L-Glutamine. For normoxic experiments cells were incubated at 37^{o}C with 20% O_2 and 5% CO_2. For hypoxia cells were grown under 1 or 3% oxygen in a humidified sealed hypoxystation (Don Whitely Scientific, UK) calibrated to 5% CO_2 and 95% N_2.

8.4 Transfections

Cells prepared 24 hours prior to the transfection stage had been grown in DMEM with 10%FBS. The incubations during this growth period were all at 37^{o}C in 20% O_2 and 5% CO_2. Before transfection began the media in each well

of the plate was replaced with pen/strep free DMEM with 3%FBS. Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions. The cells were transfected with a pGEM plasmid (Margaret Ashcroft, UCL, London). The DNA/ lipofectamine mixture is then added drop wise to the cells and incubated at 37^{o}C/ 5% CO_2 for 6-8 hours. Following this incubation period the DNA is carefully removed and the media replaced with DMEM containing 3% FBS and pen/strep. The cells are left overnight in the incubator before experimentation.

8.5 HRE Luciferase Assay

This assay is performed to ascertain the kinetics of HRE activation in cells exposed to hypoxic conditions representing HIF activity. Following transfection, the cells were split into 96 well plates for each time point and condition using standard tissue culture techniques. The contents of each well is then combined within a universal and 100ul added to appropriate wells of a 96 well plate for 5,10, 15 and 25 hour time points. The cells are then treated with the appropriate concentrations of pro-inflammatory cytokines (TNFa) or 10% DMEM (control) and then placed into either normoxic (20% O2) or hypoxic (1-3% O2) incubation. A T0 time point is taken at this point. The media is removed from each well in the 96 well plate. 50ul of 1x luciferase lysis buffer is added to each well. At appropriate times, the other plates are removed from their condition, the media removed and luciferase lysis buffer added. After the full 25 hours of the time course have passed the plates are removed from the freezer and left to thaw for a couple of hours. The luciferase assay substrate is also removed from the freezer and allowed to thaw (the contents is light sensitive and therefore

kept wrapped in foil). The 50ul of lysis buffer and lysed cells is transferred to the 96 well luminometer plates. 50ul of luciferase assay substrate is added to each well. The plate is then read using a luminometer. The kinetic setting is chosen and each well is read for 5 seconds per well.

8.6 LDH Assay

At the desired time points 50ul of supernatant from each sample was transferred to a 96 well plate (round bottomed ELISA plates). The positive controls are cell lysates prepared using freeze thaw lysis (15 minutes in -80oC, 15 minutes in 37oC, repeat). 50ul of positive control lysate is added to the same 96 well plate. Add 50ul of the substrate mix (Promega) to the supernatants and cell lysates. The plate is covered in foil and left to incubate at room temperature for 30 minutes. After incubation, 50ul of stop solution is added to each well. A slight change in the red colour should happen. Using a pipette tip, any bubbles in the wells should be popped. The plate is then measured at 490nm using a Multiskan Ascent Platereader (Thermo Electron Corporation).

9 Results

9.1 Analysis of Oxygen Concentration Effects on HIF Dependent Transcriptional Responses Over Time

Previous studies investigating the effects of HIFs within the liver have largely focused upon the activities of the HIF1α isoform. These previous studies have been conducted using a combination of 20% oxygen representing normoxia and 1% oxygen to simulate hypoxic conditions. In order to understand the role of HIFs within liver disease it is important to understand the kinetics of HIF-dependent transcriptional responses within the liver. *Stroka and colleagues* data also indicates that the activity of HIFs may be transient. This further enhances the importance of studying the kinetics of HIF dependent transcriptional activity. It is interesting that HIFs activity is considered transient; if this is the case, how are certain cells within the liver that are continually exposed to hypoxic conditions regulated. Anatomically the liver exists within an oxygen range of 3-11%. Previous data studying HIFs within the liver has primarily been conducted at 1% oxygen or less. This oxygen concentration is not normally found within the liver environment and therefore there is precedent for studying the kinetics of HIF activity under more physiologically relevant oxygen concentrations. 3% oxygen is still considered a hypoxic environment and some zones within the liver exist near continually at this oxygen concentration. Therefore the study of liver cell response to this oxygen concentration is justified and a comparison of HRE responses to each oxygen concentration was carried out.

9.1.1 Exposure to low oxygen levels of oxygen results in increased HIF activity over time

To assess the kinetics of hypoxia driven transcriptional activity we utilised a reporter system; HIFs act by binding to HRE regions in the promoters of target genes containing the sequence 5'-NCGTG-3'. A HIF promoter driven luciferase construct is inserted into the cells and drives expression of luciferase upon HIF binding to HRE (*Tong et al, 2013*). Huh 7.5 cells were transfected with an HRE luciferase reporter plasmid. These represent the stabilization of HIFs, subsequent dimerization and binding to HRE regions up stream from their targets. A luciferase reporter is expressed upon binding to the HRE region. The kinetics of HRE activation is indicative of HIF activity within the cells. Figure 6 represents the initial data generated in this project, indicating that exposure to hypoxic levels of oxygen increased HRE-driven luciferase activity over time at different oxygen concentrations. There is an increase in activity between 10-15 hours; this is followed by a decline in activity at both oxygen concentrations between 15-25 hours. The literature suggests that after 15 hours the HIF1α protein begins to degrade (*Uchida et al, 2004*). This could be responsible for the halt in increased activity. The levels of HRE activity appear comparable between different oxygen concentrations. The data generated at each oxygen concentration was plotted against normoxic values taken from their respective assays. This graph enables clear observation of a pattern in HRE activation that is similar at both oxygen concentrations. In summary, these data suggest that the exposure to low oxygen is sufficient to stabilize HIFs and to stimulate HRE transcriptional activity and luciferase reporter gene expression in Huh-7.5

hepatoma cells and that the hypoxic response is similar at both hypoxic conditions in Huh 7.5 cells.

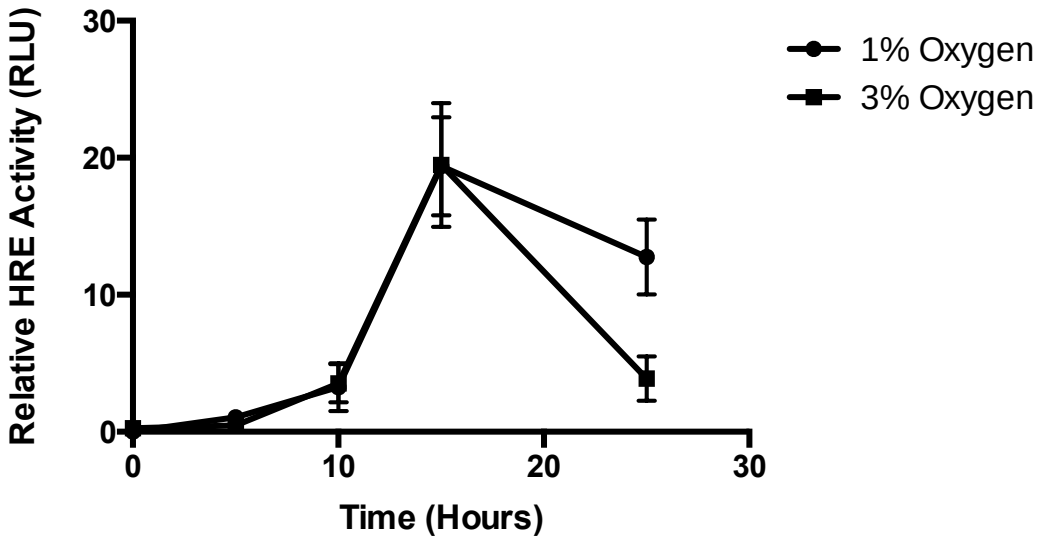


Figure 6: HRE luciferase assay showing a comparison of HIF kinetics in Huh 7.5 cells exposed to two different hypoxic oxygen concentrations

Huh 7.5 cells were seeded at 1.5x105 density and transfected with an HRE luciferase reporter gene. The cells were then placed into hypoxic or normoxic conditions. The data represents the pattern of HRE activation over 25 hours at each oxygen condition. The data is plotted as relative fold change in activity between hypoxic and normoxic conditions. The graph represents the mean values of 3 replicates per time point. A value of $P<0.008$ was determined at 25 hours using an unpaired T test with corrections for multiple comparisons.

