

**MORPHOGEN AND EPIGENETIC REGULATION OF WOUND
HEALING**

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List of Abbreviations

5MeC- 5-Methyl Cytosine
aHSC- activated Hepatic Stellate Cell
ALP - Alkaline phosphatase
BDL- Bile Duct Ligation
CaMKii- Ca²⁺ /calmodulin-dependent protein kinase II
CCl₄- Carbon tetrachloride
CHX- Cyclohexamide
CTGF - Connective tissue growth factor
Dkk- Dickkopf
DMEM - Dulbecco's Modified Eagle's Medium
DMSO - Dimethyl sulfoxide
dpf- days post fertilisation
Dvl- Dishevelled
ECM - Extracellular matrix
FCS - Fetal Calf Serum
Fzd- Frizzled
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GFP- Green Fluorescent Protein
GSK3- glycogen synthase kinase 3
H₂O₂ - Hydrogen peroxide
hpf- hours post fertilisation
HSC- Hepatic Stellate Cell
JNK - c-Jun N-terminal kinases
KC- Kupffer Cell
LEF- Lymphoid enhancer-binding factor 1
LPS – lipopolysaccharide
LRP- low density lipoprotein receptor-related proteins
MCP-1 - Monocyte chemoattractant protein-1
MHB- midbrain hindbrain boundary
MMPs - Matrix metalloproteinases

MO- Morpholino oligonucleotide
MTT - (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, a Yellow Tetrazole)
NFκB- Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT- Nuclear factor of activated T-cells, cytoplasmic 1 and 4
PCP- Planar Cell Polarity
PCR - Polymerase chain reaction
PPARγ - Peroxisome proliferator-activated receptor gamma
TIMPs - Tissue inhibitors matrix metalloproteinases
qHSCs- quiescent Hepatic Stellate cell
qRT-PCR - Real-time reverse transcription-PCR
ROR2- receptor tyrosine kinase like orphan receptor 2
sFRP- Secreted Frizzled Related Protein
SOX9- SRY (sex determinin region Y)- box 9
Std Control- Standard control morpholino
TCF – Transcription Factor
Tg- Transgenic
TGFβ- Transforming growth factor beta
Wnt - Wingless-related integration site
αSMA- alpha smooth muscle actin

Abstract

Events during wound repair are reminiscent of developmental events such as cell migration, redifferentiation and proliferation. Factors controlling these processes in the early embryo may therefore be important regulators in adult wound healing. Fibrosis is a disease of dysregulated wound healing with fibroproliferative disorders accounting for 45% of deaths in developed nations. Despite this, effective antifibrotic therapy is limited. Understanding factors regulating wound repair process will aid identification of potential therapeutic targets.

This project first explored how activation of developmental signalling pathways influences regulation of myofibroblast transdifferentiation and behaviour in liver fibrosis, focusing on the Wnt signalling pathway. Wnt signalling was upregulated in activated myofibroblasts, with significantly increased expression of non-canonical ligands. Wnt stimulus did not provoke a canonical/ β -Catenin mediated response, with myofibroblasts responding through non-canonical associated signalling instead. Inhibition of Wnt signalling reduced classic markers of fibrosis, suggesting a profibrotic role for Wnt signalling during liver fibrosis. Stimulus with the non-canonical ligand Wnt5a did not directly affect expression of fibrotic markers in myofibroblasts. Instead it appeared to act as a prosurvival factor and increased expression of profibrotic cytokines in resident liver macrophages.

Dynamic changes in DNA methylation pattern also regulate embryonic development. This project next explored whether inheritance of epigenetic marks could alter early DNA methylation patterns and affect response to injury in adult, focusing on the histone variant H2A.Z. H2A.Z is thought anti-correlative to DNA methylation and has been shown to be enriched in the sperm of injured animals in a model of transgenerational adaptation to wound healing. H2A.Z expression was depleted via morpholino injection in early zebrafish embryos, resulting in significant mortality and phenotypic abnormality. Injected animals displayed significant hypermethylation of DNA during early embryonic periods. This suggests that alteration of epigenetic marks can influence methylation status of DNA.

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Chapter 1: INTRODUCTION

1.1 Wound healing and Fibrotic Disease

Tissue injury and the resulting epithelial cell damage triggers a highly co-ordinated, multicellular response, facilitating repair and restoration (1). Wound healing can be broadly divided into three different phases: the inflammatory, the proliferative and the resolving phase and is summarised by Figure 1.1. General mechanisms of wound healing are conserved throughout the body and are vital for restoration of normal tissue architecture and protection from external pathogens. Most vertebrates cannot completely regenerate lost or damaged tissue, healing instead through the process of scar formation (2).

The initial inflammatory phase occurs rapidly after injury. Damaged epithelial cells release damage associated molecular pattern molecules (DAMPs) promoting the recruitment of inflammatory cells to the site of injury. Infiltrating immune cells clear the injury site of debris and release a multitude of growth factors and cytokines, stimulating migration of the fibroblasts and smooth muscle cells critical for the second proliferative phase(3). Major cytokines directing the wound healing response include Platelet Derived Growth Factor (PDGF) and TGF β 1(4)

Repair of injury requires increased production and deposition of extracellular matrix, producing a fibrotic scar to replace lost or damaged tissue. Fibroblasts are the major cell type involved in the secretion of matrix components with Type 1 collagen the major component of the fibrotic scar(5, 6). Upon wound closure, activated fibroblasts are cleared from the injury site and there is a shift towards the resolution phase and a gradual remodelling of the collagen scar(1).

This highly sequential process can become disrupted under conditions of chronic injury. Fibrosis is a disease of dysregulated wound healing. Repeated insult leads to the persistent activation of profibrogenic myofibroblasts, the excessive deposition of fibrotic matrix and the subsequent disruption of organ function(7). Fibroproliferative diseases involving a number of organs include idiopathic pulmonary fibrosis (IPF), diabetic nephropathy, hepatic cirrhosis and systemic scleroderma(SSC) account for approximately 45% of deaths in developed nations(8). Effective anti-fibrotic therapy is currently limited making fibrotic disease a significant health issue for the modern world

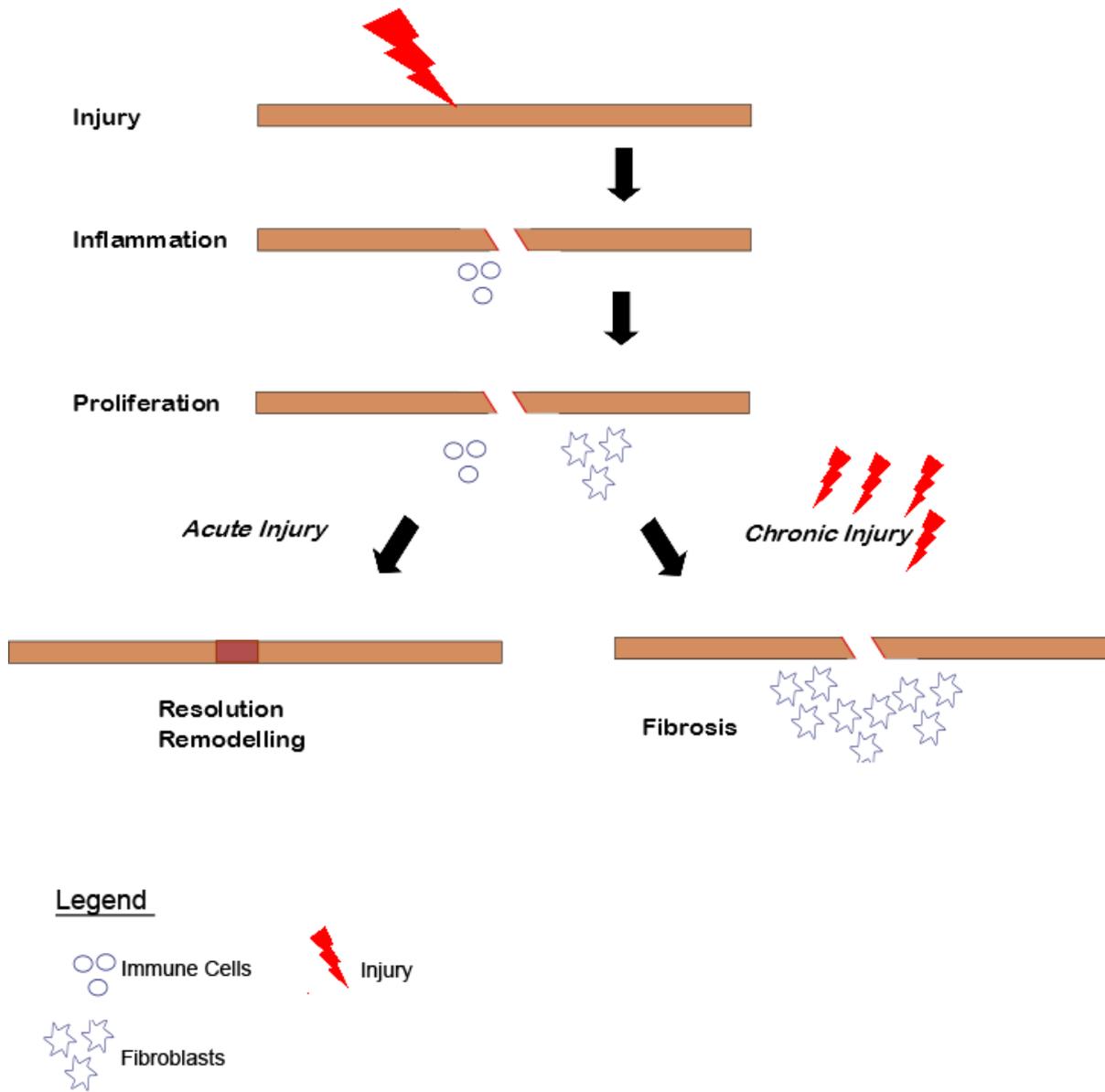


Figure 1.1 Summary of Wound Healing

Tissue injury and resulting epithelial cell damage triggers inflammation and fibroproliferation, promoting repair through deposition of scar. Chronic injury results in persistent activation of fibroblasts and excessive deposition of matrix

1.2 Myofibroblast Activity and Regulation

A hallmark of fibrosis is the activation of profibrogenic myofibroblasts. Myofibroblasts are not normally detected in healthy tissue and are activated only upon injury (7). They are characterised by high expression of alpha smooth muscle actin (α -SMA), leading to increased contractility as well as other phenotypic traits such as increased proliferation and migratory potential(7). They originate from numerous types of precursor such as endothelial cells, epithelial cells, pericytes as well as resident fibroblasts. As the major producers of extracellular matrix, myofibroblasts are the ideal target for antifibrotic therapy. Fully understanding the pathways that regulate their differentiation, activity and clearance will enable identification of targets for pharmacological modulation.

Parallels have been drawn between the events occurring during wound healing and those occurring during embryonic morphogenesis such as the highly co-ordinated movements of cells and tissues(9). Tissue morphology during embryogenesis is regulated by the morphogen families of signalling molecules such as the Hedgehog (Hh), Notch, Wnt, Fibroblast Growth Factor (FGF) and Transforming Growth Factor $-\beta$ (TGF β) families(10). Morphogens function in a concentration specific manner, establishing morphogen gradients to govern the response of receiving cells, directing migration, differentiation and cell fate specification(11).

In adults, morphogen activity in healthy tissue is limited and is only active when the need arises, during cell renewal and regeneration for example(10). If these pathways become irregularly active, the subsequent disruption of normal cell adhesion, proliferation and differentiation is a significant risk factor for pathology. Activation of embryonic developmental pathways in cancer is well established: mutations in β -Catenin for example, a downstream effector of the Wnt family, are a hallmark of numerous cancers(12).

Recent research connects morphogen signalling to wound healing and fibrosis. Reactivation of these embryonic signalling pathways has been implicated in promoting activity of myofibroblasts in numerous fibrosis models such as SSC, IPF and renal fibrosis(13-17). Furthermore, blockade of morphogen activity diminishes myofibroblast activity: knockdown of the Hh transcription factor Gli for example attenuates the fibrotic response mice after unilateral ureteral obstruction (UUO) injury(18). Manipulating morphogen signalling could therefore be an attractive possibility for modulating the fibrotic response.

1.3 Liver Fibrosis

Although the liver has remarkable potential for regeneration and repair chronic liver disease is a major health burden. Approximately one million deaths per year worldwide are attributed to cirrhosis, the end stage of liver fibrosis, making it the 14th most common cause of death(19). Liver transplant is currently the only effective treatment with over 5500 transplants performed in Europe per year (20). A shortage of donor material however significantly limits the availability and efficacy of this treatment. The major cause of liver injury is chronic alcohol abuse although other contributing factors include viral infection and metabolic syndrome, damage due to the accumulation of fat and impaired lipid metabolism(21)

The initial insult leads to inflammatory damage, death of parenchymal cells and activation of myofibroblasts. Early stages of liver fibrosis are characterised by excessive ECM deposition and scar formation but few clinical symptoms. Evidence suggests that if underlying aetiology is removed at this early fibrotic stage, reversion to normal liver function is possible (8). Continuous injury however promotes development of advanced fibrosis and cirrhosis. Cross linking of deposited matrix renders scars increasingly resistant to degradation, disordering tissue architecture and disrupting liver function(22). Increased ECM deposition distorts the hepatic vasculature, gradually increasing the hepatic venous pressure gradient (HVPG) and resulting in portal hypertension. Clinical complications

associated with cirrhosis include variceal bleeding, ascites, renal failure and hepatic encephalopathy(23). In addition, there is dysregulation of hepatocyte regeneration and the development of regenerating nodules, a significant risk factor for hepatocellular carcinoma (HCC)(24). Here, potential for reversion is limited and the only complete treatment is liver transplant.

The liver is composed of multiple cell types with hepatocytes the most abundant, comprising between 70-80% of the liver cell mass. They are the primary functional cells in the liver, involved in protein synthesis and storage, carbohydrate and lipid metabolism and synthesis and secretion of bile(25). In addition, there are Kupffer cells, the liver resident macrophages, sinusoidal endothelial cells and the mesenchymal hepatic stellate cell (HSC). The primary source of pro-fibrogenic myofibroblasts upon liver injury is thought to be the hepatic stellate cell(26).

1.4 Models of Liver Fibrosis

Numerous *in vivo* animal models aid study of these numerous aetiologies. Toxin-mediated induction of liver injury is a widely used method with intraperitoneal administration of carbon tetrachloride (CCl₄) one well established model(27). Inflammatory response is induced upon administration and fibrosis observable after 4-6 weeks of repeated injection. Resolution of scarring occurs once injections stop, making CCl₄ a good model for all stages of the fibrotic response: initial inflammation, fibrogenesis and fibrosis resolution. Bile duct ligation (BDL) is a common model of cholestatic injury(28). Non Alcoholic Fatty Liver Disease (NAFLD), a significant contributor to liver disease worldwide, can be modelled by several dietary regimes such as high fat diets or methionine - coline deficient (MCD) diets(29).

In vitro, hepatic stellate cells (HSCs) the primary source of profibrogenic myofibroblasts can be isolated by enzymatic digestion and density gradient centrifugation from liver tissue(30). However, difficulties encountered with primary cell culture such as limited amount of source tissue and

variability between preparations have led to the development of several HSCs cell lines from rat, mouse and human. The human LX-2 cell line is the most widely used, developed from spontaneously immortalised human HSCs and considered representative of fully activated myofibroblasts(31). Finally, more sophisticated *in vitro* culture methods such as precision cut tissue slices are increasingly being used. Culture of tissue slices allows study of a multicellular system *in vitro*, where tissue architecture, ECM composition and cell-cell contacts are maintained(32). This is far more physiologically relevant than the monoculture of primary HSCs or cell lines which is currently the main method of *in vitro* liver fibrosis study.

1.5 Hepatic Stellate Cells

Hepatic Stellate Cells are liver specific pericytes and reside in the space of Disse between the sinusoidal endothelial cells and the hepatocytes(26). They comprise approximately 15% of the total cell population. In normal, healthy liver they are considered 'quiescent' (qHSC). Liver injury however stimulates a significant phenotypic change and transdifferentiation into pro-fibrogenic myofibroblasts (aHSC). Their embryonic origin is not fully resolved and evidence supports HSCs deriving from the endoderm, ectoderm or mesoderm. Typical markers used to identify HSCs include vimentin, desmin and glial fibrillary acid protein (GFAP)(26). Quiescent HSCs secrete a variety of cytokines and growth factors and their major role is in the storage of vitamin A (retinol), acting as a store for 80% of the body's retinol in cytoplasmic lipid droplets. Maintenance of the quiescent phenotype involves an adipogenic transcriptional profile: qHSCs express high levels of the adipogenic master regulator PPAR γ along with other adipogenic associated transcription factors such as CCAAT/enhancer-binding protein (C/EBP) α , C/EBP β , C/EBP δ and sterol regulatory element-binding protein 1c (SREBP-1c).

Transdifferentiation to profibrogenic myofibroblasts involves a massive shift in transcriptional profile(33). Adipogenic associated gene expression is replaced by profibrogenic mediators such as

TNF α , TGF β and PDGF, several of which are known antagonists of apoptosis(33). Expression of the myofibroblast marker α -SMA is significantly upregulated and as HSCs are the only major liver cell type to express α -SMA, this is often used as a definitive identifying marker of aHSCs(34). Clarifying the factors regulating this dramatic change in HSC phenotype of critical importance. Activity of several key transcription factors during transdifferentiation has been well defined, with roles for the NF- κ B, KLF6, AP1, E Box binding proteins and the Smad families of transcription factors(35). Substantial morphological changes follow this shift in transcriptional profile with HSCs adopting typical myofibroblast characteristics including increased contractility, increased proliferation, stress fibre formation and increased migratory capacity(26) (Figure 1.2). aHSCs are the major synthesisers of ECM components involved in scar deposition.

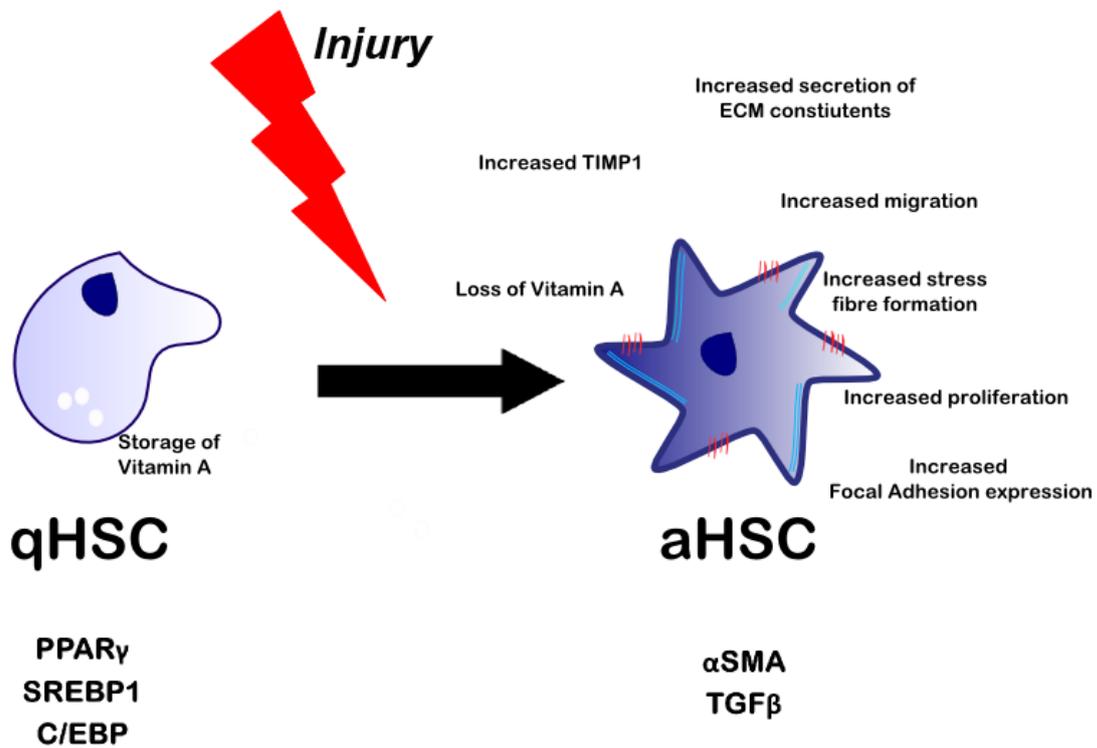


Figure 1.2 HSC transdifferentiation

Diagram detailing phenotypic and transcriptional changes occurring during HSC activation. Injury triggers transdifferentiation of the vitamin A storing, adipogenic like quiescent HSCs. They lose vitamin A storage and undergo significant phenotypic changes such as increased contractility, proliferation and migration. Expression of adipogenic related genes such as PPAR γ and SREBP1 is downregulated while expression of profibrogenic genes such as TGF β is increased.

1.6 Epigenetic regulation of HSC transdifferentiation

Recent work has highlighted the importance of epigenetic factors in regulating myofibroblast transdifferentiation and progression of liver fibrosis. Epigenetics is traditionally defined as: 'heritable changes in gene function that occur without changes in DNA sequence'(36). DNA is not found naked in the cell, packaged instead by proteins into chromatin which forms chromosomes. The three major epigenetic modifications are methylation of DNA, modification of histones and microRNA activity. Structural changes resulting from these modifications function in regulation of gene expression(37).

DNA methylation is an extensively studied epigenetic modifications, highly conserved across animals, plants and fungi(39). Methylation of DNA is the transfer of a methyl group to the fifth position of a cytosine base and in mammals is restricted to cytosines present in CpG dinucleotides. Regions of high CpG composition are termed 'CpG Islands' and are a typical feature of gene promoters(38). When DNA methylation occurs at these regions it is normally associated with transcriptional repression (39). Transfer of methyl groups is mediated by the DNA Methyltransferase (Dnmt) enzymes, divided into two functional classes. De novo methyltransferases (Dnmt3a and Dnmt3b) initiate methylation on previously unmethylated CpG sites while the maintenance methyltransferase (Dnmt1) recognises hemimethylated DNA during DNA replication and copy existing patterns(40, 41).