Following the observations of HRE activation within Huh 7.5 cells we thought it would be interesting to compare responses between different cell types in addition to different oxygen concentrations. HepG2 cells are a more differentiated hepatoma cell line with polarizing capabilities. This experiment was performed because the stabilization and activity of HIFs may be different within another cell type. Figure 7 represents the transcriptional activation in HepG2 cells exposed to hypoxia relative to normoxic control time points. The graph indicates an increase in activity over time at both oxygen concentrations.

However, the response observed under 3% oxygen appears lower. A different profile is evident over 25 hours. Whereas the response under 1% oxygen is similar to that observed in Huh 7.5 cells.

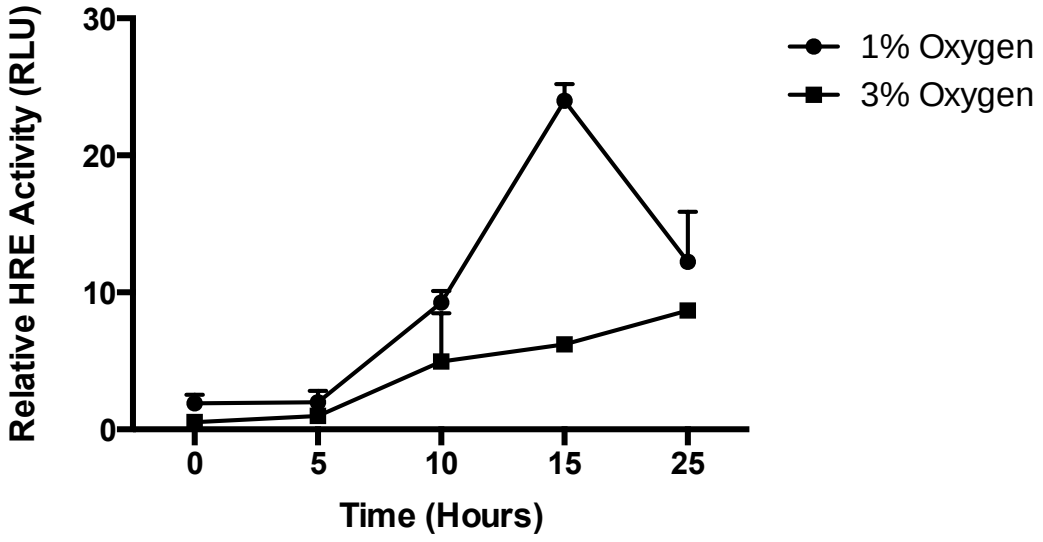


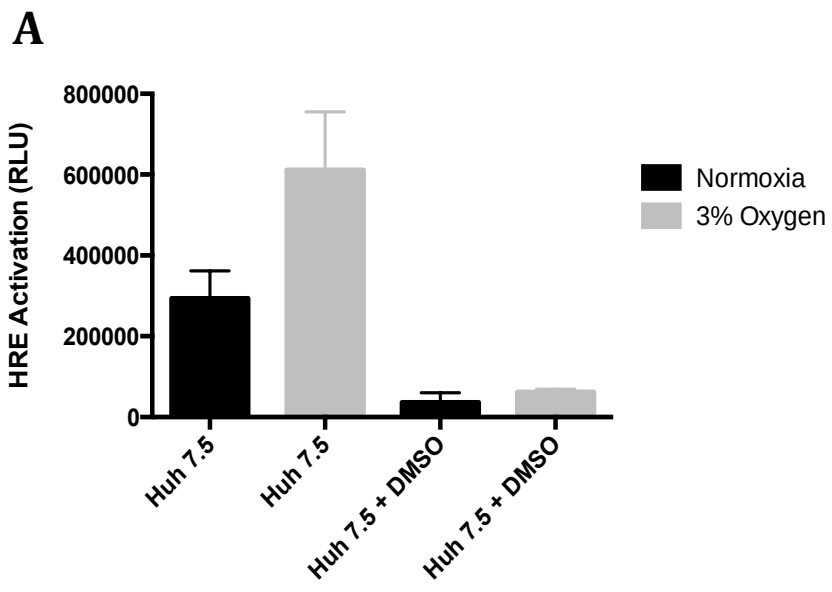
Figure 7: HRE luciferase assay showing a comparison of HIF kinetics in HepG2 cells exposed to two different hypoxic oxygen concentrations.

HepG2 cells were seeded at 1.5x105 cells/ml and transfected with an HRE luciferase reporter gene. The cells were then placed into hypoxic or normoxic conditions. Figure 7 represents the patterns of HRE activation observed at each oxygen concentration over 25 hours. The data is plotted relative to normoxic controls showing a fold change in activity. HRE response was measured as relative light units. The graph represents the mean values of 3 replicates per time point. A value of $P<0.002$ was determined at the 15 hour time point using an unpaired T test with corrections for multiple comparisons.

9.1.2 A comparison of HRE luciferase signals in naïve and highly differentiated Huh 7.5 cells

This experiment was performed in order to study the potential difference in response following DMSO mediated differentiation of Huh 7.5 hepatoma cells. DMSO treatment arrests cell division and promotes the expression of many differentiation markers expressed by primary hepatocytes isolated from human liver such as HnF4a (*Sainz & Chisari, 2006*) or CYP3a4 (*Choi et al, 2009*). This

means that these cells may be a more accurate representation of how the cells of a normal healthy liver would respond to hypoxia. Figure 9 shows that highly differentiated hepatoma cells induce a lower HRE response compared to parental untreated hepatoma cells.



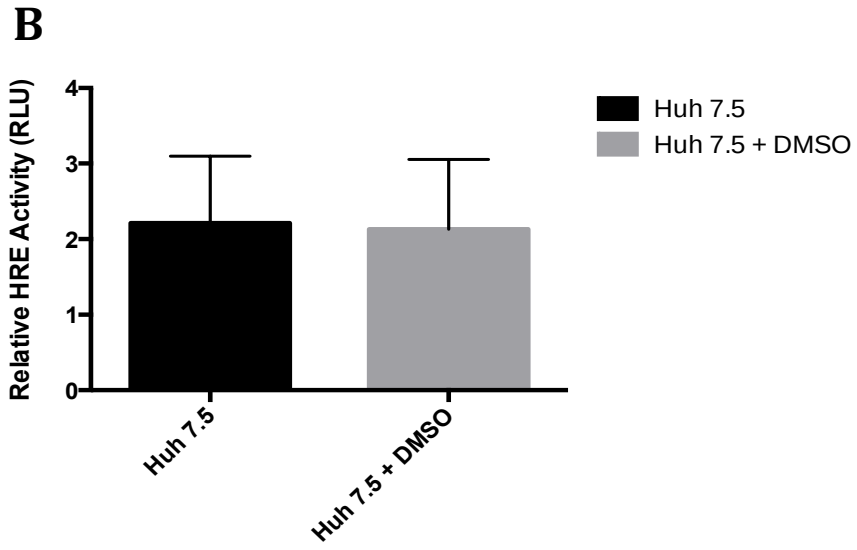


Figure 8: HRE luciferase assay showing the effect of hypoxia on HRE luciferase signals in DMSO differentiated cells.

Huh 7.5 cells were seeded at 2x105 cells/ml in 24 well plates and treated with DMSO. The cells were left for 7 days to differentiate. Naïve Huh 7.5 cells were seeded at 2x105 cells/ml after 7 days. All cells were transfected with an HRE luciferase reporter gene. The cells were placed into hypoxic or normoxic conditions overnight. **A**. The raw data comparing the HRE activation observed in naïve and highly differentiated Huh 7.5 cells. The graph represents the mean values of 3 replicates per time point. A value of $P<0.02$ was determined using a multiple T test. **B.** The data is plotted relative to normoxic controls showing a fold change in activity. The graph represents the mean values of 3 replicates per time point. A value of $P<0.92$ was determined using an unpaired T test. HRE response was measured as relative light units.

Figure 8 A indicates that DMSO differentiated cells have a reduced response to hypoxic conditions. However, when the data is plotted relative to normoxic controls we see very little difference in response between these cells. It is important to note that HRE activation appears lower in naïve Huh 7.5 cells compared to previous results.

9.2 Studying the effect of cytokines on HIF activity

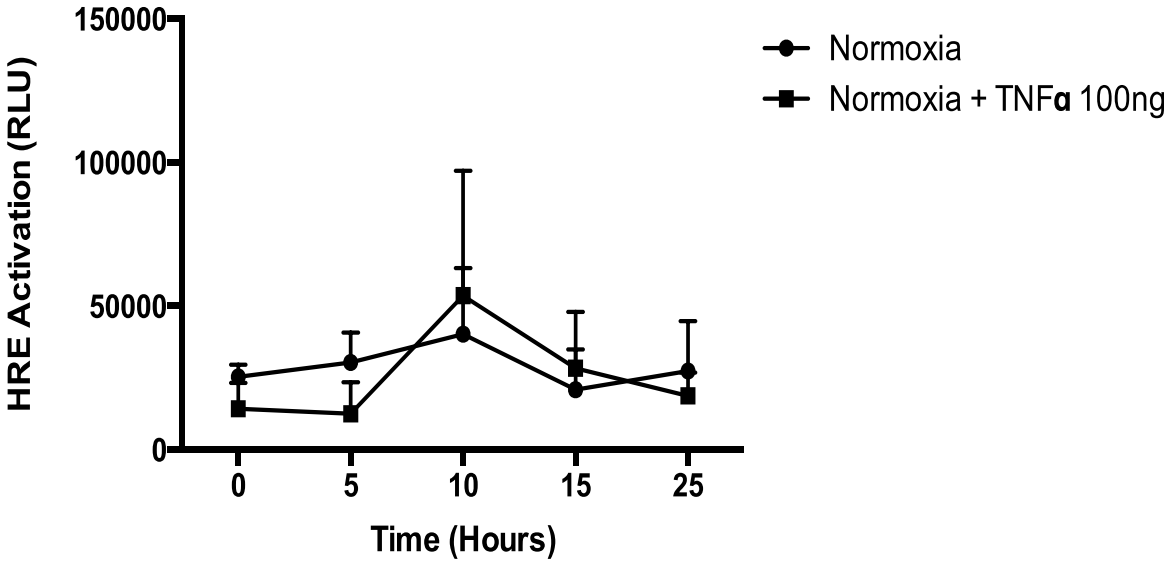
Following initial experiments studying the kinetics of HIF activity and stability, we began to investigate the effect of TNFα on HIF transcriptional activity in

Huh-7.5 and HepG2 hepatoma cells. Previous studies have shown that cytokines such as TH1 cytokine IFN-γ and TH2 cytokine IL-4 stabilize HIFs in macrophages (*Thieu et al, 2007; Rius et al, 2008*). The following experiments are performed to test whether the effects of hypoxic conditions and cytokines activate HIF differently. Specifically experiments were performed using TNFα, which has been reported to stabilize HIF through NFκB (*Görlach & Bonello, 2008*).

These experiments were performed to investigate the effect of treating Huh 7.5 cells with the pro-inflammatory cytokine TNFα. Previous work has shown that the addition of TNFα is capable of stabilizing HIF1α under normoxia and hypoxia (*Görlach & Bonello, 2008*). These experiments aim to study the kinetics of this stabilization and to determine whether TNFα may produce an HRE response beyond that of hypoxia alone. The data suggests that the addition of TNFα does not induce HRE activity beyond that of hypoxia alone.

To ensure the TNFα used during this experiment is active an assay was performed to test TNFα activity by measuring NFκB activation in both Huh 7.5 and HepG2 cells. Figure 9 B indicates that the addition of TNFα to a sample produces a higher luciferase response in cells transfected with an NFκB reporter. These are compared to transfected cells treated with media alone. The data suggests that the TNFα in use is active and should therefore produce expected responses.

A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100000
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HRE Activation (RLU)
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Normoxia + TNFα 100ng

B

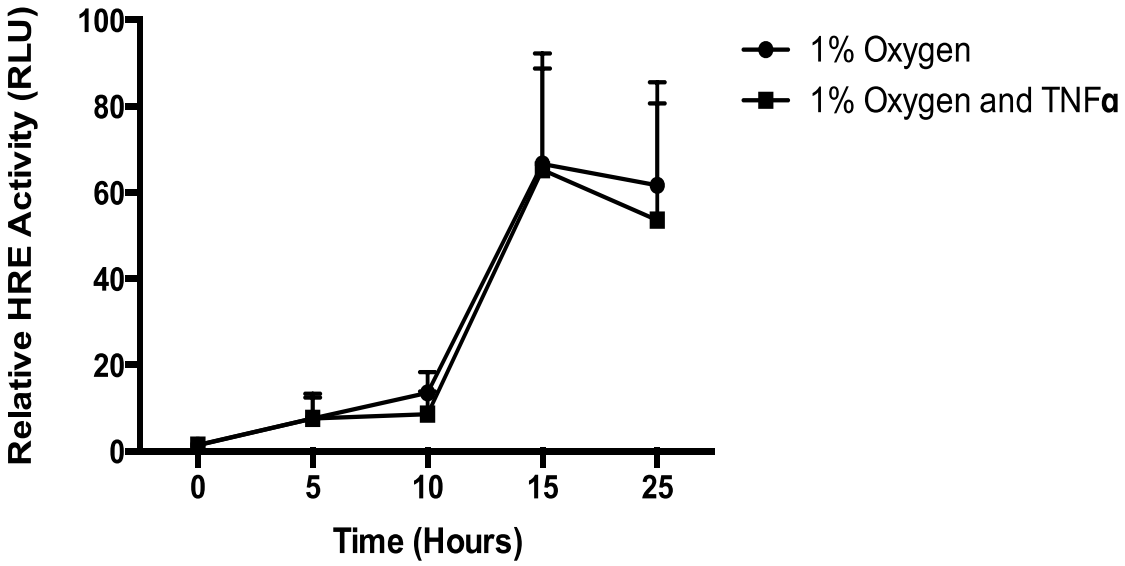

Figure 9: HRE luciferase assays representing HIF kinetics in Huh 7.5 cells following the addition of TNFα.