Disruption of methylation machinery has drastic consequences for development, depletion of Dnmt expression in mice leading to early embryonic or early postnatal death (40, 42). Likewise, in the adult perturbation of methylation patterns is implicated in disease, notably in numerous cancers(43).

Dynamic alterations of DNA methylation patterns are critical for the control of cell behaviour during morphogenesis. Epigenetic reprogramming during wound healing can therefore be considered another way in which response to injury recapitulates developmental processes(9).

DNA methylation can promote gene repression through two mechanisms: decreasing access of transcription factors to target sites or targeting repressive complexes to DNA (39). Treatment of cultured HSCs with the commonly used demethylating agent, 5-aza-2'-deoxycytidine, halts transdifferentiation of HSCs, preventing the morphological and transcriptional changes associated with an activated myofibroblast phenotype(44). DNA Methylation therefore has a critical role in regulating myofibroblast transdifferentiation.

Methyl CpG binding protein 2 (MeCP2) is one nuclear protein which preferentially binds methylated DNA and is thought to function in the recruitment of corepressor proteins(45). MeCP2 is significantly upregulated in aHSC and is shown to regulate the activity of key transcription factors involved in HSC transdifferentiation. One major factor required for HSC quiescence is PPAR γ , an adipogenic associated regulator. MeCP2 binds to the promoter of PPAR γ and mediates its repression. PPAR γ expression in MeCP2 deficient myofibroblasts is significantly increased, moreover CCl₄ injured MeCP2 knockout animals display a reduced fibrotic response(46). MeCP2 regulates several other transcriptional regulators important in HSC transdifferentiation. It is associated with repression of I κ B α , an inhibitor of the nuclear factor kappa B (NF κ B) family of transcription factors necessary for HSC transdifferentiation(44). MeCP2 also plays a role in repression of PTCH1, an inhibitory component of the morphogen Hh pathway shown important in promoting HSC transdifferentiation(47).

Progression from initial liver injury to the end stage cirrhosis can take between five to fifty years and is highly variable: only a minority of patients with chronic liver disease progress to end stage cirrhosis (8). This variability cannot be explained by genetic factors alone suggesting a complex interaction of environmental, epigenetic and genetic influences. A recent study explored multigenerational epigenetic adaptation to the wound healing response, where injury in parents led to epigenetic changes which can be stably transmitted to offspring(48). In this study, offspring of CCl₄ injured parents demonstrated decreased fibrotic response when compared with offspring of

control, non-injured groups. Differential gene expression was accompanied by corresponding changes in DNA methylation status: increased methylation of the pro-fibrogenic TGF β promoter corresponded with reduced mRNA expression whereas decreased methylation of the anti-fibrogenic PPAR γ promoter corresponded with increased mRNA expression. This suggests that ancestral injury may be one factor contributing to population variability. How this information is transmitted between parent and offspring has yet to be determined.

1.7 Process of HSC activation

Activation of HSCs is stimulated through receipt of paracrine signals from neighbouring cells. Injury dramatically alters hepatic architecture: damaged hepatocytes undergo apoptosis provoking an inflammatory stimulus with activation of the resident Kupffer cells and subsequent infiltration of circulating lymphocytes(23). In this altered environment a multitude of factors are released which promote transdifferentiation such as PDGF,EGF and production of reactive oxygen species mediated by Cyp2e1 (cytochrome P450 2E1), a member of the cytochrome P450 family of enzymes (23). Increased TGF β signalling is a major profibrotic stimulus, promoting the recruitment of inflammatory cells and fibroblasts to the site of injury as well as stimulating increased expression of extracellular matrix components(49).

This initial activation phase promotes the change in HSCs to a myofibroblast like phenotype with increased responsiveness to growth factors and environmental signals. aHSCs are characterised by expression of classic markers of activated myofibroblasts such as α -SMA, Type I collagen, Tissue Inhibitor of Metalloproteinase 1 (TIMP1) and TGF β and its associated receptors(26). Once transdifferentiation has occurred, HSCs are able to maintain their activated phenotype through autocrine signalling as well as receipt of paracrine stimulus. Environmental as well as chemical stimuli also play a role, alterations in ECM composition for example where increased amounts of fibrosis associated collagens such as Type 1 collagen cause increased stiffening of matrix and further enforce the activated phenotype(50). Placing aHSCs on a softer matrix such as Matrigel, promotes a reversion to quiescence(51).

As mentioned, aHSCs major role is in the production of ECM components. They secrete massive amounts of Type1 Collagen, one of the best characterised components of the fibrotic scar(52). aHSCs are a source of multiple members of the matrix metalloproteinases (MMPs) family involved in degradation of matrix, specifically MMP2, MMP9 and MMP13(53, 54). They also secrete increased amounts of TIMP1 involved in inhibition of MMPs as well as conferring resistance to apoptosis. This increased production of ECM components disrupts regulation of matrix composition: clearance of

matrix in early stages by increased MMP activity allows for increased deposition of collagen. This leads to a change shift in matrix from a laminin and type IV collagen rich composition to one comprising interstitial collagens such as collagens I and II(55). Degradation of excessive collagen will be prevented by increased TIMP1 activity, leading to accumulation of fibrotic matrix. The upregulation of α -SMA, a major constituent of stress fibres, enhances their contractility and promotes wound closure(56). Excessive production in the chronically injured liver however disrupts vasculature, promoting increases in the HVP. If injury is not resolved and aHSCs not cleared, this disruption of ECM will continue leading to the impairment of liver function described previously.

As well as mediating fibrogenesis, HSCs also contribute to the immune response upon injury, stimulated via TLR activity such as LPS induced TLR4 signalling (57). They are highly secretory, producing numerous chemokines to promote migration of inflammatory cells(26). Evidence even exists for them being capable of acting as antigen presenting cells (58).

Resolution of acute injury requires clearance of HSCs, allowing for degradation of excessive matrix and a return to a normal hepatic architecture. Apoptosis of aHSCs is thought to be the major mechanism of HSC clearance(59). Specifically targeting induction of apoptosis to α -SMA expressing cells in the injured liver significantly improves resolution of fibrosis after CCl₄ mediated liver injury, demonstrating its significance(60). During injury, HSCs receive pro-survival signals derived from matrix. Maintaining an intact matrix prevents apoptosis whereas matrix degradation, through recombinant MMP9 treatment for example, promotes it(61). Furthermore, increased TIMP1 expression by aHSCs acts as an autocrine anti-apoptotic signal : liver specific overexpression of TIMP1 for example impairs fibrosis resolution compared to control animals in a CCl₄ injury model in mice(62). Under conditions of chronic injury, degradation of matrix and reduction of survival signals will not occur. HSCs will therefore remain present and persistently active.

As well as clearance of HSCs by apoptosis, there is the potential for them to 'deactivate', returning to a quiescent like phenotype. Decreased expression of activation associated genes has been observed

in vitro(33). *In vivo* studies further confirmed these findings, genetic labelling experiments demonstrating cells which had previously been α SMA positive during injury remaining in liver after cessation of injury(63). These cells are termed inactivated HSCs (iHSCs) rather than fully quiescent as they demonstrated a more marked response to TGF β 1 stimulus in comparison to quiescent controls. Cellular senescence is also thought to contribute to aHSC clearance and fibrosis resolution. The accumulation of senescent cells observed in cirrhotic liver thought to be derived from HSCs and animals deficient for components of senescent pathway displaying reduced ability to resolve fibrosis(64). These studies demonstrate the importance of clearing HSCs for resolution of fibrosis.

1.8 Morphogens and HSCs

From this it is clear that HSCs are fundamental to fibrosis progression in the liver. The signalling events that regulate their activation need to be fully understood to facilitate identification of potential therapeutic targets. Regarding the potential for developmental pathway reactivation in HSCs, much work has been done on reactivation of Hh signalling observed in aHSCs. Upregulated Hh signalling occurs upon liver injury and is associated with regulating metabolism in HSCs, promoting glycolysis and stimulating the quiescent to activated HSC transdifferentiation(65). Blockade of Hh signalling is able to revert myofibroblasts to a more quiescent like phenotype as well as decreasing fibrotic response in a mouse CCl₄ injury model(65). Further work has highlighted the importance of Notch signalling, again upregulated in HSCs upon injury. Inhibition of Notch in cultured HSCs again reduces myofibroblast markers and inhibition in a BDL injury model attenuated the fibrotic response(66). The importance of cross talk between these two pathways has also been highlighted, with inhibition of Notch signalling disrupting Hh activity and vice versa(66).

Wnt is one of the major signalling pathways active during development, critical for cell differentiation and proliferation(67). However, the activity of Wnt signalling and potential contribution to HSC activation has not yet been fully explored. Wnt signalling is increasingly implicated in tissue injury and repair in the adult, observed in simple organisms such as planaria to numerous types of injury models in humans including myocardial infarction, bone fracture, skin wounding and retinal damage(68). Furthermore, blockade of Wnt signalling disrupts invertebrate regeneration and impairs tissue repair in mammals(68).

Activation of β -Catenin, a key downstream mediator of Wnt signalling is one of the first responses to tissue injury in numerous organs (69-71). Furthermore, elevated Wnt signalling is initially beneficial to repair and regeneration, increased Wnt7a expression for example promotes hair follicle neogenesis after wounding in mouse skin(72). However, in the situation of chronic injury, the promotion of cell migration and proliferation driven by Wnt activity is likely to contribute to pathology of fibrosis. The Wnt pathway is highly complex with numerous ligands, receptors and interactors. Identifying Wnt components active in HSCs may therefore identify potential targets for modulating HSC behaviour.

1.9 Wnt Signalling

Wnt proteins are a family of highly evolutionary conserved secreted glycoproteins. Wnt signalling is a key regulator of embryogenesis across the animal kingdom: from the wingless (*wg*) protein first identified in *Drosophila* to the nineteen different Wnt members currently described in mammals(67). The Wnt signalling cascade is outlined in Figure 1.3. In summary, Wnt proteins secreted by signalling cells bind to their membrane bound Frizzled (Fzd) receptors, resulting in phosphorylation of the downstream mediator Dishevelled (Dvl). Phosphorylated Dvl then propagates Wnt signalling by three potential pathways: the canonical β -Catenin associated pathway, the non-canonical Planar Cell Polarity (PCP) pathway or the non-canonical Calcium associated (Wnt/ Ca^{2+}) pathway. Wnt ligands can be broadly classified as canonical or non-canonical depending on the pathway they initiate(12).

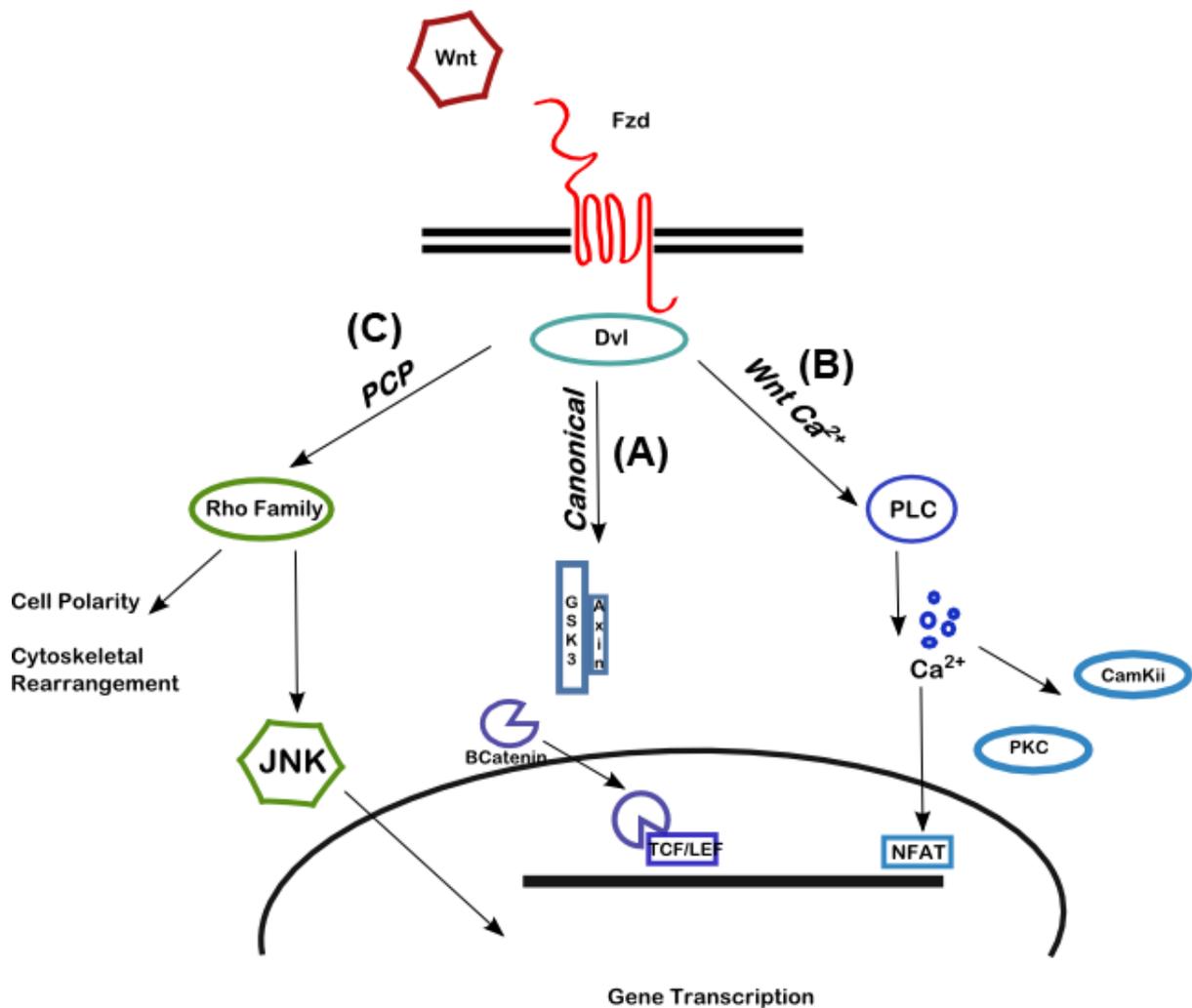


Figure 1.3 Summary of the Wnt Pathway

An overview of the Wnt signalling pathway. Wnt ligands bind to Fzd receptors to trigger phosphorylation of Dvl and propagation of signal through ‘Canonical’ or ‘Non Canonical’ branches. **(A)** Canonical signalling involves the inhibition of GSK3 β and subsequent stabilisation of β -Catenin. Stabilised β -Catenin then translocates to the nucleus to activate TCF/LEF transcription factors. ‘Non-Canonical’ signalling is independent of β -Catenin. **(B)** Wnt/Ca²⁺ associated signalling involves activation of Phospholipase C (PLC), an increase in intracellular Ca²⁺ levels and activation of downstream effectors such as calcium/calmodulin-dependent kinase II (CamKii), Protein Kinase C (PKC) and the nuclear factor of activated T cell (NFAT) family of transcription factors. **(C)**

1.10 Canonical/ β -Catenin Signalling

Canonical/ β -Catenin associated signalling was the first Wnt signalling cascade to be described and remains the most extensively studied of the branches. The canonical pathway proceeds by stabilisation of the cadherin protein β -Catenin, enabling its nuclear translocation and activation of transcriptional targets(12). β -Catenin was initially identified as a membrane bound component of adherens junctions. Cytoplasmic β -Catenin has a different role however as a transcriptional activator. Under normal conditions, cytoplasmic pools of β -Catenin are regulated through phosphorylation by a destruction complex composed of APC, Axin and GSK3 β and subsequent targeting for ubiquitination and destruction. Upon Wnt stimulus, phosphorylated Dvl is able to inhibit destruction complex function, increasing levels of non-phosphorylated β -Catenin.

In its non-phosphorylated form, β -Catenin is then able to translocate to the nucleus. β -Catenin's best known nuclear targets are the TCF/LEF family members of transcription factors. Mammals have four members: TCF1, TCF3, TCF4 and LEF1 and each member has multiple potential isoforms, adding significant complexity to the pathway(73). Nuclear β -Catenin displaces the repressor of TCF/LEFs, Groucho/TLE, forming a transcriptional activating complex with TCF/LEF members and enabling activation of target genes. Known targets of canonical Wnt signalling are summarised in Table 1. The vast diversity of targets demonstrates the wide reaching effects of canonical Wnt signalling during development. The dramatic effects of early embryonic β -Catenin disruption further enforces this, β -Catenin deletion in mouse embryos causing defective anterior-posterior axis formation along with defunct initiation of gastrulation(74).

Target Genes of Canonical Wnt			
Axin-2	FGF9	mBTEB2	RANK ligand
BMP4	FGF18	MMP2	Runx2
Brachyury	FGF20	MMP7	SALL4
bTrCP	Fibronectin	MMP9	Snail
CCN1/Cyr61	Follistatin	Movo1	Sox2
CD44	FoxN1	Nanog	Sox9
Cdx1	fra-1	NeuroD1	Sox17
Cdx4	Gastrin	Neurogenin 1	SP5
c-jun	Gbx2	Nkx2.2	Stromelysin
Claudin-1	Gremlin	n-myc	Telomerase
c-myc	Hath1	Nr-CAM	Twist
CTLA-4	Id2	Oct4	uPAR
CyclinD	Il8	Osteocalcin	VEGF
Cyclooxygenase-2	Irx3	Osteoprotegerin	
E-cadherin	Islet1	Periostin	
EGF receptor	ITF-2	Pitx2	
EphB/ephrin-B	Jagged1	PPARd	
FGF4	Keratin	Proglucagon	

Table 1.1 Target genes of β -catenin/Canonical Wnt signalling

1.11 Non-Canonical Wnt Signalling

Early experiments identified that treatment of *Xenopus* embryos with certain Wnt ligands such as Wnt1 induced secondary axis formation through β -Catenin activation. Treatment with other Wnts such as Wnts4 and Wnt5a either achieved no secondary axis induction or antagonised canonical Wnt mediated induction. This suggested certain Wnts are able to function independently of β -Catenin. β -Catenin independent Wnt activity, classed as 'Non-Canonical' is divided into two main branches: Wnt/Calcium signalling and Planar Cell Polarity

The Wnt/Calcium Pathway uses calcium as a second messenger. Here, Dvl activates phospholipase C (PLC), increasing intracellular calcium levels and stimulating activity of effectors such as calcium/calmodulin-dependent kinase II (CamKii) or Protein Kinase C (PKC). The nuclear factor of activated T cell (NFAT) family are the transcription factors associated with the calcium associated pathway(75).

The planar cell polarity pathway (PCP) is predominantly associated with regulation of convergent extension in embryogenesis. It is a key regulator during gastrulation and neurulation: perturbation of germ layer formation and defects in neural tube closure commonly observed with PCP disruption(76). In PCP signalling, phosphorylated Dvl activates members of the Rho family of small GTPases such as RhoA, Rac and cdc42. This leads to alterations of cytoskeletal arrangement along with activation of the phosphorylated JNK mediated signalling cascade(77). In addition to Wnt-Fzd-Dvl interaction, PCP signalling is regulated by numerous other factors known as the 'Core PCP genes'- CELSR1-3, VANGL1, VANGL2, DIVERSIN and PRICKLE1-4. PCP signalling is also implicated in orientation of cell division, left-right determination and orientation of cilia. Significant defects in heart, kidney and foregut development are observed in mouse and zebrafish models deficient for PCP components(78).

1.12 Wnt Ligands

Different Wnt ligands display varying specificities for different receptors and co-receptors, leading to differential activation of canonical vs non-canonical signalling. Structurally, Wnt proteins are very similar being cysteine rich, highly glycosylated and sharing a common sequence for secretion(79). Extensive post translational modification is necessary before secretion of Wnt ligands due to their greatly hydrophobic nature. An essential requirement for Wnt secretion is palmitoylation, the addition of palmitic acid onto proteins, mediated here by Porcupine (Porc) a seven pass transmembrane protein. Mutations in Porc prevent Wnt signalling and lead to early embryonic death, demonstrating its essential function(80).

There are multiple proposed methods of Wnt secretion that aid its morphogen activity, signalling over such long distances of multiple cell diameters. These include lateral diffusion of Wnt ligands

aided by heparan sulphate proteoglycans, Wnts secreted on lipoprotein particles, the formation of micelles able to shield hydrophobic regions or Wnts secreted by exosomes. The presentation of Wnts at points of cell-cell contact has also been proposed(81).

Wnts1, 3a, 10a and 10b are well known as canonical ligands while Wnt5a and Wnt11 are considered non-canonical(67). The canonical/non-canonical activities of the other Wnt family members remain poorly characterised. In most studies, Wnt3a and Wnt5a are used as prototypical canonical and non-canonical ligands(82, 83).

1.13 Wnt Receptors

Regardless of their method of secretion, Wnts function through binding to their cell surface bound receptors, triggering their intracellular signalling cascade and the uptake and degradation of Wnt ligands themselves. The main class of Wnt receptors are the Fzd receptors: seven transmembrane, G protein coupled receptors(84). They possess two functional domains: a cystine rich domain (CRD) on the N terminus involved in ligand binding and a C terminal PDZ (Postsynaptic density 95, Discs Large, Zonula occludens-1) domain which binds intracellular effectors. Like Wnts, Fzds are conserved throughout the animal kingdom and ten Fzd family members have been identified so far in mammals. Fzds are not simply restricted to Wnt ligand binding, their CRD domain allows interaction with a variety of ligands including RSpondins, Norrins, connective tissue growth factor (CTGF), Il6, Il8 and IFN γ . The two main interactors with the intracellular PDZ domain are Dvl proteins and the heterotrimeric G proteins(85).

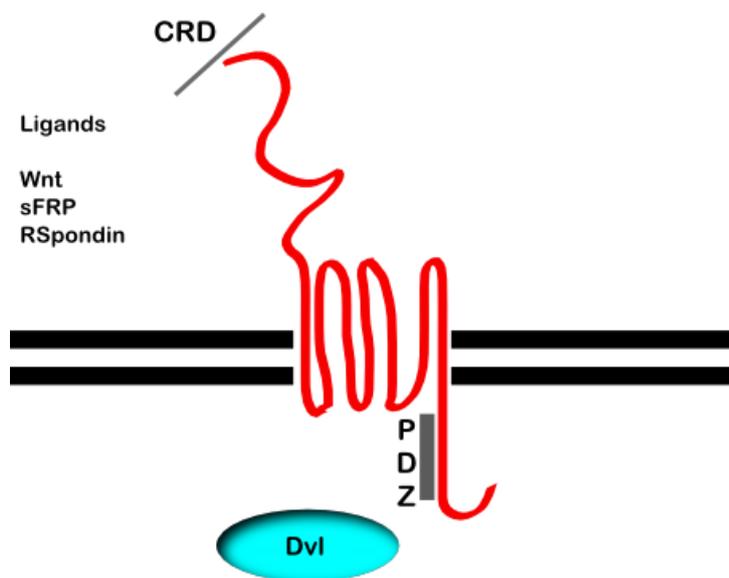


Figure 1.4 Frizzled Receptor Structure

Diagram detailing structure of the Frizzled (Fzd) receptors. The cysteine rich domain (CRD) on the N terminus functions in ligand binding including Wnts, sFRPs and RSpondins. The PDZ (Postsynaptic density 95, Discs Large, Zonula occludens-1) domain on the C terminus functions in binding and phosphorylation of Dvl.

Simple binding of Wnt ligands to Fzd receptors is not sufficient for signalling to occur. For complete transmission of signal the input of Fzd co-receptors is also required. Several members of the low-density lipoprotein receptor related protein (LRP) family (LRP5 and LRP6) are the best characterised of these Fzd coreceptors, implicated in mediating canonical Wnt signalling(86). Phosphorylation of LRP5/6 by Wnt activated Fzd is thought to contribute to the inhibition of the destruction complex and promote stabilisation of β -Catenin. Recent research has also identified numerous other proteins able to act as Fzd coreceptors such as the members of the receptor tyrosine kinase-like orphan receptor (ROR) family ROR1 and ROR2, Ryk and PTK7(87). These alternative co-receptors are predominantly associated with the non-canonical, β -Catenin independent signalling

Current thinking is that specificity of different Wnts for different receptors and co-receptors governs whether cells respond via the canonical or non-canonical pathway. HEK293 cells treated with the canonical associated Wnt3a responded through phosphorylation of LRP5/6 and activation of β -catenin. Conversely, Wnt5a treatment promoted response through phosphorylation of ROR2(82).

Phosphorylation of co-receptors was achieved through the recruitment of the same cellular machinery, suggesting that Wnts are able to instigate significantly different effects through changing only the co-receptor they phosphorylate. Rather than determined by the Wnts available, a cell response to Wnt may therefore be governed by its current expression profile of receptors and coreceptors. This will allow for the highly cell context specific nature of Wnt signalling. Known Wnt-Fzd interactions are summarised in Table 2. From this, activities of Wnts3a and 5a appear the best characterised.

Wnt	1	2	2B	3	3A	4	5A	5B	6	7A	7B	8A	8B	9A	9B	10A	10B	11	16
Fzd1	✓	✓			✓		✓				✓								
Fzd2							✓												
Fzd3		✓			✓		✓												
Fzd4		✓	✓								✓								
Fzd5					✓		✓			✓									
Fzd6					✓		✓												
Fzd7				✓	✓														
Fzd8					✓														
Fzd9		✓																	
Fzd10											✓								

Table 1.2 Known Wnt-Fzd interactions, as demonstrated by Immunoprecipitation. Adapted from (85)

1.14 Propagation of Wnt Signalling

After binding to Fzd receptors and recruitment of associated co-receptors, Wnt signal is transmitted by the phosphoprotein Dvl. Dvl has three conserved domains: an N terminal DIX (Dvl,Axin) domain, a central PDZ domain and a C terminal DEP (Dvl, Egl-10, Pleckstrin) domain. Dvl proteins interact with Fzd receptors through their shared PDZ domains while the DEP domain mediates interaction of Dvl with its target proteins(88). Dvl is recruited to Fzds and phosphorylated by the kinases Casein Kinase 1 (CK1) Casein Kinase 2 (CK2) and PAR1. Vertebrates have three Dvl members : Dvl1, Dvl2,Dvl3. Single or double null mutations in mice result in significant developmental defects, again emphasising the critical role of correct Wnt pathway function during development(89).

Dvl activity mediates of all branches of Wnt signalling. In the canonical pathway it inhibits activity of the β -Catenin destruction complex, recruiting the scaffolding protein Axin away to the plasma

membrane, preventing the GSK3- β mediated phosphorylation and destabilisation of β -Catenin(12). In the non-canonical PCP pathway, phosphorylated Dvl interacts with PCP associated proteins at the cell membrane such as Diego/Diversin, enabling its interaction with numerous downstream effectors(90). Dvl/ RhoA interaction, mediated by the adapter protein Daam1, leads to activation of ROCK kinase and instigation of cytoskeletal rearrangement, Dvl activation of RhoB is associated with influencing migration and Dvl activation of Rac1 is associated with stimulation of the JNK signalling cascade(88). In the calcium associated pathway, Dvl is attributed with activating phospholipase C (PLC) and instigating an increase in intracellular calcium levels(75).

1.15 Extracellular Interactors

In addition to the multiplicity of Wnt ligands, receptors and downstream effectors, numerous secreted interactors of Wnts also exist. This imposes a further layer of regulation to the pathway before Wnts even bind to their receptors. These include Wnt-inhibitory factors (WIF1 and 2), sclerostin, Gremlin, Wise and WNT1-inducible-signaling pathway protein 1 (WISP1) (Figure 1.5A)(91). The two major families of extracellular modulators of Wnt proteins are the secreted Frizzled related proteins (sFRPs) and the Dickkopfs (Dkks). Traditionally, these families are seen as inhibitors of Wnt signalling, although recent research suggests potential for expanded roles(92).

The Dkks are small secreted proteins able to disrupt the interaction between Fzd and the Lrp5/6 co-receptor, preventing subsequent phosphorylation of Dvl and Wnt propagation. The dkk family consists of four members, with Dkk1, Dkk2 and Dkk4 highly similar in structure and function (Figure 1.5B)(93). As Dkk1/2/4 function through binding and preventing Lrp5/6 function, they are mainly associated with blockade of canonical activity. Their effects on non-canonical signalling have not yet been explored. Dkks have key roles in modulating effects of Wnt during embryogenesis with dkk1 mutants in mice and zebrafish defective in anterior-posterior patterning and limb development(94,

95). Dkk3 is thought a divergent member of the family, both in structure and function and there is a evidence it may function in activation rather than inhibition of the pathway (96, 97).

Unlike the Dkks, sFRPs are not limited to the canonical pathway. The family consists of 5 members, sFRP1-5. They are structurally similar to the Fzds , sharing a similar CRD, allowing interaction with both Wnt ligands and Fzd receptors but lack the transmembrane domain (Figure 1.5) (98) Again, conventionally they are viewed as Wnt inhibitors: binding to either Wnts or Fzds to antagonise signalling. Recent research has challenged this and there is evidence for members able to bind and directly activate Fzds or facilitate Wnt-Fzd binding(92). One important proposed function is in aiding Wnt diffusion: sFRPs shown able to bind to Wnt ligands and increase their range of signalling, another possible mechanism for establishing morphogen gradients(99). Early papers identified them as secreted apoptosis related proteins (SARPs), with a role in modulating apoptosis separate from Wnt activity(100). sFRPs also possess a netrin like domain (NTR), found also in procollagen C proteinase enhancer proteins and tissue inhibitors of metalloproteinases (TIMPs)(101), suggesting that through this domain they may have Wnt independent roles in regulating extracellular matrix composition.

From this it is clear that Wnt signalling is far more complex than the summary diagram in Figure 1.3, with numerous potential ligands, receptors, downstream effectors and extracellular modulators. Specificities between Wnts and their receptors and Wnts and their extracellular modulators have not yet been mapped out. Nor has it been fully characterised how the presence of different Wnt ligands and extracellular interactors may affect other members of the same family. There is therefore much further work required on the mechanisms of the Wnt pathway.

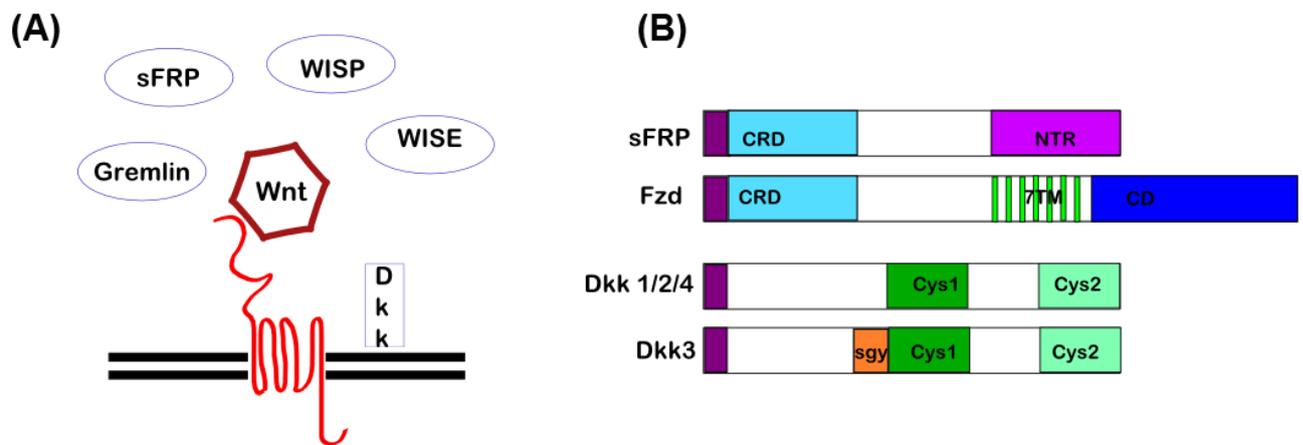


Figure 1.5 Extracellular interactors of Wnt

(A) Examples of proteins capable of interacting with Wnts or Fzd receptors including: Gremlin, Wise, WNT1-inducible-signaling pathway protein (WISP), secreted Frizzled related proteins (sFRPs) and the Dickkopfs (Dkks). **(B)** Structural comparison of sFRP, Fzd and Dkk families. sFRPs share a similar cysteine rich domain (CRD) with Fzd receptors while lacking the seven transmembrane (7TM) and cytoplasmic domains (CD). Dkk3 is considered a divergent member of the Dkk family due to the presence of the sgy domain not present in Dkks 1/2/4.

1.16 Wnts in Embryo and Adult

As indicated previously, Wnts are one of the main families of morphogens critical for governing cell fate specification and cell polarity during embryonic development. Mouse knockout models have been invaluable in clarifying the roles of specific Wnt ligands. Importance of the Wnt pathway in development is emphasised by the diverse range of phenotypes elicited upon disruption of signalling. Abnormal neurulation, gastrulation and somatogenesis are observed along with limb and bone deformities(67). Issues of redundancy between different ligands adds further complexity with some phenotypes not observed until multiple ligands are knocked down. This further complicates elucidating function of specific Wnts.

In the adult, dysregulated Wnt signalling occurs in numerous pathologies such as arthritis, cancer and metabolic disease(67, 102, 103). Regarding fibrosis, the current literature primarily focuses on the pro-fibrotic influence of canonical/ β -catenin associated signalling. Upregulated expression of classic canonical ligands such as Wnt10b and 3a is observed in multiple models of fibrosis across a variety of organs, such as the skin, lung, liver and kidney(104-106). This is accompanied by nuclear accumulation of β -catenin in fibroblasts and upregulation of β -catenin target genes such as Axin2, PAI-1 and LEF1(107-109). Ectopic Wnt signalling further promotes fibroblast activity. Human dermal and lung fibroblasts stimulated with recombinant canonical Wnts such as Wnt1 and Wnt3a increased pro-fibrotic gene expression, stress fibre formation and production of ECM(104). Overexpression of Wnt10b in mice leads to increased development of spontaneous dermal fibrosis in comparison to controls. These animals also display increased susceptibility to bleomycin injury, commonly used to model fibrosis, with an intensified fibrotic response(107). Furthermore, disruption of canonical signalling can attenuate fibrosis. The Dkk family member Dkk1 is commonly used as a method of Wnt blockade. In vivo administration of Dkk1 in a murine UUO model of kidney fibrosis decreased nuclear β -catenin accumulation and induction of target genes and subsequently reduced fibrotic response(106).

How non-canonical Wnts influence fibrosis is more enigmatic. Significant upregulation of non-canonical ligands is also observed in fibrotic kidney, liver and lung(110-112). Manipulation of non-canonical Wnt signalling suggests functional roles, for example overexpression of the non-canonical Wnt11 in renal epithelial cells increased expression of α -SMA(113). Through the PCP pathway non-canonical Wnts are able to alter cytoskeletal conformation via interaction with Rho family members(114, 115) providing great potential for influencing the increased migration and contractility characteristic of activated myofibroblasts.

There is clearly strong bias in the field towards investigating profibrotic potential of the canonical pathway. Identifying upregulated canonical signalling is straightforward, achieved by measuring nuclear translocation of β -catenin, upregulation of classic canonical targets such as Axin2 or c-Myc or using reporter assays such as the TOPFLASH luciferase assay(116, 117). Non-Canonical signalling on the other hand is mediated by effectors influenced by numerous other pathways. Therefore no non-canonical Wnt specific, direct read-out of activity exists. Despite the increased complexity of studying non-canonical Wnt signalling there is significant potential for its influence on myofibroblast activity. Focusing research only on the canonical renders a vast number of prospective therapeutic targets unexplored.

Upregulation of Wnt ligands in activated HSCs has been reported in numerous studies(110, 118-132). Wnt expression appears absent in quiescent cells, again suggesting that in healthy liver Wnt expression is minimal. However, there is significant disagreement as to which ligands are upregulated. The non-canonical ligands Wnt5a and Wnt4 are identified in the majority of studies of *in vitro* activated HSCs (110, 119, 121, 122, 130) and analysis of fibrotic ECM isolated from LX-2s detected the presence of Wnt5a protein(126). Further validation of increased Wnt5a protein expression was demonstrated in murine model of CCl₄ induced liver fibrosis, one of the few studies

to show protein data in an in-vivo model(126). In addition several studies also identify increased expression of canonical Wnts (Wnt3a and Wnt10b)(110, 132). Establishing whether Wnt activity in HSCs is occurring through canonical or non-canonical mechanisms is critically important as their signals are transduced by significantly different downstream mediators.

There appears to be general agreement that Wnt signalling is activated in myofibroblasts. How it contributes to the fibrogenic phenotype is less well explored. Most studies focus on the role of canonical ligands: recombinant Wnt3a shown to promote survival of HSCs(125) and the canonical agonist RSpondin2 shown to stimulate upregulation of fibrotic associated markers. As canonical Wnts are known to be anti-adipogenic, one group suggests a mechanism by which Wnt antagonises the adipogenic transcription factors maintaining HSC quiescence(132). In addition, inhibition of β -Catenin activity through adenovirus mediated overexpression of the canonical antagonist Dkk1, stimulates reversion of in-vitro activated cells to a 'quiescent like' phenotype and amelioration of fibrosis in a murine model of biliary fibrosis(110). The actions of several anti-fibrotic agents are suggested to work through inhibition of canonical/ β -Catenin signalling, such as the plant flavonoid Morin (124)and the AMPK activator Metformin(127). However, these agents are attributed to influencing survival of HSCs rather than the fibrogenic phenotype.

There are an equal number of studies contradicting the importance of canonical Wnt activity, instead supporting non-canonical Wnts and an absence of canonical signalling in the fibrogenic myofibroblast. One of the initial microarray studies identifying upregulated Wnt activity demonstrated no increase in expression of either total β -Catenin or its non-phosphorylated, active form in *in vitro* activated cells(121). Culture activation has shown to initiate upregulation of Wnt5a protein accompanied by downregulation of Wnt10b(122). The same study showed treatment with an agonist of canonical Wnt pathway stimulating decreased expression of fibrotic markers and a shift from a fibroblast like morphology. This suggests that active β -Catenin is associated with

maintaining quiescence, in keeping with its role in maintaining stem cell pluripotency(133).

Disruption of non-canonical Wnt signalling appears to have functional consequences: depleting

Wnt5a in the LX-2 cell line reduces Collagen I and TGF- β expression(130) and knockdown of Wnt4

causes a similar reduction of α -SMA expression in activated rat HSCs(119). Similar direct knockdown

studies of canonical Wnts have not been published.

1.17 General Hypothesis and Main Aims

General hypothesis:

Similar regulatory mechanisms co-ordinate cell behaviour during embryogenesis and cellular response to tissue injury. Exploring these events will allow insight into mechanisms regulating myofibroblasts differentiation and wound healing response

Aims

- To characterise the expression of the Wnt morphogen pathway in HSCs
- To explore the effect of canonical Wnt signalling in HSCs
- To explore the potential for non-canonical Wnt signalling in HSCs
- Development of zebrafish for study of developmental control of fibrosis.

Chapter 2: Materials and Methods

2.1 Primary cell culture

Hepatic stellate cells (HSCs) were isolated from normal livers of male Sprague-Dawley rats by a standard, previously optimised protocol(46). Rats were anaesthetised using a combination of Ketamine (2µl per gram of body weight) and Xylazine (1µl per gram of body weight). Livers were cannulated and flushed through with 300 ml Hank's Balanced Salt Solution without Calcium and Magnesium (HBSS -). Livers were then perfused with 3mg/ml Collagenase B (Roche) in HBSS containing Calcium and Magnesium(HBSS +) followed by 10mg/ml Pronase (Roche) in HBSS +. Following perfusion, livers were dissected out and incubated in 3mg/ml Pronase in HBSS+ for 15 mins. Livers were then homogenised under sterile conditions using nylon mesh, mixed with 10mg/ml DNase in HBSS and centrifuged at 1800rpm for seven minutes. Supernatant was removed, pellets resuspended in HBSS + DNase and centrifuged again. HSCs were purified from liver homogenate by discontinuous density centrifugation in 11.5% Optiprep (Sigma Aldrich D1556): pellets resuspended in a solution of HBSS+ and Optiprep and centrifuged for 23 minutes at 1500g (with an acceleration of 7 and a deceleration of 2). Upper layer containing HSCs was removed, resuspended in HBSS+ and centrifuged again at 1800rpm for seven minutes. Isolated cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) and initially plated on plastic T75 flasks for 20 minutes to allow attachment and separation of Kupffer cells. The non adhered HSCs were then washed, centrifuged and seeded onto 10cm² dishes or 6 well culture plates. Day 0, freshly isolated cells are considered quiescent HSCs (qHSCs). Cells were cultured on plastic for up to ten days and were considered activated (aHSCs) after Day 7.

Dermal fibroblasts were obtained from freshly harvested rat skin. Skin was briefly sterilised in 70% ethanol and washed in serum free medium. Skin was cut into small pieces and digested with collagenase A for 2.5 hours at 37°C. Digestion was then filtered, centrifuged, resuspended, strained to remove debris and plated onto 10cm dishes or 6 well culture plates. Primary cells were maintained in humidified atmosphere of 5% CO₂ in air at 37°C. Cells were cultured in DMEM

supplemented with 16% fetal bovine serum (FBS), 100 U/mL penicillin, 100µg/mL streptomycin and 2mM L-glutamine,

2.2 Cell line Culture

Cell lines used were the immortalised human HSC cell line, LX-2, or the human embryonic kidney (HEK 293). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100µg/mL streptomycin and 2mM L-glutamine. Again, cells were maintained in humidified atmosphere of 5% CO₂ in air at 37°C.

2.3 Cell Culture Reagents

Reagent	Source
Cyclosporin A	Tocris
SP600125	Calbiochem
Recombinant Human Dkk-2	RnD
Recombinant Human Dkk-3	RnD
Recombinant Human sFRP-1	RnD
Recombinant Human sFRP-5	RnD
Alkaine Phosphatase (CIAP)	Promega
IWP2	Sigma
ICG001	Tocris
Dvl-PDZ domain inhibitor	Calbiochem
Mitomycin C	Sigma

Table 2.1 Cell culture treatment reagents

2.4 RNA isolation and cDNA Synthesis

Cells were washed with PBS and scraped into RLT buffer (Qiagen) with added β-mercaptoethanol. Total RNA was purified from harvested cells using the RNeasy kit (Qiagen), following the manufacturer's instructions. cDNA templates were prepared using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Promega). A nanodrop was used to quantify isolated RNA and 0.5 - 1µg was incubated at 37°C for 30minutes with one unit of RQ1 RNase-Free DNase in 10X DNase reaction buffer. DNase activity was then stopped using 0.5M EGTA solution and 0.5 µg of Random Hexamers were added. After a 5 minute incubation at 70°C, samples were placed onto ice and a

reaction mixture of 20units of RNAsin, a mix of dNTPs at 10mM each and one unit of MMLV-RT in 5X reaction buffer was added. After a final incubation of 60mins at 42 °C, samples were diluted with RNase free water to 10ng/μl of cDNA

2.5 RT-PCR

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed as follows: an initial 3 minutes at 94 °C, followed by 35 cycles of denaturation for 20 seconds at 94 °C, annealing at 50 – 60 °C (depending on primer pair) for 30 seconds and elongation at 72 °C for 30 seconds, followed by a final 7 minutes at 72 °C. Reagents used were DreamTaq PCR MasterMix (Life Technologies) consisting of Dream Taq DNA polymerase in 2X DreamTaq buffer along with a mix of dNTPs at 0.4 mM each and 4 mM MgCl₂, 20ng of cDNA template and 0.1μM of primers made up to a 25μl reaction mix with RNase free water. Once amplification was complete, 3 μl of 10x loading buffer was added to products before loading onto 1% agarose gels stained with 0.5μg/ml of Ethidium Bromide . Gels were run for one hour at 0.15A and results visualised under UV.