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C

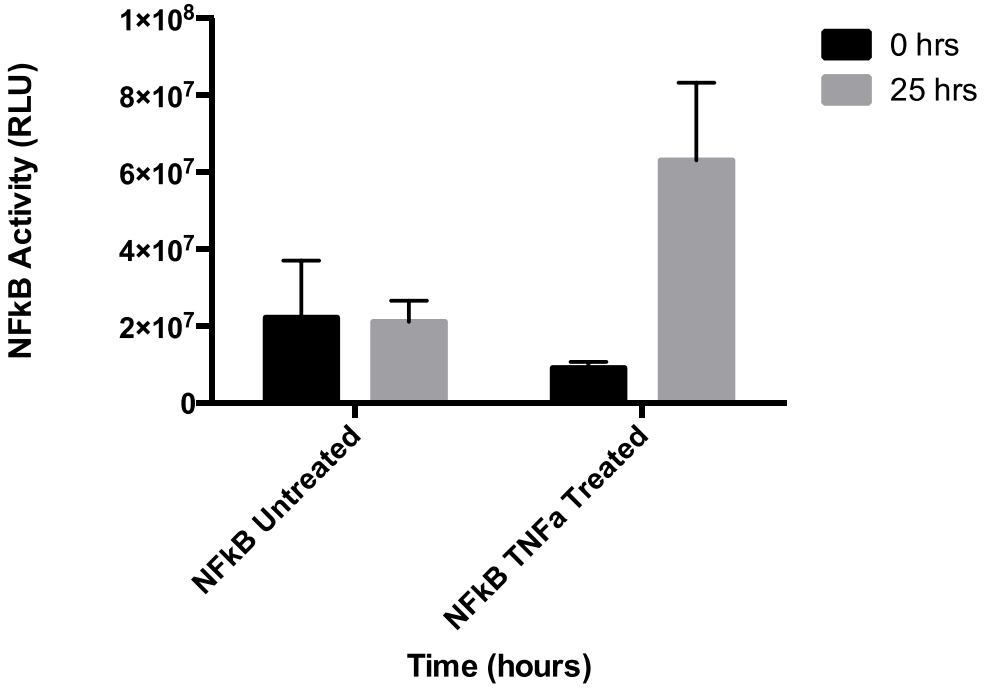
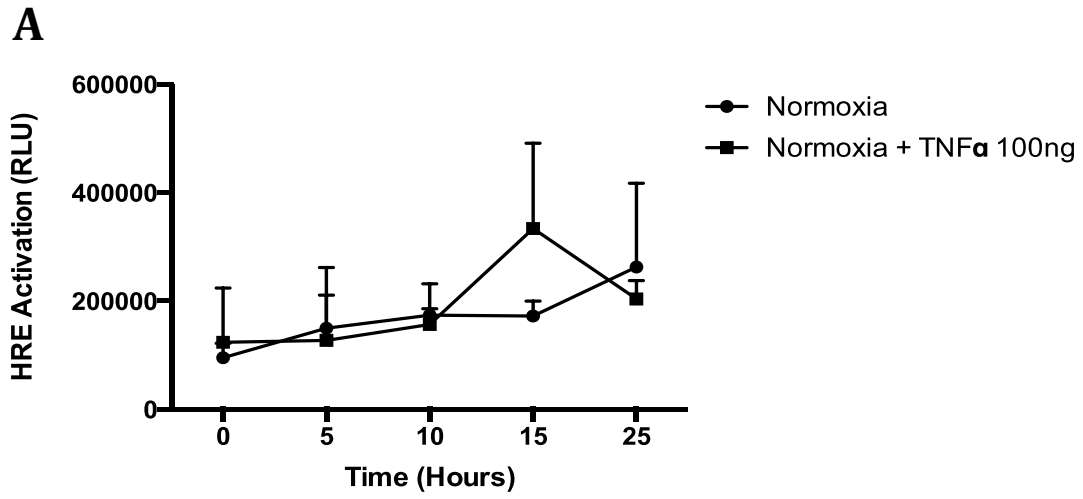


Figure 9: HRE luciferase assays representing HIF kinetics in Huh 7.5 cells following the addition of TNFα (continued).

Huh 7.5 cells were seeded at 1.5x105 cells/ml and transfected with an HRE luciferase reporter gene. Cells were treated with either 100ng of TNFα or with untreated media. The samples are then placed under normoxia or 1% oxygen. HRE activation is measured in relative light units. **A.** Figure 9 A represents HRE responses under normoxic conditions **B.** Figure 9 B represents HRE responses under 1% oxygen relative to normoxic controls, showing the fold change in activation. The graph represents the mean values of 3 replicates per time point. A value of P<0.8859 was determined using an unpaired T test. HRE response was measured as relative light units. **C.** Figure 9 C represents HRE activation through NFκB stabilization of HIFs in Huh 7.5 cells treated with TNFα compared to an untreated control. Huh 7.5 cells were seeded at 1.5x105 cells/ml and transfected with an NFκB luciferase reporter gene. The cells were then treated with 100ng of TNFα or untreated media. HRE activation is measured in relative light units.

Following the investigation into TNFα effects on Huh 7.5 cells, we studied the effect on stabilization in HepG2 cells. This experiment was performed because the stabilization and activity of HIFs may be different within another cell type. These results indicate there is no significant difference in HRE activity in cells treated with TNFα beyond that of hypoxic conditions alone. Interestingly there may be a blunted response, which may represent negative regulation caused by over stimulation. This pattern of activity is consistent with those seen in Huh 7.5 cells.



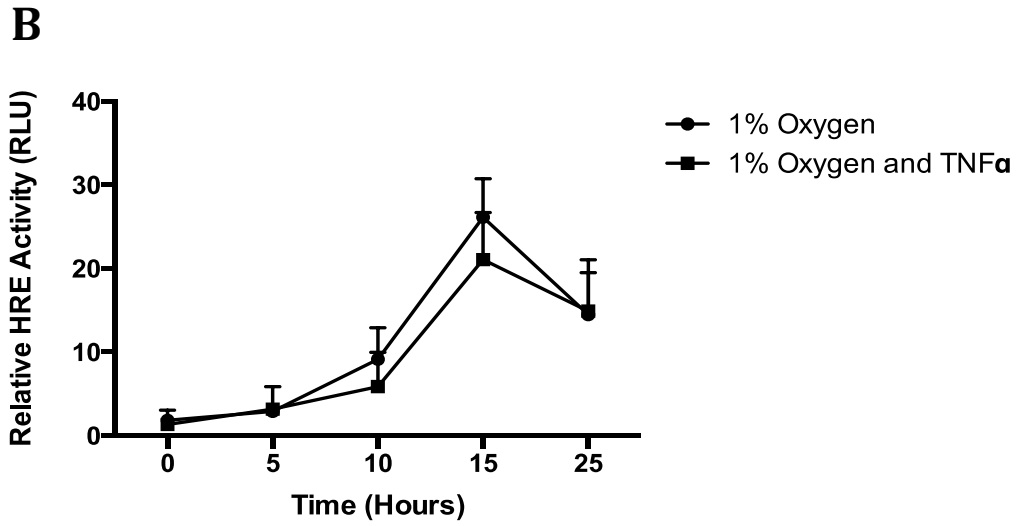


Figure 10: HRE luciferase assays representing HIF kinetics in HepG2 cells following the addition of TNFα.