2.6 qPCR

Quantitative polymerase chain reaction (qPCR) was performed using a 7500 Fast system (Applied Biosciences). Reagents used were SYBR Green JumpStart *Taq* ReadyMix (2X) (Sigma), 10ng of cDNA and 0.1 μM of primers made up to a 13 μl reaction mix. The program consisted of 40 cycles of denaturation for 10 seconds at 95 °C, annealing at 50 – 60 °C (depending on primer pair) for 30 seconds and elongation at 72 °C for 30 seconds, followed by a final dissociation curve cycle of 95 °C for 15 seconds, 60 °C for 60 seconds and 95 °C for 30 seconds. All results were normalised to a control housekeeping gene by subtracting average ct for housekeeping gene from the average ct of gene of interest, producing Δct. The average Δct obtained from experimental condition was

normalised to control conditions using the equation $1/\text{POWER}(2, \text{avg } \Delta\text{ct}^x - \text{avg } \Delta\text{ct}^y)$. Housekeeping genes used were : GAPDH (Human) , β -Actin (Rat) or rppo (Zebrafish).

2.7 Primers

Primers for target sequences were designed using Oligo Primer Analysis Software 4.0 (Molecular Biology Insights, Inc). Primers were designed to be between 20-23 base pairs in lengths, with an optimal annealing temperature between 55 and 60 °C. Ideal primers produce a single clear band following PCR amplification when visualised on agarose gel. At qRT-PCR level, they produce a uniform melting curve with single peak and no contaminating primer dimers. Figure 2.1 demonstrates examples of ideal and poor melt curve read outs.

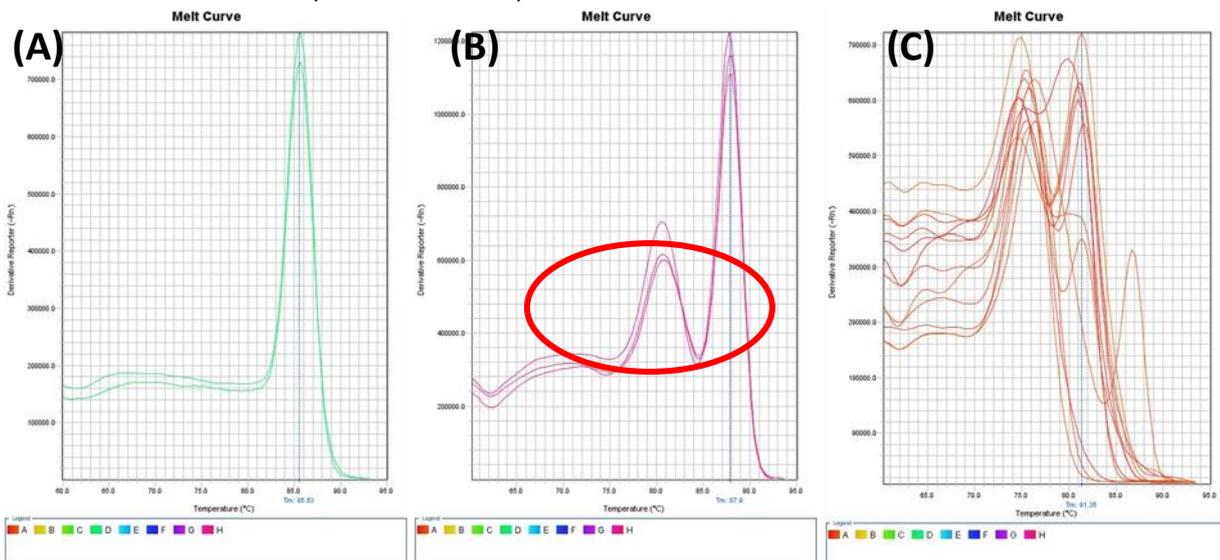


Figure 2.2 Example Melt Curves (A) Example of ideal melt curve (B) Evidence of primer dimers (C) Example of non specific binding

2.7.1 Wnt Ligands

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
rhWnt1f	GCAGTGGCCGATGGTGGGGTAT	hWnt6f	GAGAGTGCCAGTTCCAGTTCCG
rhWnt1r	CACTCACGCTGTGCAGGATCCC	hWnt6r	CAGCAGCTCGCCCATAGAACAG
rWnt3a f	TGGGTTGTCTGGCAGGTGTGAG	rhWnt7bf	GGACGGCTGTGACACCATGTGC
rWnt3a r	TCC ACCACCATCTCTGAGGCAC	rhWnt7br	GGTGAAGACCTCGGTGCGCTCG
hWnt3af	GGCTGGAAGTGGGGTGGCTGTA	rhWnt9bf	CGGCCAGCAAGTACTCACCTG
hWnt3a r	CCTCGTTGTTGTGGCGTTTCAT	rhWnt9br	GCACTCCACGTAGCAGCACCAC
rWnt4f	CTGGAGAAGTGTGGCTGTGACC	rhWnt10af	TCTTCCTACTGCTGCTGGCTGC
rWnt4r	AAGGACTGTGAGAAGGCTACGC	rhWnt10ar	CATCAGGGTGACGCACACACAC
hWnt4f	GTGGGTCACGCACTGAAGGAGA	rWnt10bf	CTCGGGATTTCTTGGATTCCAG
hWnt4r	GGGGCTAGGCTCCAAGTACACC	rWnt10br	CAGGTTTGAATTGGCAGC
rhWnt5af	AACCCTGTTTCAGATGTCAGAAG	hWnt10bf	GACACTTGCATTTCCGCTTCAG
rhWnt5ar	CTGCATGTGGTCCTGATACAAG	hWnt10br	GACACTTGCATTTCCGCTTCAG
rWnt6f	GGGAGGCTGCGGAGATGATGTC	rmhWnt11f	TCCAAGCCAATAAACTGATG
rWnt6r	CAGAGCACAGGAACCCGAAAG C	rmhWnt11r	CAGGTATCGGGTCTTGAGGTC

Table 2.2 Primer sequences for Wnt ligands

2.7.2 Wnt Receptors

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
rFzd1f	GCGACGTAAGGAGCGGAGTG	rRYK F	TCCACCCAGACGACCCAGTAT
rFzd1r	TGATGGTGCGGATGCGGAAG	rRYK R	CTTCACCTTGGCTTTGGCTTC
rFzd2f	CTCAAGGTGCCGTCCTATCTCAG	rROR1 F	CAGGGGGAAATAGAAAATCAG
rFzd2r	GCAGCACAACACCGACCATG	rROR1 R	ACAGTATGGGAAGGCGTAGTG
rFZD4f	CCCCACAAAACCTCCCATCCAGC	rROR2 f	CGTGCGGTGGCTGAAGAATG
rFZD4r	CCACACGGCCATCCAAATATCC	rROR2 r	GCCCGTGTCTGTCTGTGTTCA
rFZD5f	CTGGAGTCTTGGCGGCGCTTCA	rPTK7 F	CCCCTCAGCACCAAACAGAAG
rFZD5r	GCCCGTCTTGGCAGTGAGTGC	rPTK7 R	GGACACCTTACCTGCCTCTG
rFZD6f	AGACCTCCCAGAAGCATCCCT		
rFZD6r	TTCTCCTTACGGTGCCTGCTC		
rFZD8f	GCGAGTACGAGGAGCTGGGTGC		
rFZD8r	CCGCAAGAACCACGTGAGAGA		

Table 2. 3 Primer Sequences for Fzd receptors

2.7.3 Extracellular Interactors

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
rSfrp1F	GCTCAACAAGAA CTGCCACATG	rDkk1F	GTGCTTTCAACGATGGCTCTCT
rSfrp1R	CCAGTAGAAGCCGAAGAACTGC	rDkk1R	TACTTGTTCCCGCCCTCATAGA
rSfrp2F	CCTGGAGACAAAGAGCAAGACC	rDkk2F	ATTGATGGGTTTTGTTGTGCTG
rSfrp2R	TGACCAGATAGGGAGCGT TGAT	rDkk2R	GGTAGCATCCTTCCACACTTTG
rSfrp4F	CTTCCCCTGTGTGGACCTT	rDkk3F	ACAACGAGA CCA ACA CGGAGAC
rSfrp4R	CTATCCCTCGAACGCAAGTC	rDkk3R	CTGGAG AACTGG CAG TAC CTGG
rSfrp5F	GAGATGCTGCACTGCCACAAGT	rDkk4F	GGTCTGCGTGAATGATGTTTGC
rSfrp5R	ACGAAGTCGCTGGAGCACATCT	rDkk4R	CCTGTCCCTTAT TGCTTTGTGA

Table 2.4 Primer Sequences for Wnt Extracellular interactors

2.7.4 Pathway Components

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
rhBcatF	AAGGTGCTGTCTGTCTGCTCTA	rDvl1f	CAGATGAGGAGGACAACACGA
rhBcatR	TTCCATCCCTTCTGCTTAGTC	rDvl1r	GTGATGCTGCTGAAGGAGGAT
rGSK3F	CGAGTGGCGAGAAGAAAGATGA	rDvl2f	GATTTTGGGGTGGTGAAGGAA
rGSK3R	GTGGTTTAATGTCTCGATGGCA	rDvl2r	AAGGGGGTAATGGTGGAGGTG
rAPCF	CGCTCCCACACACATCCAAACA	rDvl3f	CACGCCTAATGAGAAGACACA
rAPCR	ATGGGCTAGGTCGGCAGGATAC	rDvl3r	AGTGGAGTCTGTGATGCTGCT
rAxiF	GCAGGCGTT TGGAGGAAGAAGA		
rAxiR	GGGTCCGGTAGGGTATTGGTT C		
rLrp5F	AAGTGGACAGGGGAGAAAAGGA		
rLrp5R	TTGTCCTGCAACTGTACACCAG		
rLrp6F	ACCATCTAATCTCGTCACATCA		
rLrp6R	TACTGGATTGACTCTCGACAAA		

Table 2.5 Primer Sequences for Wnt pathway components

2.7.5 Wnt Targets

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
rSOX9 F	TGGCAGAGGGTGGCAGACAGC	hAxi2 1 f	CGAGATCCAGTCCGGTGATGG
rSOX9R	TTGGGCGGCAGGTATTGGTCA	hAxi2 r	CCACACACGACCTTTAGGCT
hSOX9 F	AGCGAACGCACATCAAGAC	rAXin2f	TCCTTACCGCATGGGGAGTA
hSOX9 R	CTGTAGGCGATCTGTTGGGG	rAXin2r	GTGGGTTCTCGGGAAGTGAG
rCCND1 F	CTACCGCACAACGCACTTTCT	rNFATc1 f	GCAAAGCCCAAGTCTCTATCC
rCCND1 R	TTCTTCTCCACTTCCCCTTC	rNFATc1 r	CATAGTGAGCCCTGTGGTGAG
hCCND1 F	CCAGAGGCGGAGGAGAACAAA	rNFATc2 f	ACAGTTCCGCTCAGGGCTCTC
hCCND1 R	AGCGTGTGAGGCGGTAGTAGG	rNFATc2 r	AAGAGGGACGGCTGCTGGTAG
rcMYC F	CCACAGCAAACCTCCACACA	rNFATc3 f	GAGAAGAGTCTGGCATCAAC
rcMyc R	ACCCTGCCACTGTCCAATT	rNFATc3 r	GAGTGATGGGGTGAAGAGAG
rEVI F	ATTGGGTCACTGGCTCATAAG	rNFATc4 f	ATCAGTATGGAGGACAGGGCT
rEVI R	GATGAAGATGCTGGGTGTGAG	rNFATc4 r	GAGTGGTGGGGAGGAAGGTAA

Table 2.6 Primer Sequences for typical Wnt target genes

2.7.6 β -Catenin Interactors

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
hChibbyf	GGTTAGTGGCGGTGTGGAC	hPygopus2f	GTGAACGATGACCAGGATG
hChibbyr	AAGTGGGATTACAGCAGTGG	hPygopus2r	TGCCCTCACGGATGTAGAC
rChibbyf	CCGAAGAAGACTCCTCCTC	hrBCL9f	GGTCCGTCCCCCTACAGTGAT
rChibbyr	CACACCCCACTAATCACT	hrBCL9r	CATTTCAGCCCCATTCTTCA
hPygopusf	GTGGACTGGATGGGTTAGGAG	hBCL92F	TCTGGGCTGAGGGAGGTGGAC
hPygopusr	GATTCGGTGGTGGAGCATACT	hBCL92R	TCTGCTGCTGCGGGGTCTATCT

Table 2.7 Primer sequences for nuclear β -Catenin interactors

2.7.6 Zebrafish Germ Layer Markers

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
drneurog1F	TCCTTTTCGCACACGGATGA	drpitx3F	CCGTTATCCCGACATGAGCA
drneurog1R	GTGCACAGTGGTTTCGTTCC	drpitx3R	CCACACCCGTA CTCTTGCTT
drspry1F	CGTGTCTCTCAGGCTGTCA	drcxcr4aF	TTTCTCCCAACGGTGTACGG
drspry1R	GGGCCCTCAGTGTATTCGTT	drcxcr4aR	TACCAGTCTTTGGCCACGTC
drntlaF	CCACAAGGAAGTCCCAGACC	drmyoDF	CTCCGAGGTCCTGCGAATTTT
drntlaR	ATTGAACTGAGGAGGGCTGC	drmyoDR	GGGAAGGGGATATCCGACAA
drsnai1aF	CCCGGTGCCTGTTATACCTC	drctnnb1F	ACCAAGAGCAAGTAGCAGACA
drsnai1aR	GCAGGTTGCTGGTAGTCCAT	drctnnb1R	CGAACTGTGTGGAAGGTATCT
drfoxa1F	CCCCAAACTCGGAACTGAA	drcnd1F	CACTTCCTTGCCAAACTGCC
drfoxa1R	ATGGGGGTCTCCTTTGAGGT	drcnd1R	ATGAGAGGCAACTGTCCGGTG
drfoxa2F	TCAATGACAGGCATGTCCCC	drsox9aF	GCATGAATCTCCTCGACCCC
drfoxa2R	TAGCTCCTGCGGTAGGTCTT	drsox9aR	CTGGGTGCATCAGACAGACA
drsox17F	GCATCCGAAGCCAATGAAC	drrpoF	CTGAACATCTCGCCCTTCTC
drsox17R	GCTTTCATGACTTACCAAGC	drrpoR	TAGCCGATCTGCAGACACAC

Table 2.8 Primer sequences for zebrafish germ layer markers

2.8 Primers Used

2.8.1 Fibrogenic Targets

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
rBActinf	AGAGGGAAATCGTGCCTGACA	hGAPDHf	GTCAGTGGTGGACCTGACCT
rBActinr	ACATCTGCTGGAAGGTGGACA	hGAPDHR	TGAGCTTGACAAAGTGGTCCG
rαSMAf	CATGTCGTCCCAGTTGGTGAT	hαSMAf	CACTGTTCTCTGTGCCTAAAC
rαSMAr	CGAAGCGCAGAGCAAGAGA	hαSMAr	GGTTGGCCTTTCCCAGTG
rTGFβf	GAACCCCATTTGCTGTCC	hTGFβf	TGACAGCAGGGATAACACACT
rTGFβr	TCCGTCTCCTTGGTTCAG	hTGFβr	CGCACGCAGCAGTTCTTCTCC
rTIMP1f	GCATGGACATTTATTCTCCAC	hTIMP1f	ACTTCCACAGGTCCACAAC
rTIMP1r	TCTCTAGGAGCCCCGATCTG	hTIMP1r	CATTCTCACAGCCAACAGT
rCollagen1f	TTCACCTACAGCAGCTTGTG	hCollagen1f	CAAGAGGAAGGCCAAGTCGAGG
rCollagen1r	GATGACTGTCTTGCCCCAAGTT	hCollagen1r	CGTTGTCGCAGACGCAGAT
rPPARyf	ATTTCTGCTCCACACTAT	hPPARyf	GGGATCAGCTCCGTGGA
rPPARyr	GCTTTATCCCCACAGACT	hPPARyr	TGCACTTTGGTACTCTTGAAGTT

Table 2.9 Primer sequences for human and rat fibrogenic targets

2.9 Protein Lysate Preparation

Cells were washed with cold PBS and scraped into Radio-Immunoprecipitation (RIPA) lysis buffer (20 mM Tris-HCL, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10% glycerol) with added protease inhibitors (1 cOmplete Protease Inhibitor Cocktail Tablet (Roche) per 50 mL buffer) and phosphatase inhibitors (200µl Phosphatase Inhibitor Cocktail (Pierce) per 50 mL buffer). Samples were sonicated for six minutes then centrifuged at 4 °C for 15mins at 13000xg. The supernatant was aspirated and stored and the pellet discarded. Protein concentrations were then determined using the Bradford DC assay kit (BioRad). Equal amounts of protein sample were added to 5X Laemmli buffer and made up to a 20µl reaction mixture with RIPA.

2.10 Cytoplasmic and Nuclear Fractionation.

For cytoplasmic fractionation cells were washed, scraped into PBS, pelleted by centrifugation for 10 minutes (6000g) at 4 °C and resuspended in 650µl of fractionation buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% Igepal, 1mM DTT and 1 mM PMSF, adjusted to pH 7.6). Samples were incubated on rotary wheel at 4 °C for 10 minutes. Samples were then vortexed, centrifuged at maximum speed for 30 seconds and the resulting supernatant taken. For nuclear fractionation cells were washed, scraped into PBS and then pelleted by centrifugation (6000g, 2minutes). Pellet was resuspended in 75µl of Dignam A buffer (10mM HEPES (pH7.9), 1.5mM MgCl₂, 10mM KCl, 0.5mM DTT, 0.2% NP-40), spun at 3000rpm for 10secs and supernatant discarded. This wash was repeated twice and pellet then resuspended in 20µl of Dignam C buffer (2mM HEPES(pH7.9), 25 % glycerol, 420mM NaCl, 1.5mM MgCl₂, 0.5mM DTT, 200nM EDTA). Samples were incubated on ice for 10minutes and vortexed frequently. After incubation, samples were spun for 1 minute at 1300rpm. Supernatant was taken and pellet discarded.

2.11 Alkaline Phosphatase (ALP) Treatment for dephosphorylation of protein

Protein lysates from culture activated rat HSCs were incubated with 100 units of ALP (Promega) in reaction buffer (5 mM Tris pH 7.9, 10 mM NaCl, 1 mM MgCl₂, and 0.1 mM DTT) for 30 minutes at room temperature. Samples were then diluted with 5X Laemelli buffer and used for Western Blotting.

2.12 FLAG Immunoprecipitation

Medium from LX-2 cells overexpressing Flag tagged sFRP4 subjected to immunoprecipitation using the FLAG Tagged Protein Immunoprecipitation Kit (Sigma). 1ml of medium was added to 40µl of Anti-FLAG M2 affinity gel resin and incubated at 4 °C overnight. After incubation, resin was centrifuged, supernatant removed and washed 3 times with 0.5ml of kit specific Wash buffer. Protein was then eluted with 3X FLAG Peptide following manufacturer's instructions. Eluted protein then subjected to SDS-PAGE and immunoblotting.

2.13 SDS PAGE and Western Blotting

Samples were denatured by incubation at 95 °C for 5 minutes and then separated by electrophoresis on 10% sodium dodecylsulfate–polyacrylamide gels. Gels were run for approximately 2 hours at 100 volts then transferred to nitrocellulose membranes (Amersham). Membranes were first blocked to minimise non specific binding using 5% Milk in Tris-buffered saline (TBS)/Tween 20 (TBS-T) solution. After 1 hour blocking, membranes were incubated with antibody overnight at 4 °C. Membranes were washed three times with TBS-T solution before a 1 hour incubation at room temperature with horseradish peroxidase (HRP) conjugated secondary antibody at a 1:5000 dilution. Membranes were again washed with TBS-T before visualisation by chemiluminescence reaction (Pierce) followed by exposure to film.

2.13.1 Primary Antibodies Used

Antibody (Clone Number)	Dilution	Source	Catalogue #	Predicted MW (kDa)
CaMKII (pan)	1 in 1000	Cell Signal	3362S	60, 50
c-Myc (9E10)	1 in 1000	Pierce	A1-980	45
Cyclin D1 (92G2)	1 in 1000	Cell Signal	#2978	36
Dvl2	1 in 1000	Cell Signal	#3216	90-95
DNMT1 (60B1220.1)	1 in 500	Genetex	GTX48548	183
FLAG	1 in 500	Sigma	F3165	13
Fzd4	1 in 500	Abcam	ab83042	65
Fzd8	1 in 1000	Abcam	ab75235	73
GAPDH	1 in 2000	Abcam	ab9485	34
LEF1 (C12A5)	1 in 1000	Cell Signal	#2230	25-58
H2A.Z	1 in 1000	Abcam	ab4174	14
H3K27me3	1 in 1000	Abcam	ab6002	17
p84 (5E10)	1 in 1000	Genetex	GTX70220	84
PJNK Thr183/Tyr185)	1 in 1000	Cell Signal	#9251	46, 53
sFRP1	1 in 1000	Abcam	ab4193	37
sFRP4	1 in 500	Abcam	ab32784	50
TCF1 (C63D9)	1 in 1000	Cell Signal	#2203	48, 50
TCF3 (D15G11)	1 in 1000	Cell Signal	#2883	78
TCF4 (C48H11)	1 in 1000	Cell Signal	#2569	58, 79
TGFβ (56E4)	1 in 1000	Cell Signal	#3709	45 to 65
TJNK	1 in 1000	Cell Signal	#9252	46, 54
Wnt5a	1 in 1000	Abcam	ab174100	45
Wnt5a (C27E8)	1 in 1000	Cell Signal	#2530	45

αTubulin	1 in 1000	Cell Signal	#2144	52
Bactin (AC-15)	1 in 2000	Abcam	(ab6276	45
β-Catenin (Active) (8E7)	1 in 500	Millipore	05-665	92
β-Catenin (Total)	1 in 1000	Abcam	ab6302	92

Table 2.10 Primary antibodies used

2.13.2 Secondary Antibodies Used

Antibody	Dilution	Source
Goat Anti-Rabbit	1 in 5000	Cell Signal
Rabbit Anti-Mouse	1 in 5000	Sigma

Table 2.11 Secondary Antibodies used

2.14 ELISA for TGF β 1

Supernatants harvested from primary rat HSCs and assayed using TGF β 1 Quantikine[®] ELISA kit (R&D Systems) as per manufacturer's instructions. Reagents, standard dilutions, control and experimental samples were prepared as directed. 50 μ L of Assay Diluent RD1-21 added to 50 μ L of Standard, Control or experimental samples and incubated for 2 hours at room temperature. Plate washed with wash buffer and 100 μ L of TGF- β 1 Conjugate added to each well. Samples were incubated for a further 2 hours at room temperature and washed again. 100 μ L of Substrate Solution was then added to each well and samples incubated for 30 minutes at room temperature in the dark. Finally, 100 μ L of Stop solution was added and optical density of each well determined using a microplate reader set to 450 nm with correction at 540nm. Supernatants from two separate preparation of HSCs used for assay.

2.15 Transformation of competent bacteria

Plasmid DNA was mixed with 100 μ L of JM109 competent bacteria and 0.1M CaCl₂. After 30 minutes incubation on ice, bacteria were heat shocked for 45 seconds at 42 °C, returned to ice for five

minutes then incubated for 1 hour in 500µl of Luria broth (LB) medium (Sigma) under gentle agitation. 100µl of cell suspension was spread onto LB agar plates containing selection antibiotic and incubated at 37°C. After overnight incubation, single colonies were picked, placed into 5mls of LB medium containing selection antibiotic and again incubated overnight at 37°C under agitation.

2.16 Purification of Plasmids

Plasmid DNA was purified via alkaline lysis. QIAprep Spin Miniprep Kit (Qiagen) was used for mini-preparations of DNA, following manufacturer’s instructions. For maxi-preparations, 1 µl of transformed bacteria was added to 300ml LB medium containing selection antibiotic and incubated at 37°C under agitation. After overnight incubation, suspension was centrifuged at 4500 g for 15 minutes. NucleoBond Xtra Maxi kit (Clontech) was used for purification of plasmid, as per manufacturer’s instructions. Plasmid DNA was quantified using a Nanodrop.

2.17 Cell Transfection

Cells were transfected with plasmid DNA 24 hours after passage at approximately 40-60% confluency. Transfection was mediated via Effectene transfection reagent (Qiagen) following manufacturer’s instruction. Standard transfection period was 48 hours before harvest. DNA concentration was dependent on vessel size: 1 µg in 10cm dishes, 0.5 µg per well of 6 well plate, 0.25 µg per well of 12 well plate.

2.17.1 Constructs used

Construct	Source	Resistance
TOPFLASH	Millipore	Ampicillin (50µg/ml)
FOPFLASH	Millipore	Ampicillin (50µg/ml)
Renilla Luciferase	Promega	Ampicillin (50µg/ml)
hWnt3a	Origene	Ampicillin (50µg/ml)
hWnt5a	Origene	Ampicillin (50µg/ml)

hWnt10b	Origene	Ampicillin (50µg/ml)
hFzd4	Origene	Ampicillin (50µg/ml)
hFzd8	Origene	Ampicillin (50µg/ml)
hROR2	Origene	Ampicillin (50µg/ml)
hsFRP4	Origene	Kanamycin (50ug/ml)
hBcatenin (S37A)	Orford <i>et al</i> (134)	Ampicillin (50µg/ml)
hLEF1	Origene	Ampicillin (50µg/ml)
NFAT Luciferase	Jans et al (135)	Ampicillin (50µg/ml)
pGL3 Control Luciferase (empty)	Promega	Ampicillin (50µg/ml)
pcDNA3.1	Invitrogen	Ampicillin (50µg/ml)

Table 2.12 Constructs used

2.18 Luciferase Assay

β-Catenin activity was measured by transfection of the TOPFLASH firefly luciferase TCF-reporter construct. NFAT transcriptional activity was measured by transfection of an NFAT firefly luciferase reporter plasmid containing nine copies of an NFAT-binding site from the IL-4 promoter. For luciferase assays, 0.5µg of plasmid was cotransfected with 0.05 µg of *Renilla* luciferase plasmid serving as a transfection control. TOPFLASH assays were accompanied by a further negative control with separate transfection of FOPFLASH reporter constructs with mutant TCF binding sites. TOPflash activity was then normalised to the ratio of FOPflash activity. After 48 hours transfection, cells were scraped into 1 x Passive Lysis Buffer (Promega), freeze thawed and luminescence measured using the Dual Luciferase Reporter Kit (Promega) and a luminometer. Transfections were performed in duplicate and data presented as average ratios of firefly to *Renilla* luciferase activity

2.19 siRNA Knockdown

sFRP4 oligo nucleotide sequences

Oligonucleotide	Sequence
siGENOME siRNA Rat sFRP4 (89803)	GGAUAGACAUCACACCAGA
siGENOME siRNA Rat sFRP4(89803)	GCGAUGAGCUGCCUGUCUA

sFRP4 siRNA oligonucleotides designed and ordered from Thermo Scientific. Rat HSCs (passage 2 – 3) transfected with a combination of siRNA at increasing doses using Interferin transfection reagent (Polyplus). Cells subjected to second dose of siRNA 24 hours after first and harvested for protein 72 hours after initial dose. Nonspecific siRNA used as a control.

2.20 Scratch Wound Assay to assess migration

90% confluent LX-2 cells seeded in 6 well plates were treated with Mitomycin-C (Sigma) for 30 minutes to inhibit proliferation and then a single scratch made in each well using a sterile 200 μ l pipette tip. Bright field images of each well were taken using a Zeiss Axio Observer over a period of 8 hours. At each time point, three fields per scratch were analysed and results presented are averages of ten measurements of wound width per field. Experiments were performed in triplicate. For Wnt overexpression studies, LX-2s were transfected with Wnt5a or empty control expression vectors 48 hours before scratch injury. For IWP2 and Wnt Conditioned medium treatment, LX-2s were pre-treated with IWP2/Wnt conditioned media for 30 minutes before scratch injury.

2.21 Acridine Orange staining to assess apoptosis

Apoptosis assays were conducted on cells seeded in 12-well plates at 20 – 30% confluency. Apoptotic cells were detected by staining with Acridine Orange (1 μ g/ml) and visualisation under a FITC filter using a Zeiss Axio Observer. Five random fields per well were counted at x20 magnification in duplicate wells.

2.22 MTT Assay to assess proliferation

Cells were seeded in 96 well plates. After 24 hours treatment, cells were treated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5mg/ml, Sigma) for 4 hours. Medium was

then aspirated and cells incubated with DMSO (Sigma) for 30 minutes. Plate was read using plate reader at 570nm absorbance.

2.23 Zebrafish Maintenance

Wild type zebrafish line used was the AB line. Transgenic lines used were the LiPan

(*Tg(fabp10:dsRed, elavl3:GFP)* *Tg(ngn1:GFP)* and *Tg(isl1:GFP)*). Adult fish were maintained on a light dark cycle of 14:10 hours at 28°C. Embryos were collected after spawning and incubated at 28°C in fish water containing 0.6g/L salt (Crystal Sea Marinemix; Marine Enterprises International) and methylene blue (0.002 g/L) (Sigma).

2.24 Morpholino injections

A morpholino was obtained from Gene Tools, designed using their provided software to target the *h2afv* transcript (CTTTTCCTGCTTTGCCTCCTGCCAT) and obtained from Gene Tools. A standard control morpholino (CCTCTTACCTCAGTTACAATTTATA) with no known targets in the zebrafish genome was used as a control. Morpholinos were injected at the 1 – 8 cell stage using a microinjector with needles calibrated to inject 4nl per embryo. Viable larvae were defined as normal, severe or mildly affected as demonstrated. For coinjection experiments morpholinos targeting *dnmt1* or *p53* were used.

2.25 Genomic DNA Isolation and Slot Blot to assess DNA methylation

Larvae were collected in lysis buffer (10mM TRIS, 100mM EDTA, 0.5% SDS) with added 10ng/ml Proteinase K and incubated at 55 °C for 2hours. Samples were centrifuged at maximum speed for five minutes and supernatant used for DNA isolation via Phenol Chloroform extraction. DNA was precipitated using 100% ethanol. Pellets were then washed with 75% ethanol, air dried and resuspended in 1 x Tris-EDTA (TE) buffer. 600ng of DNA was used for slot blot analysis. DNA was diluted in 20µl of TE buffer and denatured with equal volume of denaturing solution at 95 °C. After

10min incubation equal volume of ice cold 2M ammonium acetate was added and samples were loaded onto a nitrocellulose membrane pre-soaked in 2X SSC buffer. Membrane was then dried and baked in vacuum oven at 80 °C. After one hour membrane was blocked in 5% milk/TBST for a further hour and then incubated in primary antibody overnight at 4 °C. After primary incubation, membrane was washed 3 times in TBST, incubated with secondary antibody for a further hour then washed and visualised using chemiluminescence reaction. Antibodies used were anti-5-MeC (Eurogentech) (1 in 2000) and rabbit anti-mouse (1 in 5000). Samples blotted in parallel were stained with 0.2% Methylene blue in 0.3M NaOAc to detect total DNA. The freeware programme GelAnalyzer was used to quantify 5MeC and methylene blue intensity and 5MeC levels were determined by normalising to total DNA.

2.26 Touch Response Assay to assess motility

A single two day post fertilisation (dpf) fish was placed in a dish marked with concentric rings 1cm apart as illustrated by Figure 6.5. Fish were touched once lightly on the head and the zone they swam into recorded (Zone 1, 2 or 3).

2.27 Statistical Analysis

GraphPad Prism 6 was used to calculate mean and standard error values. Data presented as averages \pm standard error of the mean (SEM). Statistical analysis was performed using a student's T-Test (paired or unpaired where appropriate). A P value of <0.05 was considered statistically significant. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

CHAPTER 3: Wnt Pathway Characterisation in Hepatic Stellate Cells

3.1 Introduction

From the current literature there is considerable conflict regarding Wnt signalling and HSCs. This first Chapter intended to decipher which patterns of Wnt expression and activity were observable here in HSCs. Many previous studies focus predominantly on Wnt ligand expression in HSCs, overlooking the potential effects of Wnt receptors and extracellular modulator. A thorough study of how the whole Wnt pathway responds upon transdifferentiation will be far more informative. This will enable identification of the ligands expressed by HSCs, the receptors which mediate their signal and other components able to enhance or repress signalling.

3.2 Increased Wnt activity accompanies HSC activation

All current literature at least agrees that increased Wnt signalling accompanies HSC activation(110, 119-122, 126, 130, 132). These studies predominantly infer activation of the Wnt pathway from upregulation of Wnt ligand mRNA expression. However the complexity of the pathway means that this may not necessarily equate with upregulated Wnt activity. To begin, I first wanted to determine increased active Wnt signalling in HSCs based on a reliable downstream indicator. I selected phosphorylation of the downstream protein Dvl as a putative marker, Dvl being the first signal mediator in the pathway after Wnt/Fzd binding. It mediates all branches of Wnt signalling and should therefore function as a marker of both canonical and non-canonical activity(88). When visualised on Western blot, two Dvl bands are apparent: a phosphorylated, higher weight form and a non-phosphorylated lower weight form. A shift to a phosphorylated form of Dvl has previously been

used as a marker of active Wnt and I aimed to reproduce this in primary HSCs(136). Cell lysates from activated rat HSCs (seven days in culture) show a clear double band when immunoblotted with an anti-Dvl antibody (Figure3.1A). Treatment of lysates with alkaline phosphatase (ALP), a dephosphorylating agent, removes the top band, demonstrating its specificity to phosphorylation(Figure3.1B). mRNA expression of all members of the Dvl family (Dvl1,Dvl2, Dvl3) is significantly increased in aHSCs when compared to qHSCs by qRT-PCR (Figure3.1C).

In vitro culture promotes transdifferentiation of freshly isolated primary HSCs, resulting in myofibroblast like cells by Day 7. This spontaneous activation is a commonly used method of studying HSC behaviour(30). Protein expression of Dvl is visible by Day 3 of a time course of *in vitro* activation and expression of hyperphosphorylated Dvl is visible by Days 5 and 7 as cells approach a fully activated phenotype. I was unable to detect Dvl expression in quiescent HSCs suggesting that Wnt signalling is active only upon HSC activation (Figure3.1D).

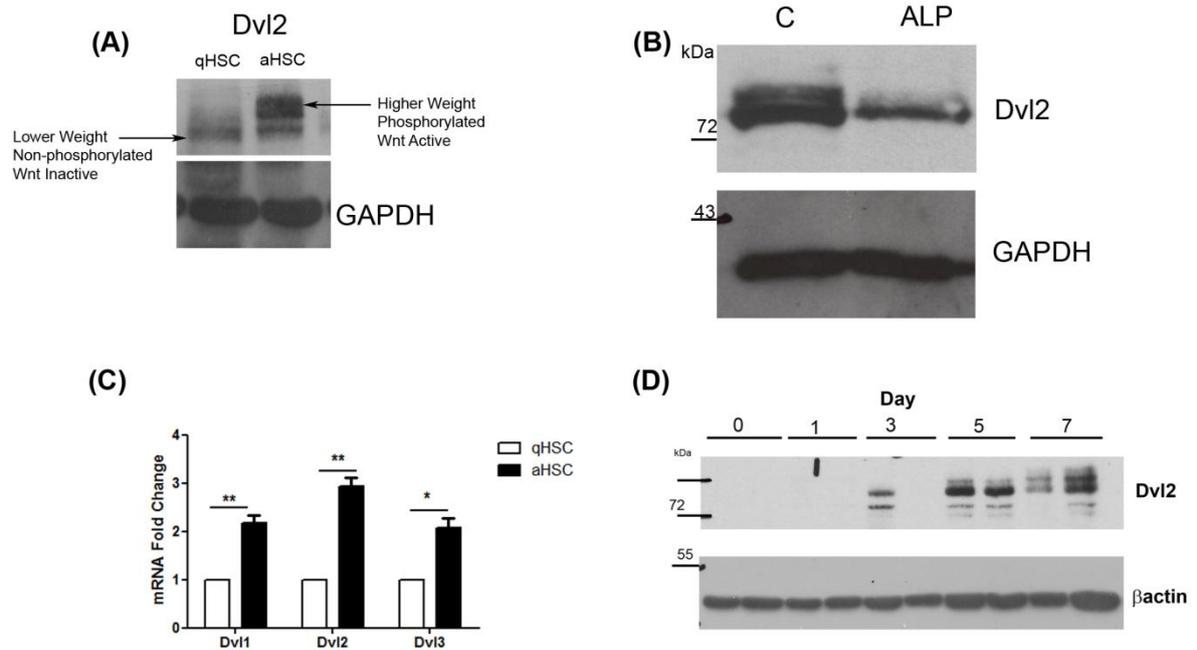


Figure 3.1 Increased expression of phosphorylated Dvl in aHSCs

(A) Two bands are distinguishable upon immunoblot for Dvl2: a lower and a higher weight band corresponding to non-phosphorylated, Wnt inactive and phosphorylated, Wnt active forms respectively. GAPDH serves as a loading control **(B)** The higher band can be attributed to phosphorylation as treatment of protein lysates with the dephosphorylating agent Alkaline Phosphatase (ALP) removed the higher band. GAPDH serves as a loading control **(C)** mRNA levels of Dvl family members Dvl1, Dvl2, Dvl3 were quantified by qRT-PCR in quiescent rat HSCs (qHSC) and HSCs activated after seven days in culture (aHSC) (n=3). **(D)** Protein lysates from two different preparations of rat HSCs at different time points in culture were immunoblotted for Dvl2. Expression of Dvl2 is detectable by Day3 and phosphorylated form visible at Day5. beta-actin serves as a loading control. qRT-PCR results are expressed as fold change normalised to control \pm SEM *p<0.05, **p<0.01. Student's *t* Test (unpaired).

3.3 HSCs express predominantly non-canonical ligands

Expression of phosphorylated Dvl convincingly implies active Wnt signalling in aHSCs. I next wanted to demonstrate that increased Wnt ligand expression accompanies phosphorylation of Dvl and assessed expression of a variety of canonical and non-canonical ligands by RT-PCR in aHSCs and whole rat liver (Figure 3.2A). Rat brain was used as a positive control due to its diversity in Wnt expression (137). Only non-canonical ligands could be detected in aHSCs: Wnt4, Wnt5a and Wnt6. Wnt7b and Wnt9b expression was present in whole liver only, suggesting different Wnt expression profiles of different liver cell types as previously described (138). Wnt6 expression intriguingly appears expressed selectively in aHSCs and not detectable in either whole liver or rat brain.

Expression of Wnt 4, 5a and 6 were significantly upregulated upon activation when quantified by qRT-PCR. (Figure 3.2B). Wnt expression in quiescent HSCs was low ($Ct > 30$) again suggesting that Wnt signalling is activated only during transdifferentiation. Further analysis across a time-course of in-vitro activation suggests temporal differences in Wnt expression: Wnt4 upregulated at Day 4 well before an increase in Wnt5a at Day 8 (Figure 3.2C). This may suggest different requirements for specific Wnt ligands at different stages of activation.

Knowledge of the non-canonical branches of Wnt signalling is lacking in comparison to the well-defined non-canonical branches. Wnt6 appears to be an interesting candidate for further study due to its selective expression in HSCs. However, literature on its targets and mechanisms are limited and reagents such as antibodies are of poor quality. Wnt5a however is one of the few examples of a well studied non-canonical ligand with a large amount of literature on its roles in influencing cell behaviour (103). Furthermore, significant upregulation of Wnt5a by HSCs has previously been reported in both *in vitro* and *in vivo* models (110, 122, 126, 130). Based on this, I selected Wnt5a as a target for investigating the role of non-canonical Wnt signalling in HSCs, despite increases in Wnt4 and Wnt6 expression appearing more profound. Wnt5a protein expression across a time course of activation could not be analysed due to the poor cross reactivity of Wnt5a antibodies with rat.

However Wnt5a protein expression and secretion are evident upon analysis of whole cell lysates and concentrated medium from the LX-2 cell line (Figure3.2D).

My data so far correlate with the several reports of increased non-canonical Wnt ligands contributing to HSC activation. One group has suggested the alternative: that the increased canonical activity is important(110, 132). Canonical ligands in rat HSCs above could not be detected and to further investigate this possibility, mRNA expression levels of canonical Wnt3a and Wnt10b were assessed in human HSCs by RT-PCR . Wnt overexpressing LX-2 cells were used as a positive control (Figure3.3A). Minimal expression of canonical ligands was detected when compared with the non-canonical Wnt5a. This was further confirmed by quantifying differences through qRT-PCR (Figure3.3B). These data suggests that both rat and human HSCs express a predominantly non-canonical range of Wnt ligands.

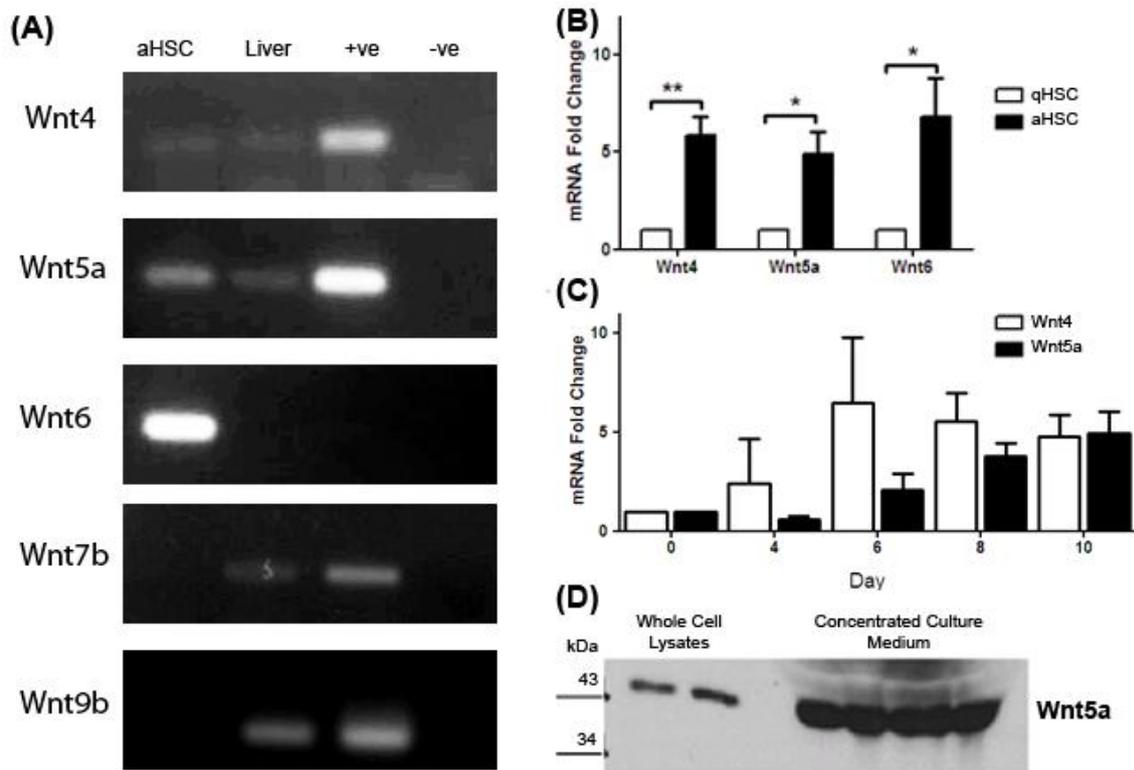


Figure 3.2 HSCs increase Wnt ligand expression upon activation

(A) Wnt ligand expression was analysed and compared by RT-PCR between rat aHSCs, rat whole liver, rat brain (+ve) and water only (-ve). Expression of Wnts 4, Wnt5a, Wnt6 only detected in HSCs. **(B)** mRNA levels of Wnt4, Wnt5a and Wnt6 were by qRT-PCR in quiescent rat HSCs (qHSC) and HSCs activated after seven days in culture (aHSC) (n=5) **(C)** mRNA levels of Wnt4 and Wnt5a were quantified by qRT-PCR across a time course of HSC activation (n=3). **(D)** Wnt5a is detectable in both whole cell protein lysates and concentrated culture medium from the LX-2 cell line when analysed by Western Blot. qRT-PCR results are expressed as fold change normalised to control \pm SEM *p<0.05, **p<0.01. Student's *t* Test (unpaired).