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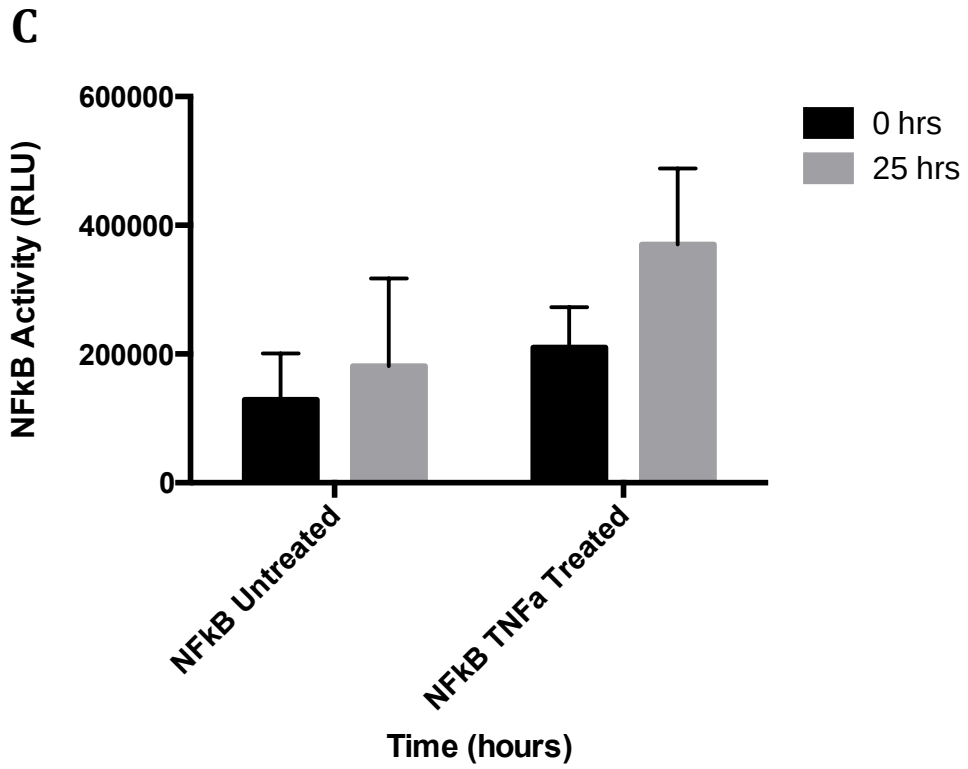

Figure 10: HRE luciferase assays representing HIF kinetics in HepG2 cells following the addition of TNFα (continued).

HepG2 cells were seeded at 1.5x105 cells/ml and transfected with an HRE luciferase reporter gene. Cells were treated with either 100ng of TNFα or with untreated media. The samples are then placed under normoxia or 1% oxygen.

HRE activation is measured in relative light units. **A.** Figure 10A represents HRE responses under normoxic conditions. **B.** Figure 10 B represents HRE responses under 1% oxygen relative to normoxic controls, showing the fold change in activation. The graph represents the mean values of 3 replicates per time point. A value of P<0.7878 was determined using an unpaired T test. **C.** Figure 10 C represents HRE activation through NFκB stabilization of HIFs in HepG2 cells treated with TNFα compared to an untreated control. This data shows the activity of TNFα. HRE activation is measured in relative light units.

9.2.1 Comparison of TNFα effects on DMSO differentiated Huh 7.5 cells under different oxygen conditions

This experiment was performed to determine if TNFα produced a difference in HRE activation in DMSO differentiated cells. Cells were left to differentiate for 7 days and were transfected using an HRE luciferase reporter gene plasmid. The samples were treated with 100ng of TNFα and placed under hypoxia or normoxia overnight. Cells treated with media are used as a control. The data in figure 12 indicates no difference in HRE activation between differentiated cells that have been treated with TNFα or with media. The result suggests that TNFα is not stimulating HIF stabilization within these highly differentiated cells.

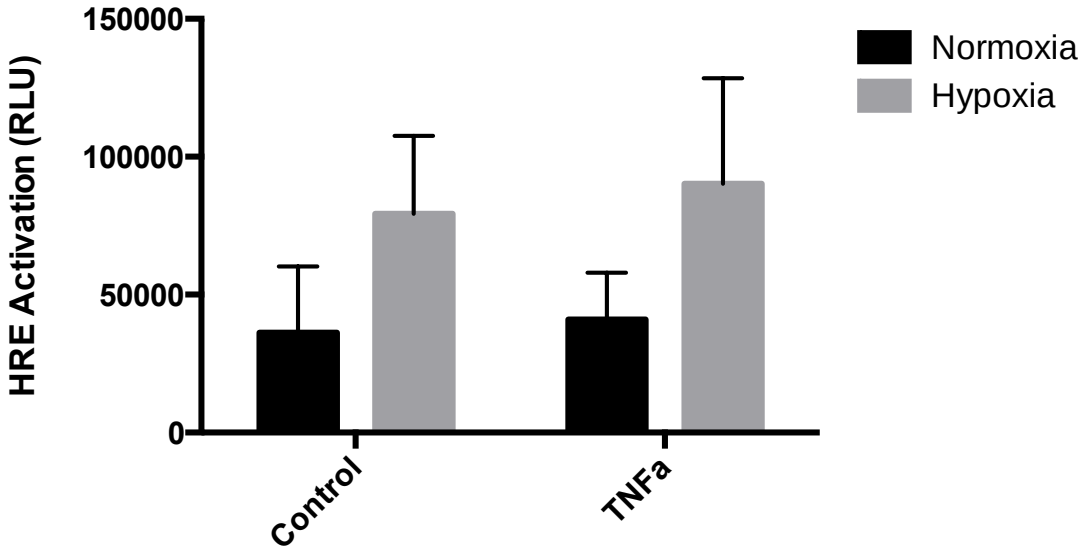

Figure 11: The effects of TNFα on HRE luciferase signals in DMSO differentiated Huh 7.5 cells

Huh 7.5 cells were seeded at 2x105 cells/ml in 24 well plates and treated with DMSO. The cells were left for 7 days to differentiate. The cells were transfected with an HRE luciferase reporter gene. The cells were placed into hypoxic (1% oxygen) or normoxic conditions overnight. HRE activation was measured in relative light units. The control cells are untreated DMSO differentiated cells. The graph represents the mean values of 3 replicates per time point. A value of $P<0.11$ was determined using an unpaired T test indicating no significant difference in activity between control and treated cells.

Following these experiments that showed no significant difference in HRE activity upon addition of TNFα while under hypoxia. An HRE luciferase experiment was performed to study the effect of Lipopolysaccharide (LPS) stimulated macrophage conditioned media on HRE responses. LPS has been shown to cause an up-regulation in HIF1α expression in macrophages under normoxia (*Blouin et al, 2004*). LPS stimulates HIF1α stabilization in a TLR4 and MyD88 dependent manor. The binding of LPS causes this signalling cascade to TLR4. The pathway may involve a number of different factors including reactive oxygen species, inhibition of PHDs and MAPK or NFκB signalling (*Imtiyaz & Simon, 2010*). This experiment was performed to test the kinetics of HRE transcriptional activity in HepG2 cells in response to the conditioned media collected from LPS stimulated macrophages. Macrophages were stimulated with increasing concentrations of LPS (0.1, 1 and 10ug/ml) and incubated overnight and the conditioned media harvested and used to treat HepG2 cells transfected with HRE under normoxia or hypoxia. As a control, conditioned media from un-stimulated macrophages was used. The data suggests that LPS conditioned media activated HRE-Luc under both hypoxia and normoxia. Coupled with previous results showing that TNFa stimulates no additional response; this suggests that there may be additional cytokines or activating

factors within LPS conditioned media that induce HIF stabilization. Garrick Wilson, who has kindly allowed the use of this data, performed this experiment.