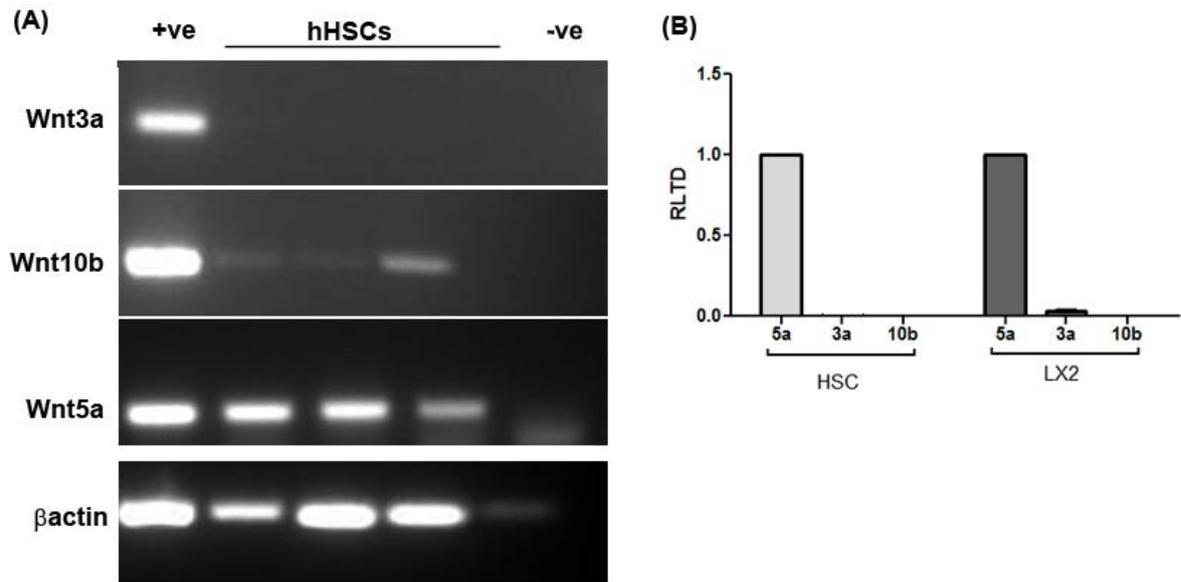


Figure 3.3 Canonical ligand expression is minimal in aHSCs

(A) mRNA expression of Wnts3a, 10b and 5a were analysed by RT-PCR in three different preparations of human HSCs. **(B)** mRNA transcript levels of Wnts3a and 10b were quantified by qRT-PCR in LX-2 and human HSCs and compared relative to transcript levels of Wnt5a (relative level of transcriptional difference (RLTD)).

3.4 HSCs express a variety of Wnt receptors

Data so far demonstrates non-canonical ligand expression in HSCs. Available receptors next needed to be assessed. The Wnt pathway has 10 different Fzd receptors, each possessing different specificities of ligand binding and different associations with canonical and non-canonical activity(85). The literature is not consistent when defining the expression patterns of Fzds in HSCs: some studies suggesting uniform expression of all ten(138) while others suggest differential expression of a select few(110, 121). Here, RT-PCR analysis confirms expression of Fzds1,2,4,5,6 and 8 in HSCs (Figure3.4A). Expression of a variety of Fzd receptors suggests potential for response to a number of different Wnt ligands despite HSCs themselves appearing to only express non canonical Wnts.

How Fzd receptors respond to transdifferentiation was next quantified. Surprisingly aHSCs appear to downregulate the majority of Fzd receptors, significant decreases occurring for Fzd1, Fzd4 and Fzd8 (Figure3.4B). Fzd2 is the only receptor upregulated, although the modest two fold increase was not statistically significant. Fzd2 is one of the better characterised Fzd receptors, and frequently associated with non-canonical ligands in comparison with the canonical associated Fzd1(139-142). This fits with the predominantly non-canonical expression profile of aHSCs. Based on this, I next looked at expression of the non-canonical associated Wnt receptors and co-receptors: ROR1, ROR2, Ryk, Ptk7(87), predicting a similar increase in expression due to the non-canonical associations seen so far. Expression of all four receptors was detected in HSCs by RT-PCR (Figure3.4C). However, transdifferentiation was associated with a reduction in expression, similar to the majority of the Fzds (Figure3.4D).

I next asked when this decrease in receptor expression occurs: whether it is early and a contributing factor to the activation of HSCs or later on, a possible consequence of HSCs becoming refractory to

Wnt signalling. I quantified the expression patterns of the most significantly decreased receptors Fzd4 and Fzd8 in more detail across a time course of activation (Figure3.5A) . The decrease in expression appears to occur early, reduced substantially by day 3 with expression remaining low throughout the time course. Western Blot analysis confirms a decrease in protein expression with activation (Figure3.5B). Despite a decrease in expression, the majority of Fzds are still noticeably present in aHSCs when visualised by RT-PCR and presumably still able to function in Wnt signalling.

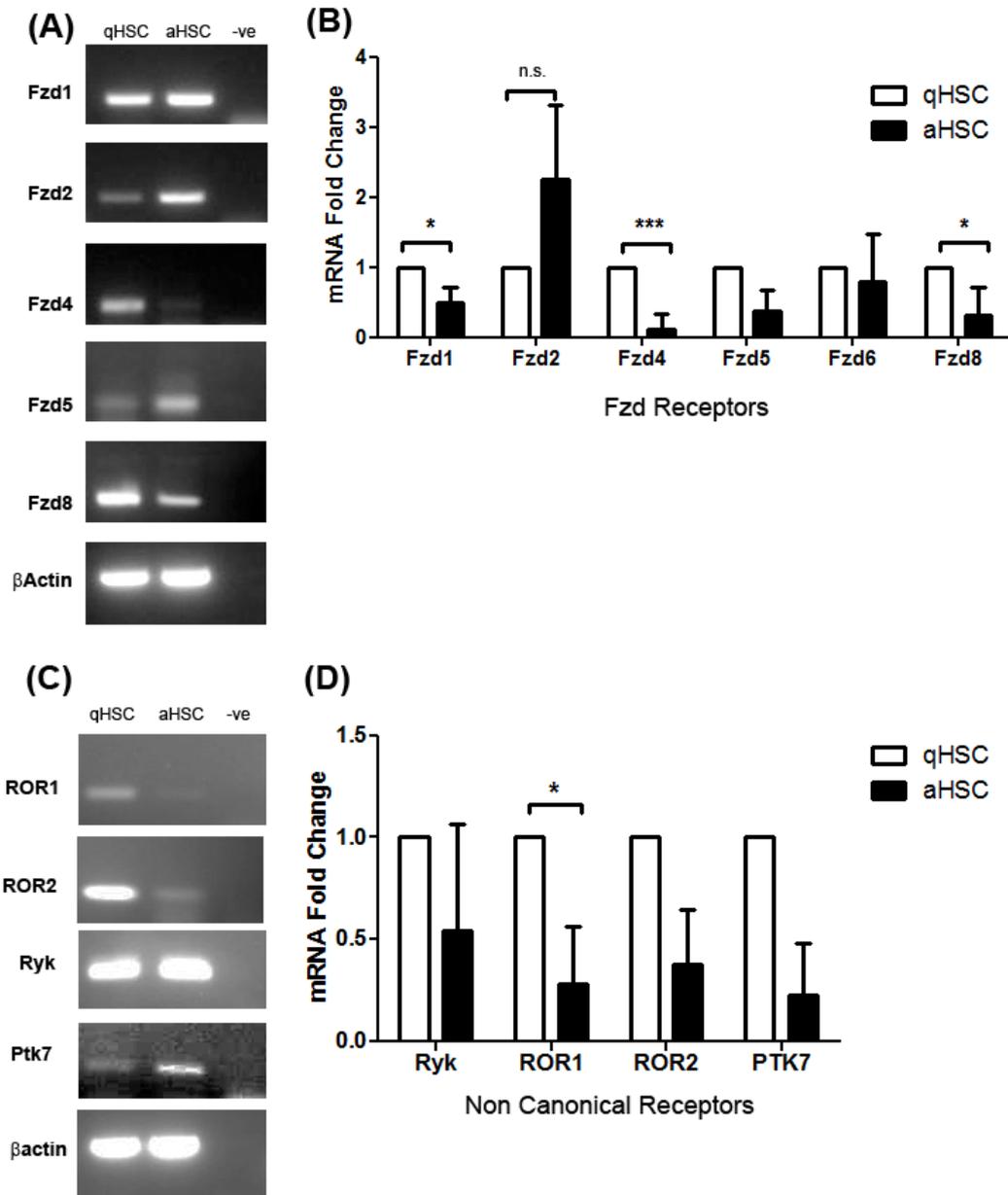


Figure 3.4 HSCs decrease Wnt receptor expression upon activation

(A) mRNA expression of Fzds 1,2,4,5,6 and 8 can be detected in both quiescent (qHSC) and active (aHSCs) HSCs by RT-PCR. β Actin serves as a loading control. Water only (-ve) serves as a negative control. **(B)** mRNA levels of Fzd receptors were then quantified in quiescent rat HSCs (qHSC) and HSCs activated after seven days in culture (aHSC) by qRT-PCR. (n=4) **(C)** mRNA expression of ROR1, ROR2, Ryk and Ptk7 can be detected in both quiescent and aHSCs by RT-PCR. β Actin serves as a loading control. Water only (-ve) serves as a negative control. **(D)** mRNA levels of non-canonical receptors were then quantified in quiescent (qHSC) and active (aHSC) HSCs by qRT-PCR (n=3). qRT-PCR results are expressed as fold change normalised to control \pm SEM * $p < 0.05$, *** $p < 0.001$. Student's *t* Test (unpaired).

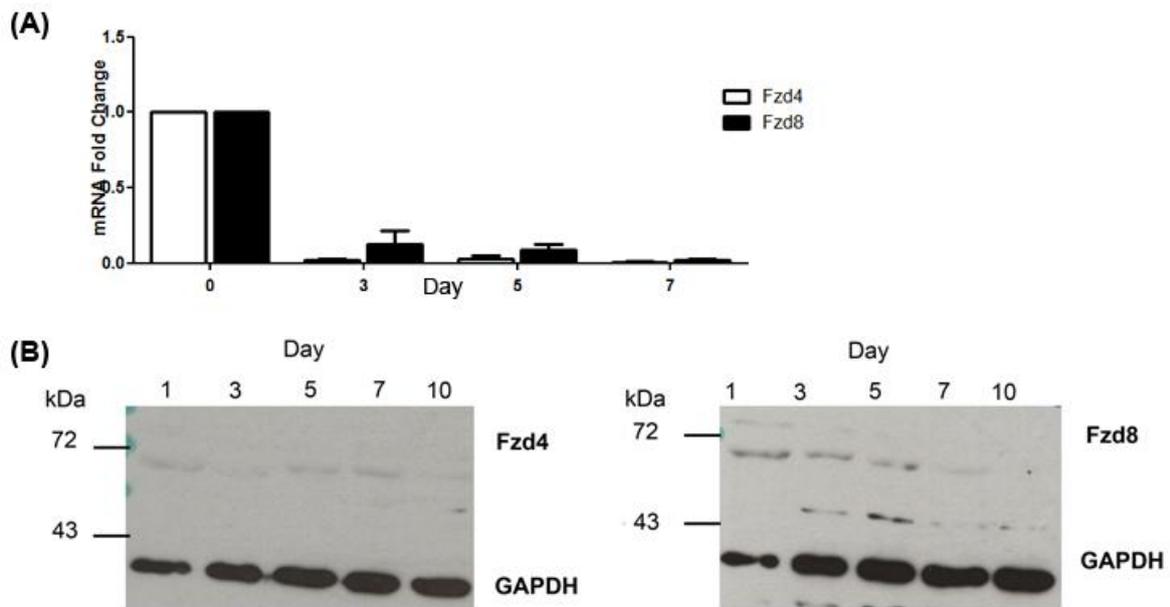


Figure 3.5 Decrease in Fzd expression occurs early in activation

(A) mRNA levels of Fzd4 and Fzd8 were quantified by qRT-PCR across a time course of culture activation of rat HSCs (n=3). **(B)** Protein expression of Fzd4 and Fzd8 was analysed by Western Blot across a time course of culture activation of rat HSCs. GAPDH serves as a loading control. qRT-PCR results are expressed as fold change normalised to control \pm SEM

3.5. HSCs increase expression of extracellular interactors

Expression and function of extracellular interactors of Wnt such as the sFRPs or the Dkk families has not been fully explored in either HSCs or the liver as a whole. In contrast to the decreased expression of Fzd receptors, mRNA expression of sFRPs 1,2 and 4 and Dkk3 in aHSCs significantly increased in HSCs (Figure 3.6A and B). Expression of sFRPs was undetectable in qHSCs by RT-PCR (Figure 3.6A), again suggesting that sFRPs function only in aHSCs. Dkk1, Dkk2 and Dkk4 expression could not be detected in either qHSCs or aHSCs while sFRP5 expression was highly variable. sFRP4 appears to be the most extensively upregulated sFRP. Analysis of protein expression across a time course further confirms this, with expression substantially increased by day 3 (Figure 3.6C).

As mentioned previously, the role of these Wnt interacting proteins has not yet been resolved and there is evidence for them either promoting or repressing Wnt signalling depending on cell context (92, 93). Significant increases in expression with HSC activation have not yet been reported and I aimed to explore their effect on HSC biology and behaviour more thoroughly. Sources indicate that the enhancing or inhibitory effect of these proteins may be influenced by concentration (99, 143). They are proposed to function as biphasic modulators of Wnt signalling: inhibitory at high concentrations and stimulatory at lower. Based on this, I treated LX-2 cells with low concentrations of recombinant sFRPs and Dkks, aiming to stimulate WNT signalling.

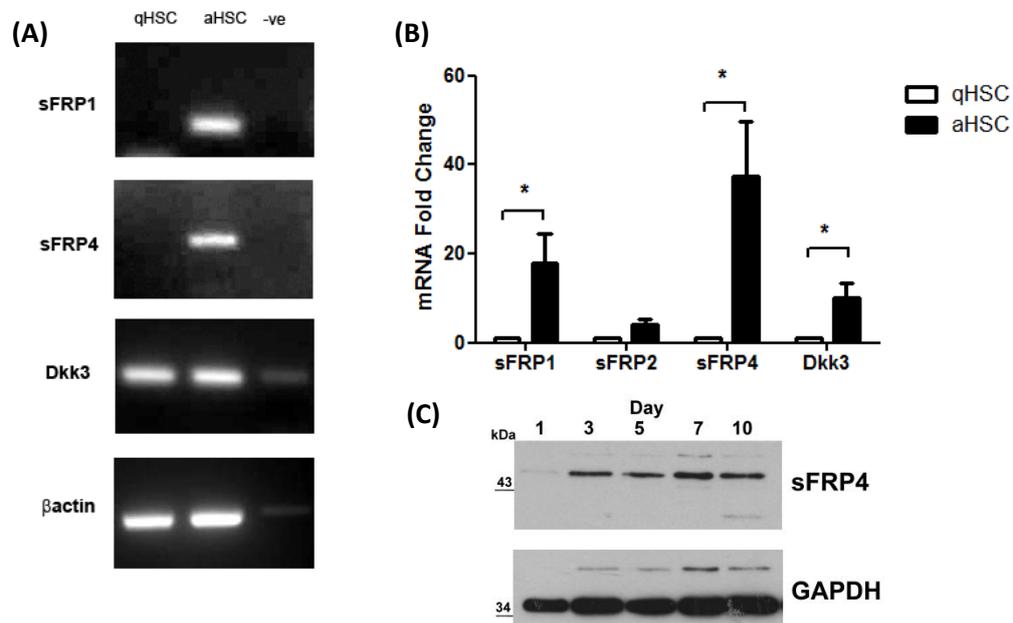


Figure 3.6 HSCs increase expression of several extracellular interactors of Wnt.

(A) mRNA expression of sFRP family members and Dkk3 was analysed in both quiescent (qHSC) and active (aHSCs) HSCs by RT-PCR. β Actin serves as a loading control. Water only (-ve) serves as a negative control.

(B) mRNA expression levels of sFRPs and Dkk3 were then quantified in quiescent rat HSCs (qHSC) and HSCs activated after seven days in culture (aHSC) ($n=4$) **(C)** Protein lysates across a time course of *in vitro* activation were immunoblotted for sFRP4 expression. GAPDH serves as a loading control. qRT-PCR results are expressed as fold change normalised to control \pm SEM * $p < 0.05$ Student's *t* Test (unpaired).

I began with Dkk3 treatment at time points of two or six hours and used Dvl2 phosphorylation as an indicator of increased Wnt activity. Dkk3 is considered a divergent member of the predominantly inhibitory Dkk family with evidence suggesting its ability to enhance Wnt signalling (96, 97). Here, treatment of LX-2 cells with 5nM recombinant Dkk3 appeared to increase hyper-phosphorylated Dvl expression at the two hour time point (Figure 3.7A). I decided to use 5nM as a concentration potentially able to promote Wnt stimulation.

Serum starved LX-2s were treated for 24 hours with 5nM Dkk2, Dkk3, sFRP1 or sFRP5 and qRT-PCR used to assess effects on transcript levels of fibrogenic markers such as α -Sma, Collagen I or TGF β or downstream markers of canonical Wnt signalling. Dkk2 treatment resulted in no significant change in either set of markers. A small but significant upregulation of β -Catenin canonical targets Axin2 and cMYC occurred upon Dkk3 treatment along with a small upregulation of Collagen1 expression (Figure 3.7C). This could imply upregulation of Wnt activity, corresponding with the increased phosphorylation of Dvl observed by Western Blot. sFRP treatment appeared to have an opposite effect. Although sFRP1 treatment had no consistent trend, sFRP5 treatment suppressed all 3 fibrogenic markers analysed as well as cMyc and CyclinD expression (Figure 3.7B). sFRP5 expression in HSCs was highly variable, significantly increased in some preparations whereas unchanged in others suggesting it may not be a consistent factor in HSC activation. From this it appears that these secreted factors have opposing effects on fibrotic potential of HSCs. As aHSCs express substantially more sFRPs than Dkk3 it could be concluded that their role in primary cells is one of inhibition.

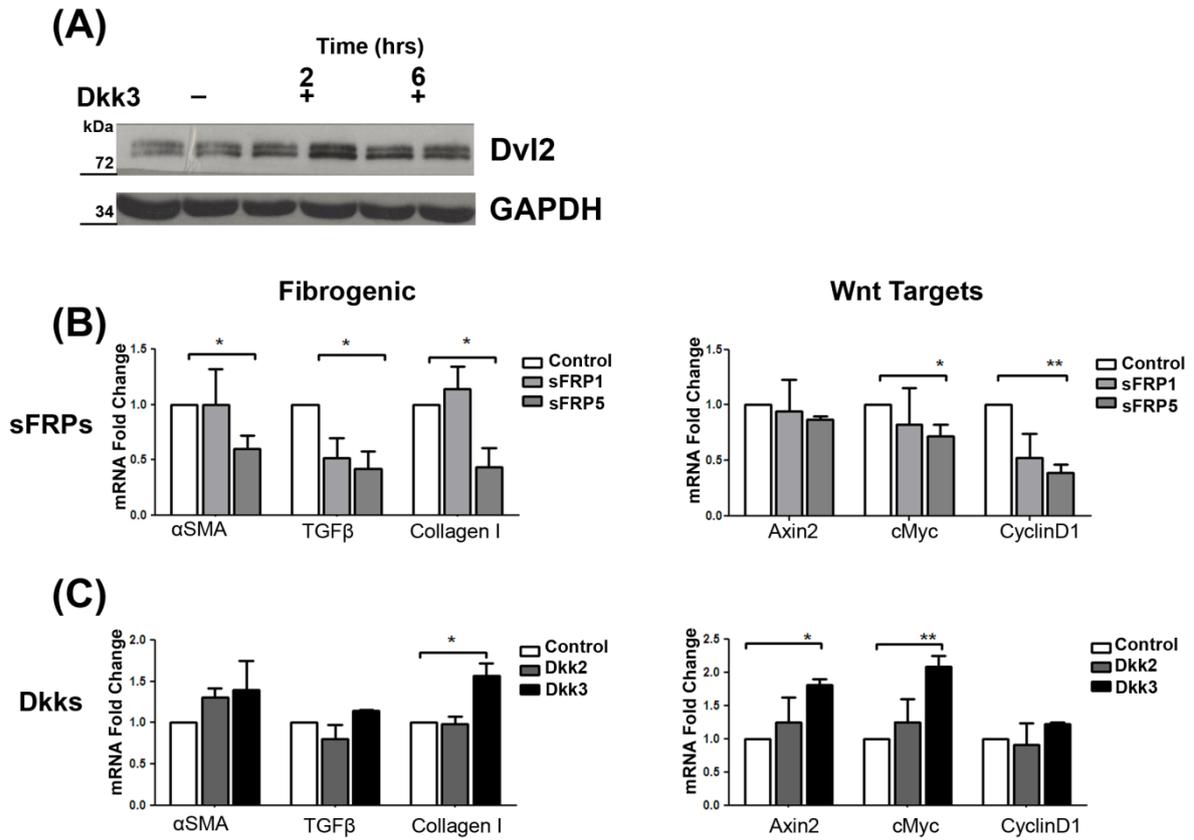
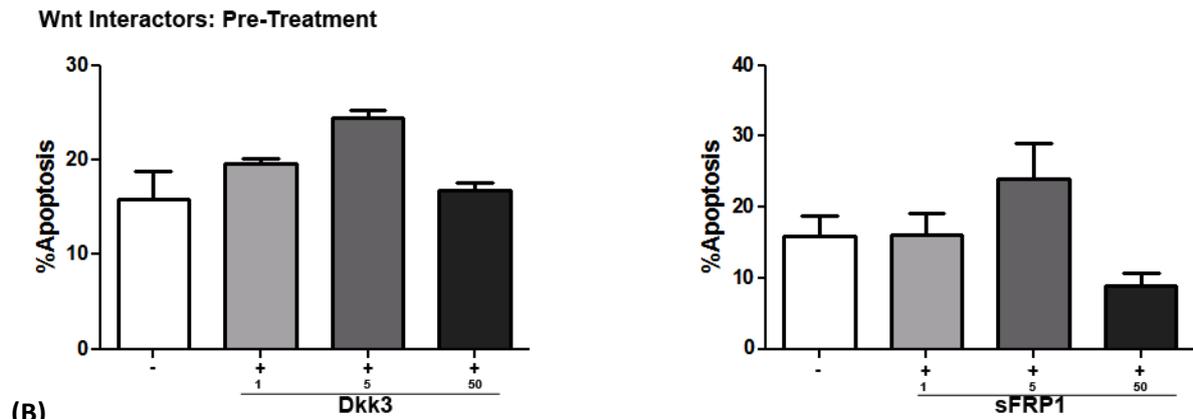


Figure 3.7 sFRPs and Dkks promote opposing effects on HSC gene expression.

(A) Protein lysates from either untreated controls or LX-2 cells treated with 5nM recombinant Dkk3 for 2 or 6 hours were immunoblotted for Dvl2 expression. GAPDH serves as a loading control. mRNA of fibrogenic markers or Wnt targets genes were quantified in LX-2 cells treated for 24 hours with 5nM sFRP1 and sFRP5 **(B)** or Dkk2 and Dkk3 **(C)** (n=3) qRT-PCR results are expressed as fold change normalised to control \pm SEM *p<0.05 Student's *t* Test (unpaired).

sFRPs were originally identified as SARPs (secreted apoptotic related proteins) able to modulate the apoptotic response in cells(100). Based on this I questioned whether their role could be mediation of apoptosis in HSCs. I aimed to stimulate apoptosis in LX-2s and either pre-treat or co-treat with recombinant sFRPs and Dkks. I chose sFRP1 and Dkk3 as they are reliably increased in aHSCs and used a range of concentrations between 1 – 50nM. 50 μ M cyclohexamide in low serum medium (0.5%) for 16 hours was used as a stimulator of apoptosis, quantifying apoptosis using acridine orange assay. Firstly, LX-2 cells were pre-treated for 6 hours with sFRP1 or Dkk3 before cyclohexamide mediated induction of apoptosis. Apoptotic cell percentage appeared to vary with concentration(Figure 3.8A). Both Dkk3 and sFRP1 appeared to induce apoptosis upon low dose (5nM) whereas the higher dose (50nM) treatment reduced apoptosis percentage, although changes did not reach statistical significance. Simultaneous treatment of cyclohexamide and Dkk3 did not show the same trend. Simultaneous sFRP1 and cyclohexamide resulted in a similar reduction at 50nM (Figure 3.8B). This may propose a role for sFRP1 in modulating response of HSCs to apoptotic stimulus instead of directly affecting fibrotic gene expression.

(A)



(B)

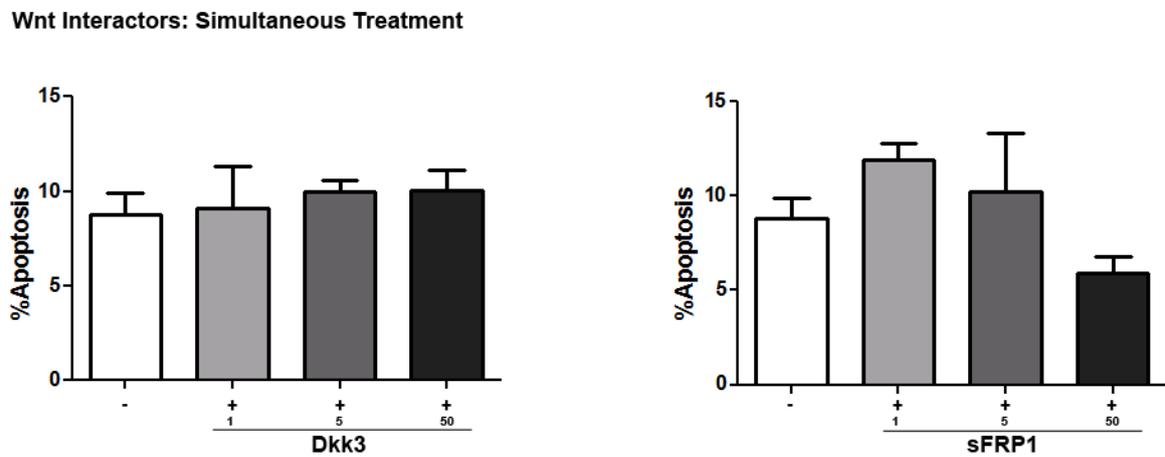


Figure 3.8 sFRP1 and Dkk3 treatment mildly influences apoptotic response

Apoptosis was induced in serum starved LX-2 cells by 50 μ M cyclohexamide (CHX) treatment . Effect of varying concentrations of recombinant Dkk3 and sFRP1 on the apoptotic response was analysed by acridine orange staining in two separate treatments. Cells treated with 1, 5 or 50nM recombinant protein either 6 hours before ((A) Pre-treatment) or in conjunction with 50 μ M CHX ((B) Simultaneous) (n=2)

Figure 3.6B identifies sFRP4 as the most significantly upregulated secreted factor, both at mRNA and protein level. As a first step in identifying its function in HSCs I attempted to deplete expression via siRNA transfection. A combination of siRNAs specific to sFRP4 were transfected into primary rat aHSCs using Inteferin mediated transfection. However, despite multiple attempts using a variety of concentrations and conditions successful knockdown could not be achieved (Figure 3.9A).

Transfection via electroporation was also attempted but resulted in highly stressed cells and no significant knockdown (Figure 3.9B). siRNA mediated knockdown of sFRP4 was therefore not further pursued.

I next attempted overexpression of sFRP4 protein in LX-2s reasoning that this may be a more permanent source of stimulus. Overexpressing cells will consistently produce sFRP4 rather than undergoing a brief stimulus with recombinant protein. LX-2s transfected with 1 μ g of sFRP4 expression vector clearly upregulate sFRP4 protein expression, as demonstrated by Western Blot analysis (Figure 3.10A). The expression vector used promotes translation of sFRP4 proteins tagged with a FLAG epitope. This provides a simple way of demonstrating successful secretion of sFRP4 by performing FLAG IPs on medium collected from overexpressing cells. Figure 3.10B demonstrates substantial expression of FLAG in medium from sFRP4 overexpressing cells. Despite this increased sFRP4 protein expression and secretion, no significant alterations in fibrogenic gene expression or canonical Wnt targets were observed when analysed by qRT-PCR (Figure 3.10C).

I next questioned whether sFRP4 overexpression may provide a similar protective effect upon induction of apoptosis as suggested by sFRP1 recombinant protein treatment. I used similar experimental conditions: low serum medium (0.5%) combined with 50 μ M Cyclohexamide treatment. However sFRP4 overexpression resulted in no change in apoptotic response (Figure 3.11)

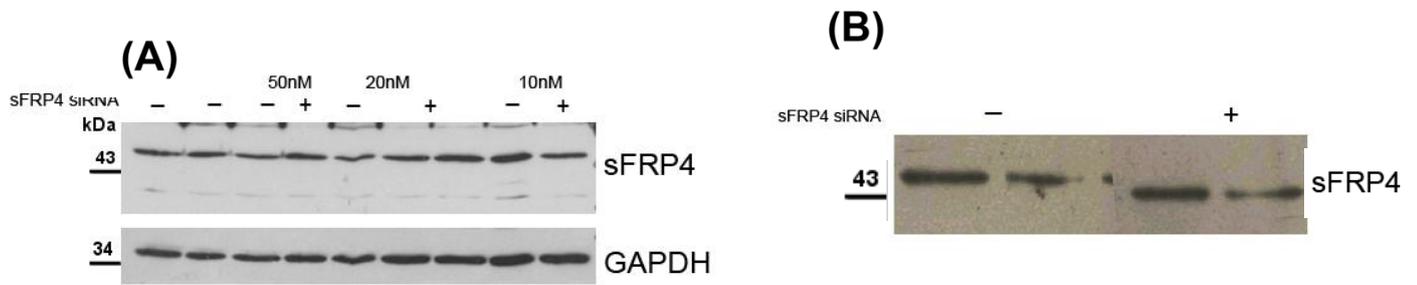


Figure 3.9 Attempted siRNA knockdown of sFRP4

(A) Western blot analysis of sFRP4 expression in primary rat aHSCs transfected with increasing concentrations of sFRP4 targeting siRNA via Interferin mediated transfection. GAPDH serves as a loading control. **(B)** Western blot analysis of sFRP4 expression in primary rat aHSCs transfected with sFRP4 targeting siRNA by electroporation.

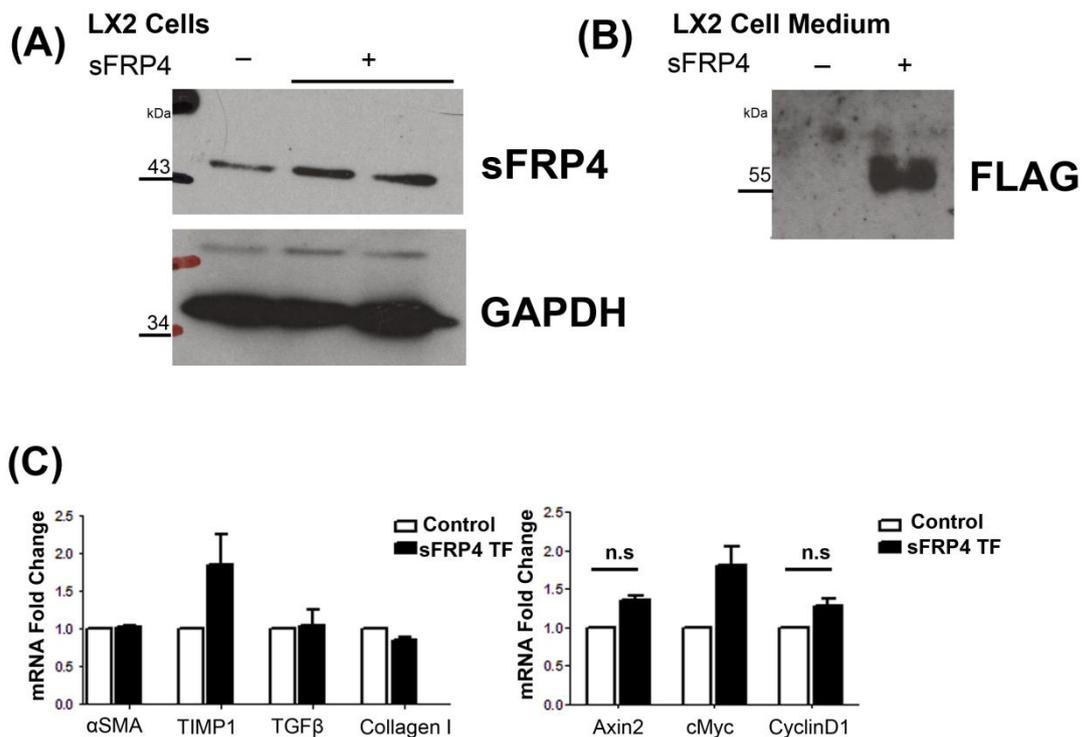


Figure 3.10 sFRP4 overexpression in LX-2 cell line has minimal effect.

(A) Protein expression of sFRP4 was analysed by Western blot in control (-) and sFRP4 overexpressing LX-2 cells (+). GAPDH serves as a loading control. **(B)** FLAG IP of medium from in control (-) and sFRP4 overexpressing LX-2 cells (+) analysed by Western Blot. mRNA levels of fibrogenic markers **(C)** or Wnt targets **(D)** quantified by qRT-PCR in sFRP4 overexpressing LX-2 cells compared to empty vector controls (n=3). qRT-PCR results are expressed as fold change normalised to control ± SEM

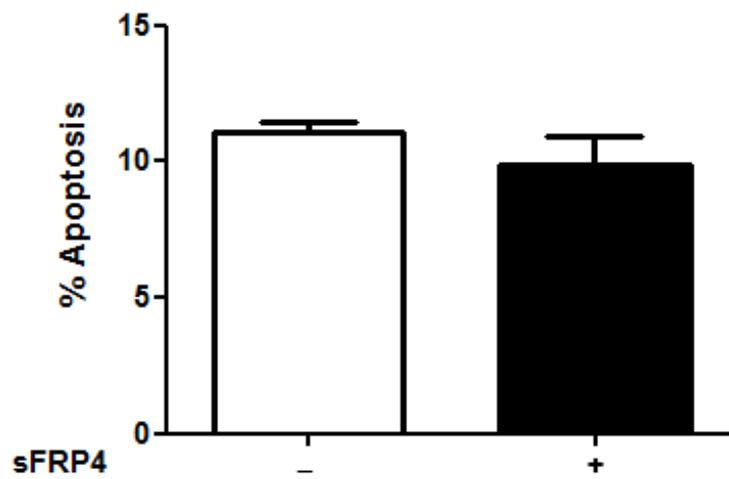


Figure 3.11 sFRP4 overexpression has minimal effect on apoptosis

% Apoptosis as assessed by Acridine Orange staining compared between control (-) and SFRP-4 overexpressing LX-2 cells (+) treated for 24hrs with 50µM cyclohexamide (CHX) under serum free conditions (n=3).

3.6 Discussion

Increased Wnt activity in myofibroblasts is implicated in numerous models of fibrosis, suggested to be one of the developmental signalling pathways reactivated upon injury (107, 111, 112). Early studies in HSCs also support a role for Wnt signalling in contributing to the transdifferentiation of qHSCs. Evidence from this Chapter agrees with all current literature, demonstrating increased Wnt activity and ligand expression in aHSCs as well as increased activity of the downstream marker Dvl . aHSCs express a variety of Wnt ligands, receptors and extracellular interactors, suggesting the potential for Wnt signalling to regulate activation of HSCs. The absence of Dvl expression and low levels of Wnts in quiescent HSCs suggests HSC derived Wnt signalling is minimal in healthy liver, as observed for other morphogens.

This Chapter offers several unexpected findings. Firstly, the predominantly non-canonical range of ligands expressed by LX-2 cells and HSCs. Figure 3.3 highlights the extremely limited expression of the canonical Wnts 3a and 10b when compared to the highly expressed Wnt5a. This appears to contrast with several previous studies, Cheng *et al.* for example purport that the canonical Wnt3a and Wnt10b are significant contributors to HSC activation(110). However this study also reported a greater upregulation of non-canonical ligands at the mRNA level: Wnt4 and Wnt5a increased by 12 fold rather than the 3 fold observed for Wnts 3a and 10b. Functional study of β -Catenin /Canonical Wnt signalling is facilitated by numerous techniques allowing direct read out of β -Catenin activity and a greater number of targeted reagents. This may explain why many of the studies pursued canonical signalling rather than the more convoluted non-canonical pathways. Despite this lack of canonical ligand expression, HSCs still express a variety of canonical associated receptors and therefore the potential to respond to canonical Wnts via paracrine signalling. Figure 3.2 indicates that peak expression of different Wnts occurs at different times. This may translate into different functions: Wnt4 expressed early and contributing to initiation of activation whereas the later expressed Wnt5a may enhance behaviour of activated myofibroblasts. Wnt expression during

development is subject to strict temporal regulation. During liver development for example, early time points are associated with high expression of Wnt3 and Wnt6 whereas later time points are associated with Wnt2 and Wnt5a(144). Temporal differences in Wnt expression during HSC activation is therefore a significant possibility. Ideally, analysis of protein expression levels would have been conducted to further confirm this. Unfortunately, due to poor cross reactivity of Wnt antibodies for rat protein samples this could not be achieved.

HSCs express eight out of ten Fzd receptors identified in mammals as well as a variety of non-canonical associated receptors. Another surprising finding was the significant downregulation in mRNA and protein expression for the majority of receptors. Visualisation of Fzd mRNA by RT-PCR still shows high expression of Fzd1, Fzd5 and Fzd8 however, despite the significant decrease when quantified by qRT-PCR. This suggests that Fzds are still present at a reduced level in aHSCs, As overactive Wnt signalling is associated with cancer and numerous other pathologies(67), this downregulation may be a regulatory mechanism, a way of modulating response to Wnts produced, preventing over stimulation. The non-canonical associated Fzd2 is the only Fzd which appears upregulated albeit not to a statistically significant extent. Downregulation of accompanying Fzds may be a mechanism of directing Wnt signalling through this receptor alone. Wnt5a is commonly attributed as a binding partner of Fzd2 and increased expression of these two pathway components again supports the non-canonical theme of Wnt signalling in HSCs.

Decreased Fzd expression occurs early in activation by day 3 as demonstrated by Figure 3.5, implying that high expression of Fzds is associated with quiescent cells only. This appears contradictory as minimal levels of Wnt ligands are present at this time. As mentioned in the Introduction, Fzd receptors are able to bind numerous ligands through their CRD domain, not merely restricted to Wnt ligands(84). Functional data outlining Wnt independent Fzd behaviour is limited but the high Fzd expression in quiescent, non-Wnt signalling cells may imply the possibility of Wnt independent function.

The increased expression of several extracellular interactors of Wnt is a novel finding, not previously reported in aHSCs. High expression of sFRPs and Dkks has been observed during early mouse development in whole liver tissue and is significantly reduced at later stages(144). Increased expression occurring upon HSC activation further supports the idea of reactivated developmental pathways contributing to fibrosis. How these proteins influence HSCs however cannot be confidently determined from this data.

One simple suggestion could be that increased expression serves as a regulatory mechanism, accompanying the decreased expression of Fzd receptors to prevent over- stimulation of Wnt. Hepatocellular carcinoma (HCC) is one cancer particularly associated with increased Wnt activity, with 25% of HCCs contain at least one activating β -Catenin mutation(145). Methylation mediated inactivation of sFRPs is a distinguishing feature of HCC cell lines and was detected in 42 – 63% of nineteen primary patient samples analysed, supports a role for sFRPs in liver having a Wnt/ β -catenin inhibitory role(146). Here, treatment of LX-2 cells with recombinant protein suggests potential Wnt enhancing roles for Dkk3 and inhibitory roles for sFRP1 and sFRP5. However as the endogenous concentrations of sFRPs and Dkk3 in primary HSCs are not known, it is difficult to determine whether the 5nM treatment outlined in Figure 3.7 is physiologically relevant.

Little effect on classic fibrogenic targets were observed upon sFRP and Dkk treatment and other aspects of the active myofibroblasts phenotype were examined instead. Alterations in apoptosis response were observed upon sFRP1 treatment with higher concentrations of recombinant sFRP1 (50nM) potentially conferring mild protection upon apoptotic stimulus with effect more pronounced with pre-treatment. This would correspond with the original role of sFRPs, identified as apoptosis related proteins(100). Clearance of HSCs by apoptosis is essential for fibrosis resolution and the survival of HSCs during fibrogenesis is dependent on matrix derived survival signals(59). sFRP secretion may be one of these factors promoting HSC survival.

Although the significant upregulation of expression with HSC transdifferentiation is exciting, further in-depth study of these proteins may be difficult. Interactions between individual members are not known and stimulus or depletion of one member could promote compensatory behaviour in the others(147). Moreover, there is possibility of functional redundancy between members and over expression or deletion of multiple members may be required before functional effect is observed. Another difficulty in studying extracellular Wnt modulators is that concentrations produced by HSCs are not known. Differences in mRNA expression are not necessarily translatable into the amounts of functional protein secreted. An extensive characterisation of sFRP and dkk concentration at each stage of a time course of HSC activation would be ideal before continuing study. This would allow identification of the most highly secreted proteins as well as giving indication as to their function: lower expression may suggest enhancement of Wnt signalling. Until the mechanics of these proteins have been more thoroughly elucidated, they remain a highly convoluted system to approach.

One of the aims of this Chapter was to identify a possible target for further study of the Wnt pathway in HSCs. Of the three Wnts expressed by aHSCs, Wnt5a is the most extensively studied and is a well-defined non-canonical ligand(103). It possess great potential for regulating behaviour of HSCs as it has been shown to regulate proliferation, migration, apoptosis resistance and contractility in fibroblasts from numerous sources(148). Wnt5a therefore appeared to be an attractive target for further study of the non-canonical pathway in HSCs. Despite no apparent canonical ligand expression detectable in HSCs, the possibility for canonical activity through paracrine stimulus cannot be ignored. Exploration of non-canonical and canonical behaviour in HSCs will be the next step to build on data presented in this Chapter.

3.7 Conclusions

In summary, this Chapter demonstrates:

- Increased Wnt activity accompanying HSC activation
- A non-canonical ligand expression profile in aHSCs
- Decreased Fzd receptor expression in aHSCs
- Increased expression of sFRP1, sFRP2, sFRP4 and Dkk3 in aHSCs

Chapter 4: Canonical vs Non-Canonical Signalling

4.1 Introduction

Chapter 3 identified multiple ligands, receptors and extracellular interactors present in HSCs, depicting a complex Wnt environment. This Chapter aims to determine how Wnts function in HSCs by exploring markers of canonical or non-canonical activity. As aHSCs express non-canonical ligands and increase expression of the non-canonical receptor Fzd2, signalling through non-canonical effectors is the predicted mechanism. Several canonical associated receptors are also expressed, requiring investigation of the potential for canonical signalling also.

4.2 HSCs express components of the canonical pathway

As mentioned in the previous chapters, canonical signalling is the most extensively described branch of the Wnt pathway. The majority of studies correlating Wnt and fibrosis focus on fibroblasts upregulating active β -Catenin. This chapter therefore begins with profiling the canonical pathway in HSCs.