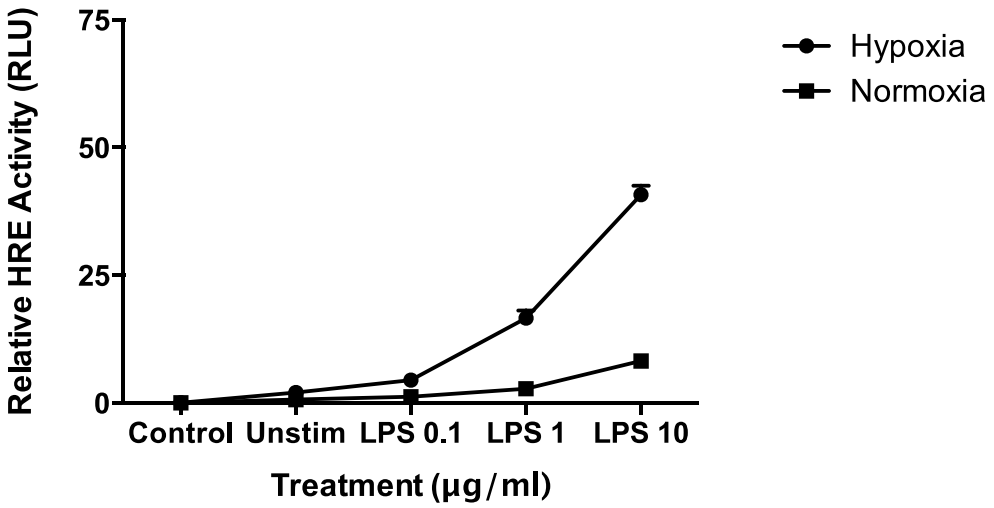

Figure 12: A dose response representing HRE activation after treatments with lipopolysaccharide stimulate macrophage-conditioned media.

HepG2 cells were seeded at 1.5x105 cells/ml and transfected with an HRE luciferase reporter gene. The cells treated with increasing concentrations of LPS and left overnight. Untreated and un-stimulated cell conditioned media are used as controls in this experiment. The samples are placed under hypoxia or normoxia. Relative light units represent HRE activation. An HRE response is observed under both hypoxia and normoxia. Each point represents the relative fold increase in activity compared to untreated controls. Each point shows the mean value of 3 replicates. A value of $P<0.0001$ was obtained at LPS 0.1. $P<0.005$ for LPS 1 and $P<0.002$ for LPS 10 using a multiple T test.

9.3 Chronic exposure to hypoxia

The majority of experiments conducted studying the effects of hypoxia in the liver have been under normoxia or acute hypoxia. Acute hypoxia is between 0-24 hours. Chronic hypoxia is exposure to low oxygen conditions beyond 24 hours (*Lin et al, 2011*). It has been shown that exposure to chronic hypoxic conditions can alter cell morphology, proliferation, biomarkers and protein expression with in some cell types (*Zhdanov et al, 2013*). It would be interesting to study this within hepatocytes; of which some may be exposed to hypoxic

conditions regularly. *Zhdanov and colleagues* also suggest that the exposure to chronic hypoxia can alter the regulation of HIFs; specifically they suggest that chronic exposure will result in reduced HIF2a activity. Therefore it was decided that an experiment studying the effects of chronic exposure on HIF activity and stabilization would be performed. Previous data (figure 7B and 8B) suggests that the signal begins to decline after 15 hours of exposure to hypoxic conditions. It would be interesting to establish kinetics at later time points to determine if this transient activation of stress response genes becomes reactivated after time.

This experiment was performed to determine if there is a difference in HRE activation between parental and DMSO differentiated Huh-7.5 cells after chronic exposure to hypoxic conditions. The data suggests that after 24 hours there is HRE activation, with the levels of activation comparable to previous assays in all conditions. The results indicate a fold change in activity compared to normoxic controls. Naïve Huh 7.5 cells exposed to hypoxic conditions for 36 hours produced a significantly higher HRE response compared to previous assay. There is approximately a 10-fold difference in activation between the 24 and 36-hour time points. Comparatively, the DMSO differentiated cells produced no reactivation in HRE response after 36 hours. This suggests a difference in how HIF activity is regulated between naïve hepatoma cells and highly differentiated cells.

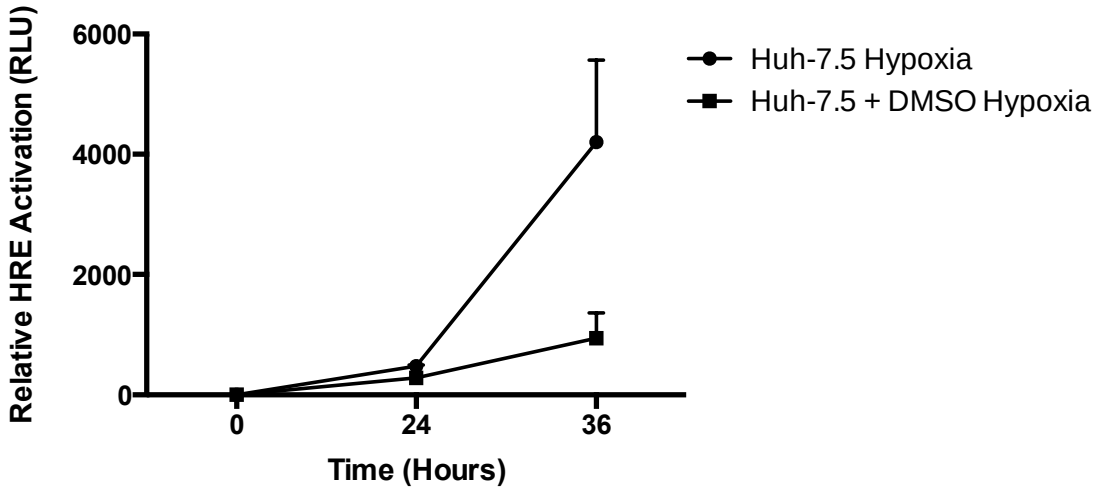

Figure 13: A time course of HRE activation over chronic exposure to hypoxic conditions.

Huh 7.5 cells were seeded at 2x105 cells/ml in 24 well plates and treated with DMSO. The cells were left for 7 days to differentiate. Naïve Huh 7.5 cells were seeded at 2x105 cells/ml after 7 days. All cells were transfected with an HRE luciferase reporter gene. The cells were placed into hypoxic or normoxic conditions over 36 hours. The data is plotted relative to normoxic controls; each time point represents the mean values of 3 replicates. A value of $P<0.197$ was obtained at 24 hours using a multiple row T test indicating no significant difference in activity at this time. A value of $P<0.016$ was determined at 36 hours using a multiple row T test indicating a significant difference in activity between naïve and differentiated cells.

Following experimentation with parental and DMSO differentiated cells over chronic exposure to hypoxic conditions, it is important to determine the viability of cells. To assess cell viability we measured cell membrane integrity through the presence of lactate dehydrogenase. Cell supernatants were collected from cells exposed to chronic hypoxic conditions (see section 1.3). A total cell lysis control obtained through freeze-thaw lysis of each sample was used for comparison with supernatants. The values obtained from cell lysis controls represent 100% cell death. The respective supernatants for each sample are then measured and the result shown as a percentage of cell death compared to the control (Figure 14).

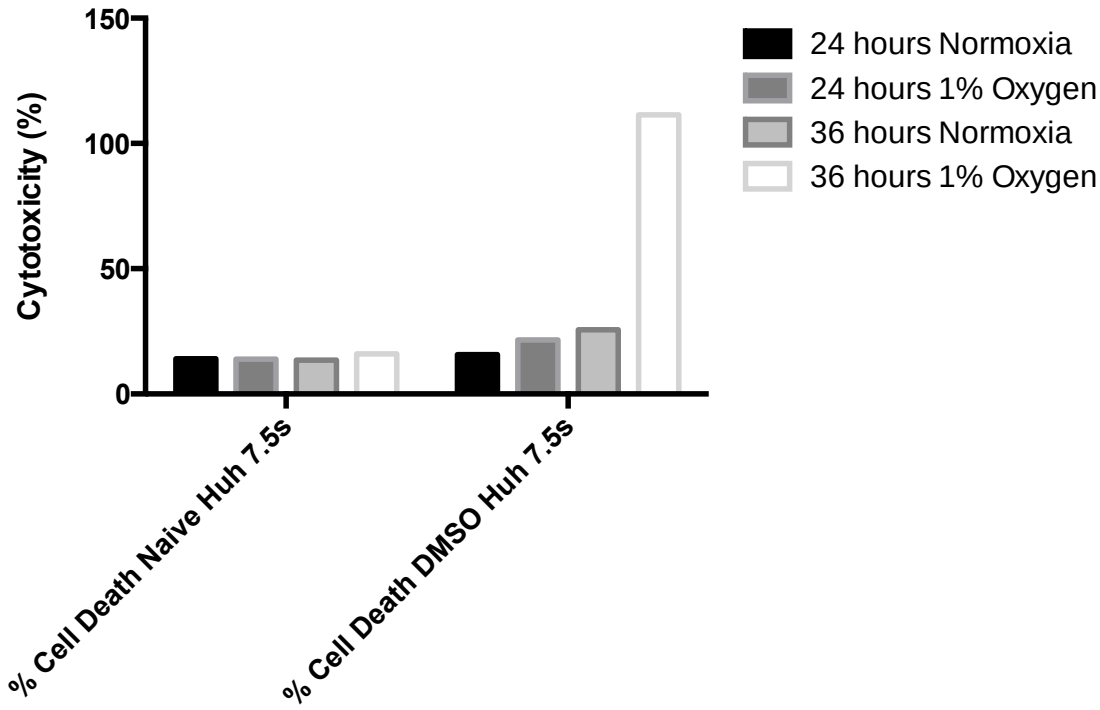

Figure 14: An LDH assay comparing cell viability of naïve and highly differentiated Huh 7.5 cells exposed to hypoxia or normoxia over time.

Huh 7.5 cells were seeded at 2x105 cells/ml in 24 well plates and treated with DMSO. The cells were left for 7 days to differentiate. Naïve Huh 7.5 cells were seeded at 2x105 cells/ml after 7 days. The cells were transfected with an HRE luciferase reporter. Samples were placed into hypoxia or normoxia. Following incubation cell supernatants were removed from each time point. A total lysis control of each sample was obtained through freeze thaw lysis. Figure 14 represents an LDH assay comparing cell viability in both naïve and highly differentiated Huh 7.5 cells exposed to hypoxic conditions over 36 hours. Each bar represents the mean values of 3 replicates.

10 Discussion

Ultimately the purpose of the investigation was to study the hypoxia inducible factors and their effects within HCV biology and liver injury. In particular, this project focused on the kinetics involved in HIF-dependent transcriptional activity. This project is part of a larger on going study into HCV and hypoxia. The results generated during this project provide good preliminary data for a larger study into HIFs and HCV. The HIF1α pathways have been thoroughly studied in the past under normoxic conditions and more recently under hypoxia in the liver. However, this project focuses on the kinetics of HIF activity and stabilisation under both oxygen conditions in two independent hepatoma cell lines. Based upon the results obtained throughout the course of this project we can draw some conclusions with regards to HIF-dependent transcriptional activity.

Firstly, these data suggest that the exposure to low oxygen is sufficient to stabilise HIFs and to stimulate HRE transcriptional activity and luciferase reporter gene expression in Huh-7.5 hepatoma cells (Figure 6). A similar pattern in HRE activation is observed in HepG2 cells exposed to low oxygen. Both cell lines when placed under 1% oxygen produce HRE activity 20-fold higher than those under normoxic conditions (Figure 7). The results indicate a difference in activity in cells exposed to 3% oxygen. A faster decline in activity is observed in Huh 7.5 cells exposed to 3% oxygen compared to 1% oxygen; this difference was significant with a value of $P<0.008$ (Figure 6). *Uchida and colleagues* describe an antisense HIF1α (aHIF) transcript, which accumulates over time

under hypoxia and is involved in regulation of HIFs by inhibiting activity; perhaps this is involved in the decline in activity seen after 15 hours. It is also suggested that accumulation of this transcript destabilises HIFα subunit mRNA (*Uchida et al, 2004*). Figure 7 indicates a difference in activity between 1% and 3% oxygen in HepG2 cells; a 4-fold difference in activity can be observed at the point of highest activity with a value of $P<0.002$ obtained here. *Holmquist-Mengelbier and colleagues* show that varying oxygen concentrations can determine which HIFα subunit is expressed in neuroblastoma cells. The difference in activity observed within these cells (Figure 7) could be due to the differential expression of these transcription factors. The same effect has been in observed in HeLa, glioma and lung epithelial cells (*Uchida et al, 2004; Nilsson et al, 2005; Li et al, 2009, Keith et al, 2012*). These papers suggest that HIF1α is expressed between 0-2% oxygen and HIF2α is expressed between 2-5% oxygen. Following these experiments, assays were conducted to observe the effect of low oxygen on highly DMSO differentiated Huh 7.5 cells. These data initially indicated reduced activity within DMSO treated cells (Figure 8A). However, when plotted relative to normoxic controls the fold change in activity compared to controls shows no significant difference (Figure 8B). Also, it is important to note that the relative fold change in activity observed within naïve Huh 7.5 cells is significantly lower than observed in previous experiments. This suggests it may be necessary to repeat the assay to confirm the results observed in DMSO treated cells. Experiments were then performed to study the effect of pro-inflammatory cytokines on HIF-dependent transcriptional activity. These have been shown to induce transcriptional activity in a number of cells

types under normoxia previously in an NFκB dependent manor (*Frede et al, 2005; Bonello et al, 2007; Jiang et al, 2010*). Assays using both Huh 7.5 and HepG2 cells indicate no significant difference in activity between cells exposed to low oxygen over 25 hours and the same cells exposed to low oxygen and treated with TNFα (Figures 9A and 10A). This data is plotted relative to normoxic controls and shows TNFα cannot induce an HRE response beyond that of hypoxia alone. Both figures indicate that there may be a lower HIF dependent transcriptional activation under hypoxia in cells treated with TNFα; however statistical analyses indicate no significant difference. *Tsapournioti and colleagues* (2013) have shown that TNFα induces expression of HIF1α mRNA and proteins in an NFκB dependent manor. However, they also show that TNFα inhibits the hypoxic stimulation of HIF1 dependent transcriptional activity in Airway Smooth Muscle Cells (ASMCs). They show this inhibition occurs by preventing dimerization of HIF1α with HIFB. Could the lack of additional activity and slight decrease observed in these hepatoma cell lines be due to similar TNFα activity?

Figure 12 represents results of an HRE luciferase assay studying HRE response to increasing concentrations of LPS stimulated macrophage conditioned media. Under hypoxic conditions the graph indicates an increase in activity with increasing concentrations. This data coupled with previous results showing TNFα produces no response beyond hypoxia alone suggests there may be another cytokine or factor present within LPS conditioned media responsible for activating the HRE response. Statistical analyses show a significant difference in activity with values of $P<0.0001$, $P<0.005$ and $P<0.002$

for concentrations LPS 0.1, 1.0 and 10 respectively between treated and untreated cells.

Figure 13 represents an HRE luciferase time course assay investigating HIF-dependent transcriptional activity within naïve and highly DMSO differentiated Huh 7.5 cells exposed to chronic hypoxia. This data indicates that chronic exposure to hypoxia (24<hours) results in activation of HIFs significantly higher than observed at 24 hours or earlier with approximately 10-fold difference in activation observable in figure 13. Indeed, results in figures 6 and 7 indicate that activity decreases after 15 hours. However, it has been shown that chronic exposure to low oxygen can alter cell morphology, protein expression and proliferation (*Lin et al, 2011; Zhdanov et al, 2013*). They also suggest that HIFs may be regulated differently during chronic exposure via different signalling pathways. An LDH assay was performed to check the cell viability; this was used to confirm that the cells exposed to low oxygen for more than 24 hours had not simply died.

The data generated throughout this project was largely gathered using an HRE luciferase reporter gene. Due to the nature of the assay, the experiment could be adapted to study a number of aspects in the HIF pathway. The literature shows that most experiments studying HIF activity within the liver are conducted using either normoxic conditions with 20% oxygen or hypoxic conditions of 1% oxygen or less. Neither of these conditions is found within the liver, which normally exists between 3-11% oxygen (Figure 1). Consequently, we conducted assays to compare the difference in HIF stabilisation and activity between previously used 1% oxygen and the more anatomically correct 3% oxygen. The

results shown in figures 6 and 7 indicate that there is minimal difference in HIF stabilisation between these hypoxic conditions and suggests that the use of 1% oxygen will produce accurate results in additional experiments. However, some previous papers suggest that the level of oxygen is important in determining which HIFs become stabilised (*Uchida et al, 2004; Nilsson et al, 2005; Li et al, 2009*). They suggest that between 0-2% oxygen HIF1α is stabilised and that between 2-5% oxygen, HIF2α become stabilised. This suggests that the kinetics displayed at 3% oxygen might represent those of HIF2α. If this is the case then the kinetics displayed by both HIFs are very similar suggesting similar methods of regulation. To this effect it is important to establish the transcription factor being studies using Western Blotting; however to date these experiments have been unsuccessful, perhaps due to the instability of HIFs when removed from hypoxic conditions. It has been shown that DMSO differentiated Huh 7.5 cells express a number of biomarkers similar to those of primary human hepatocytes (*Sainz & Chisari, 2006*) and stop dividing like cells within the liver. These therefore provide an interesting basis for studying the kinetics of HIF activity in cells similar to PHH cells.

The most widely used assay was the HRE luciferase reporter gene assay to study HIF kinetics in a couple of different cell types exposed to different levels of oxygen and cytokine treatments. This assay has a number of strengths and weaknesses that should be discussed. In an attempt to optimize this assay in future and hopefully produce cleaner results, the seeding of cells and transfections will now occur on the same day. Splitting into 96 well plates must still occur on day 2 however. Another limitation of this technique is that it does

not allow for distinction between the isoform of HIF responsible for the HRE response. This is particularly important because there is literature, which suggests that different isoforms of HIFs are expressed under different oxygen concentrations. The assay is versatile, which enables the study of multiple facets of HIF kinetics in liver cells.

Following these results it would be interesting to run a similar assay at higher oxygen concentrations representative of other regions in the liver to determine if any HRE signals are visible. Previous experiments studying the effects of HIF1α have also been conducted using 5% and 10% oxygen. This would be interesting as a comparison to the patterns after exposure to low oxygen levels observed already. Also, while a minimal or non-existent signal might be expected because HIFs are not stabilised under higher oxygen concentrations; it would be interesting to see the effect of cytokines that may stabilise HIFs over the same time course. Additionally, the production of a successful HIF1α or HIF2α Western Blot is essential for continued study in this area. These would show the protein expression and enable the study of HIF1α or HIF2α kinetics after silencing the other. It would be particularly interesting to study the endogenous HIF protein levels found within these cell lines under varying oxygen tensions. Other studies have suggested that upon the silencing of one, the other becomes over expressed (*Schulz et al, 2012*). The silencing of one subunit could cause the up regulation of the other as a compensation mechanism. It has been shown previously that within kidney cells there is overlap in the targets of each subunit (*Majmundar et al, 2010. Keith et al, 2012.*

Schulz et al, 2012). It would be interesting to determine how many genes overlap between each subunit in hepatocytes.

Further study into the effects of chronic exposure to low oxygen would also be really interesting. The data generated throughout this project shows that the HRE signal starts to decline after 15 hours, this is in keeping with the literature, which suggests that the protein starts to degrade after this time. However, it would be interesting to know whether these responses are reactivated after longer exposure to hypoxia. In addition, it would be interesting to establish whether a specific isoform is responsible for activation beyond 24 hours; it has been suggested that HIF2α activity is reduced after chronic exposure to low oxygen (*Zhdanov et al, 2013*).

Based on the results obtained during this project and the objectives outlined at the beginning there are a number of points that would be interesting to study. HIF2α is the immediate target for study; it would be interesting to know how the kinetics differs from HIF1α. This will require targeted silencing of both HIF isoforms and the use of both HRE luciferase reporter assays and Western blotting. Is expression of a specific subunit linked to oxygen concentration in the liver? Does HIF2α respond to different cytokines in hepatocytes? Does HIF2α dependent transcriptional activation work through the same signalling pathways (NFκB, TNF receptors etc). HIF3α is also apparently stabilised under low oxygen. Does this apply in the liver? Does it regulate stress responses like 1α and 2α? Or does it just regulate 1α and 2α? This could be studied through the use of co-localisation studies and dual reporter assays.

The experiments performed as part of this project may help in the future development of treatments for HCV and other members of the flavivirus family. They provide the groundwork for a larger study into HIF physiology and pathobiology in the liver with a range of oxygen tensions. This work is important because it touches upon the role that HIFs might play in regulating different metabolic processes under different prevailing oxygen tensions in certain regions of the liver. In addition studying the hepatocyte responses to hypoxia, the stability and activity of HIFs may lead to a better understanding of the normal host response to hypoxia in the liver and give greater insight into how HCV can utilize this stress response to aid in its viral lifecycle.

11 Acknowledgments

I would like to thank everyone in the McKeating lab for their help and supervision throughout the time spent in the laboratory; in particular I would like to thank Garrick Wilson for his help throughout the experiments and both Garrick Wilson and Jane McKeating, for their good advice and great teaching.

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