HSCs express the necessary components of the canonical pathway as demonstrated by RT-PCR, with expression of destruction complex components (GSK3 β , Axin and APC), canonical co-receptors (Lrp6) and downstream targets (Axin2). (Figure 4.1A). Differential expression upon activation was then determined by qRT-PCR. mRNA expression of β -Catenin is significantly upregulated by approximately 3.5 fold. Surprisingly, this is accompanied by significantly decreased expression of its downstream transcriptional target Axin2. (Figure 4.1B) Changes in co-receptor and destruction complex components were limited. However, as these are regulated by post-translational modifications changes may not be reflected at the mRNA level. Across a time course of activation a substantial

increase in protein expression of β -Catenin and downstream TCF/LEF targets can be visualised by Western Blot (Figure 4.1C).

Despite limited canonical ligand expression, HSCs clearly express major components of the canonical pathway along with canonical associated Fzd receptors as demonstrated by Figure 3.4. In order to verify whether HSCs can respond to exogenously produced Wnts, I incubated LX-2 cells with conditioned medium taken from Wnt3a and 10b overexpressing LX-2 cells. A three hour treatment significantly increased expression of β -Catenin and downstream target TCF4, suggesting canonical responsiveness in HSCs (Figure 4.2).

4.3 Canonical pathway appears inactive in HSCs

However the presence of β -Catenin does not equal active canonical signalling. β -Catenin must translocate to the nucleus and bind with target transcription factors in order to stimulate upregulation of target genes(12). The best known β -Catenin targets are members of the TCF/LEF transcription factor family(73). I used transfection of the TOPFLASH luciferase reporter as a marker of TCF/LEF dependent transcriptional activity. LX-2 cells were transfected with the TOPFLASH expression vector and either Wnt3a or Wnt10b as a canonical stimulus. A *Renilla* luciferase expression vector was also co-transfected as a control of transfection efficiency and data are presented as a ratio of firefly to *Renilla* luciferase activity. Endogenous luciferase activity in LX-2s was limited and did not increase upon with canonical Wnt stimulation (Figure4.3A). Wnt overexpression in HEK293 cells served as a positive control with a significant increase in TOPFLASH activity in comparison to LX-2s. Furthermore, transcript levels in LX-2 cells of Axin2, a classic β -Catenin target(117), were unchanged upon Wnt overexpression in LX-2 cells but significantly increased in Wnt overexpressing HEK293 cells (Figure4.3B). LX-2s therefore appear unresponsive to canonical Wnt stimulus.

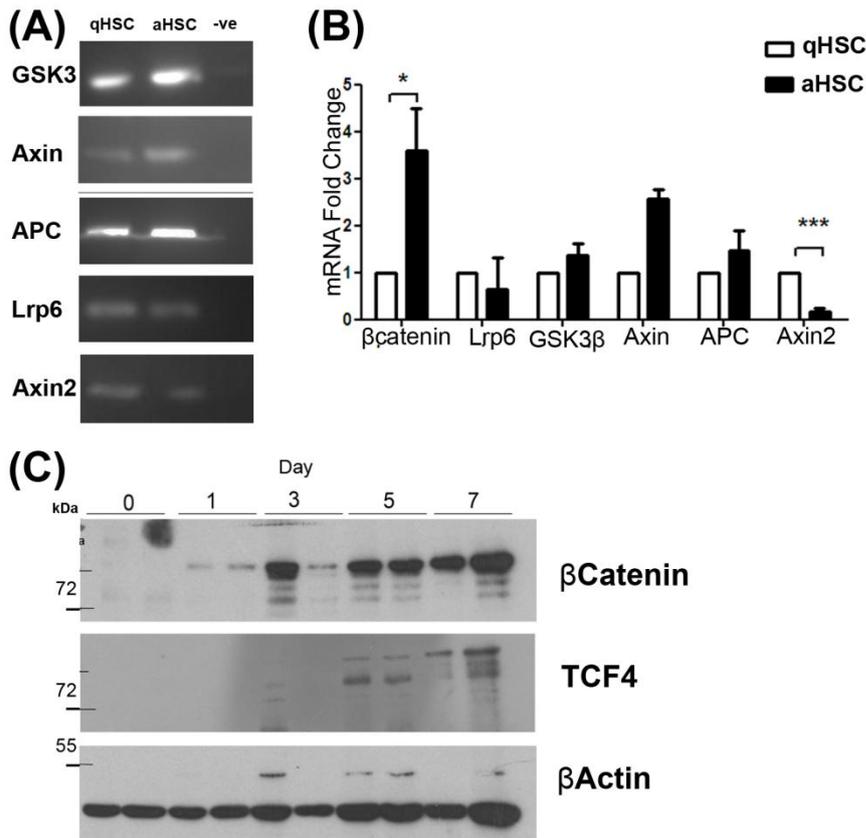


Figure 4. 1 Canonical pathway expression in HSCs.

(A) mRNA expression of canonical pathway components is detectable in both rat in quiescent HSCs (qHSC) and HSCs activated by seven days in culture (aHSCs) by RT-PCR. **(B)** Canonical pathway mRNA levels were quantified by qRT-PCR in qHSC and aHSC (n=4) **(C)** Western Blot for protein expression of β-Catenin and its target TCF4 across a time course of rat HSC culture activation (7 days in culture). βActin serves as a loading control. qRT-PCR results are expressed as fold change normalised to control ± SEM. *p<0.05 Student's *t* Test (unpaired).

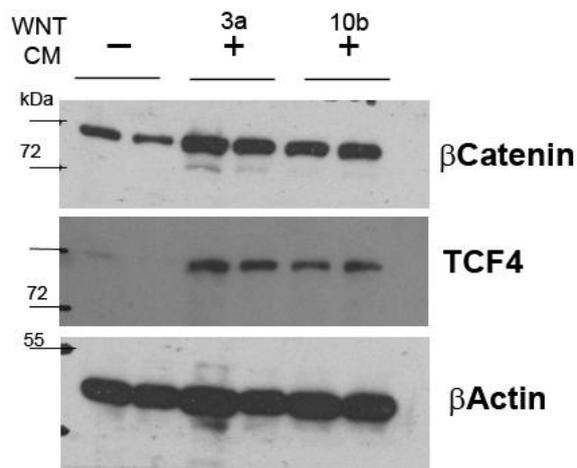


Figure 4. 2 HSCs upregulate β-Catenin in response to stimulus.

Western Blot for protein expression of β-Catenin and its target TCF4 in LX-2 cells treated for three hours with conditioned medium taken from LX-2 cells overexpressing canonical Wnt 3a (3a+), Wnt10b (10b+) or an empty control expression vector (-). GAPDH serves as a loading control

I next attempted a more direct source of stimulus and overexpressed a constitutively active form of β -Catenin, with a serine to alanine mutation at the serine 37 (Ser37) position, a GSK3 β phosphorylation site. This mutant will be resistant to GSK3 β mediated phosphorylation and subsequent degradation and will therefore always be able to localise to the nucleus and to bind TCF/LEF targets(134). TOPFLASH luciferase activity in β -Catenin overexpressing LX-2s and rat HSCs remained unchanged while HEK293 and control primary cells (rat dermal fibroblasts) significantly increased luciferase activity. (Figure 4.4A) Ser37 β -Catenin overexpression induces a robust response in HEK293s, increasing luciferase activity over 20 fold, demonstrating its efficacy as a method of stimulus. Overexpression of mutant β -Catenin increases active β -Catenin protein levels in LX-2s to a similar extent of that in HEK 293s when visualised by Western Blot, demonstrating no difficulties in LX-2s overexpressing active β -Catenin (Figure 4.4B). Transcript levels of the β -Catenin target Axin2 was again unchanged in LX-2s while substantially increased in HEK293s (Figure 4.4C). Finally I assessed the effect of canonical stimulus on the expression of fibrogenic markers such as alpha smooth muscle actin (α -Sma) and type I Collagen. However, neither Wnt10b nor active β -Catenin in LX-2s generated significant differences. (Figure 4.4D). From this, aHSCs appear again unresponsive to canonical Wnt stimulus.

One study suggests that canonical signalling may instead be active in maintaining quiescence and links the stem cell like phenotype of quiescent HSCs to β -Catenin's role in regulating pluripotency and self-renewal(122, 133). I explored this further by attempting a TOPFLASH assay in early rat HSCs, at day 2 of an *in vitro* time course. No TOPFLASH luciferase activity could be detected. However, Renilla luciferase activity acting as a control of transfection efficiency was measurable (approximately 20 relative light units (RLU)) indicating that transfection of early HSCs was low but still successful. Protein expression of β -Catenin is minimal in quiescent day 0 HSCs, only detectable at day 1 and active, dephosphorylated β -Catenin cannot be detected at either of these early time points (Figure4.5). These data strongly suggest that β -Catenin does not play a role in maintaining quiescence

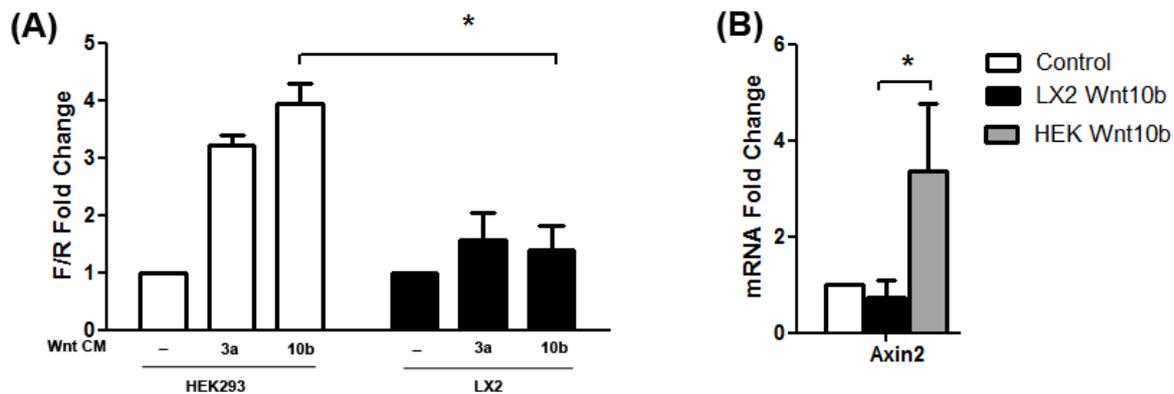


Figure 4.3 Canonical stimulus of HSCs does not result in a transcriptional response.

(A) LX-2 and HEK293 cells were transfected with a TOPFLASH luciferase expression vector and co-transfected with Wnt 3a or 10b as a canonical stimulus. Cotransfection of Renilla luciferase served as a positive control. Data presented as fold change of firefly to *renilla* ratio (F/R) \pm SEM. (n=2) **(B)** Axin2 mRNA expression levels Wnt10b overexpressing LX-2 or HEK293 cells compared by qRT-PCR (n=3) qRT-PCR results are expressed as fold change normalised to control \pm SEM. *p<0.05 Student's *t* Test

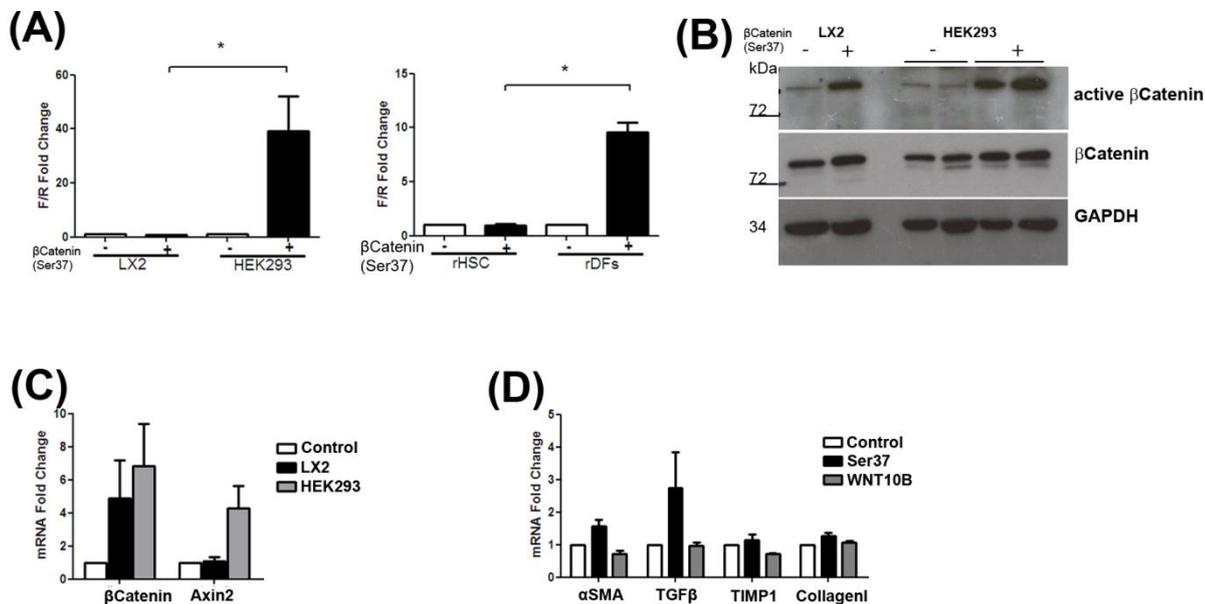


Figure 4.4 βCatenin stimulus of HSCs does not result in a transcriptional response.

(A) LX-2 and HEK293 cells or rat HSCs and dermal fibroblasts (rDFs) were transfected with a constitutively active βCatenin (Ser37) or empty control expression vector. Cotransfection of Renilla luciferase served as a control of transfection efficiency. Data presented as fold change of firefly to renilla ratio (F/R) \pm SEM. **(B)** Expression of Active and total β-Catenin analysed by Western Blot in protein lysates from control or βCatenin(Ser37) overexpressing LX-2 and HEK293 cells. GAPDH serves as a loading control. **(C)** Axin2 mRNA expression levels of Ser37 βCatenin overexpressing LX-2 or HEK293 cells compared by qRT-PCR (n=3). **(D)** mRNA expression levels of fibrogenic makers compared by qRT-PCR in βCatenin (Ser37) or Wnt10b overexpressing LX-2 cells. qRT-PCR results are expressed as fold change normalised to control \pm SEM. *p<0.05 Student's *t* Test (unpaired).

4.4 β -Catenin is correctly localised to the nucleus of HSCs

From these findings it appears that canonical signalling is deficient in LX-2s and primary HSCs even under conditions able to effectively stimulate a canonical response in other cell types. The complexity of the Wnt pathway gives rise to many potential points at which a defect could occur. I first addressed whether active β -Catenin was able to correctly localise in HSCs.

In order for nuclear localisation to occur, the phosphorylation of β -Catenin by the kinase GSK3 β must be disrupted. In the absence of Wnt signalling β -Catenin is phosphorylated by the destruction complex and degraded. Wnt signalling promotes destabilisation of the destruction complex, preventing phosphorylation and enabling translocation of β -Catenin to the nucleus(12). I first assessed whether HSCs may have a deficiency in GSK3 β inhibition. Day7 HSCs were treated with CT99021, a commercially available inhibitor of GSK3 β , reasoning that increasing inhibition of GSK3 β would yield a larger pool of non-phosphorylated, potentially active β -Catenin. Western Blots for active and total β -Catenin demonstrate several points. Firstly that again no active or total β -Catenin can be detected in Day 0 quiescent cells. By Day 7 however an upregulation of both total and active β -Catenin is observed (Figure4.6A). This suggests that there is a potentially active pool of non phosphorylated β -Catenin present in HSCs. Treatment of Day7 aHSCs with CT99021 did not increase mRNA expression of fibrogenic genes or β -Catenin target Axin2 (Figure4.6B). This suggests that the canonical pathway deficiency is not at the point of destruction complex inhibition.

β -Catenin binds to TCF/LEF transcription factors confined to the nucleus. Correct nuclear localisation of β -Catenin is therefore critical to its canonical Wnt signalling function. I separated protein lysates from LX-2 cells into cytoplasmic and nuclear fractions and assessed the expression and location of β -Catenin by Western Blot. p84 and α Tubulin were used as markers of nuclear and cytoplasmic purity respectively. LX-2s express high levels of β -Catenin, located both in the cytoplasm and in the nucleus. Furthermore, expression in both compartments is increased upon overexpression of β -Catenin (Figure 4.7) From this HSCs appear to correctly target β -Catenin to the nucleus.

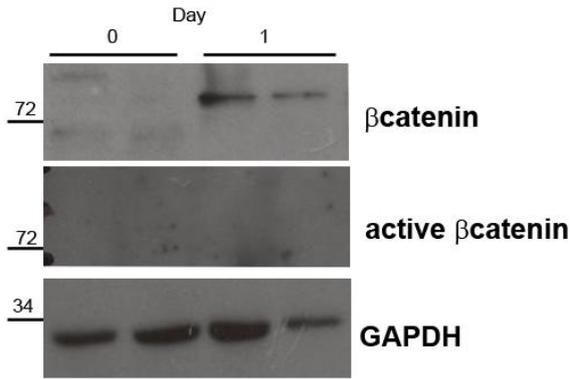


Figure 4. 5 Active β -Catenin is not detectable in qHSCs

Protein lysates from freshly isolated HSCs (Day 0) and after 24 hours of culture (Day 1) immunoblotted for active and total β -Catenin expression. Total β -Catenin only expressed after 24 hours of culture. GAPDH serves as a loading control.

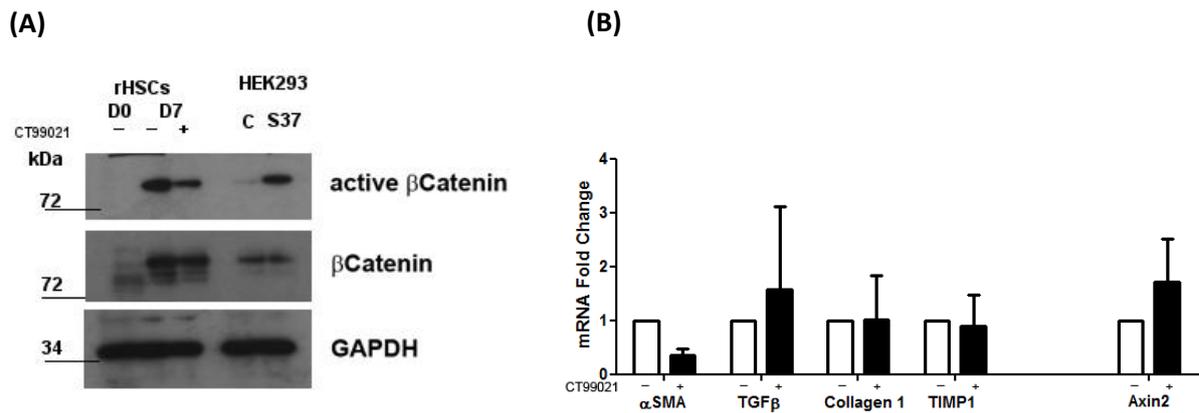


Figure 4. 6 GSK3 β inhibition does not stimulate canonical response

(A) aHSCs (seven days in culture (D7)) were treated with 2 μ M of CT99021. Active and total β -Catenin expression was compared by Western Blot between quiescent (D0) and active (D7) HSCs treated with either vehicle control (-) or 2 μ M CT99021 (+) for 24 hours. HEK293 cells overexpressing constitutively active β -Catenin (S37) were used as a positive control for active β -Catenin expression. GAPDH serves as a loading control. **(B)** mRNA levels of fibrogenic markers and the β -Catenin target Axin2 were compared by qRT-PCR between vehicle control (DMSO) or CT99021 treated aHSCs (24hrs) (n=2). qRT-PCR results are expressed as fold change normalised to control \pm SEM.

4.5 Nuclear Interactors of β -Catenin

Once in the nucleus, the stability of β -Catenin and its ability to bind TCF/LEF targets depends on interaction with a multitude of other proteins. I next hypothesised that HSCs may have a deficiency in a key nuclear interactor of β -Catenin and thus impair binding to TCF/LEF targets.

Pygopus and BCL9 are two nuclear proteins implicated in a 'scaffolding role', required to tether β -Catenin to TCF family members (149-151). Deficiencies in these scaffolding proteins can lead to impaired signalling and they are considered essential for correct canonical function(152, 153). Expression of their different forms (Pyg1, Pyg2, BCL9, BCL92) was analysed by RT-PCR in LX-2 cells with HEK293 cells again used as a positive control. However, LX-2 cells displayed no obvious deficiency in mRNA expression (Figure 4.8A).

Repressors of β -Catenin activity are also active in the nucleus. Chibby is one well documented such repressor, able to bind β -Catenin and compete with TCF/LEF targets(154). RT-PCR analysis suggests an increased amount of Chibby expression by LX-2s, although this could not be verified by qRT-PCR (Figure4.8B). Chibby expression over a time course of rHSC activation was next established, hypothesising that an increase in expression may occur to counteract increased β -Catenin (Figure 4.8C). However, again mRNA expression decreases with activation. This expression data suggests that there are no obvious deficiencies in mRNA expression of several key β -Catenin nuclear interactors. Ideally protein expression of Pyg, BCL9 and Chibby would also have been assessed to strengthen these findings. However due to poor quality of antibodies this could not be achieved.

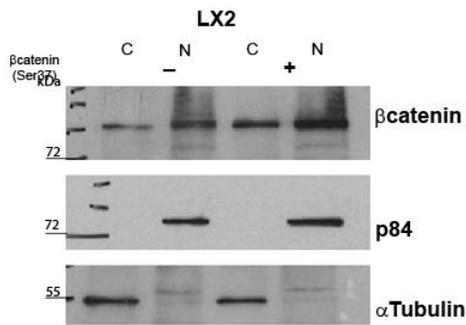


Figure 4.7 βCatenin is targeted to the nucleus of HSCs

Protein lysates from LX-2s transfected with constitutively active mutant β-Catenin (Ser37 +) or empty control expression vectors (-) separated into cytoplasmic (C) or nuclear (N) fractions. β-Catenin expression detected in both fractions by Western Blot. P84 and αTubulin serve as controls of nuclear and cytoplasmic purity respectively.

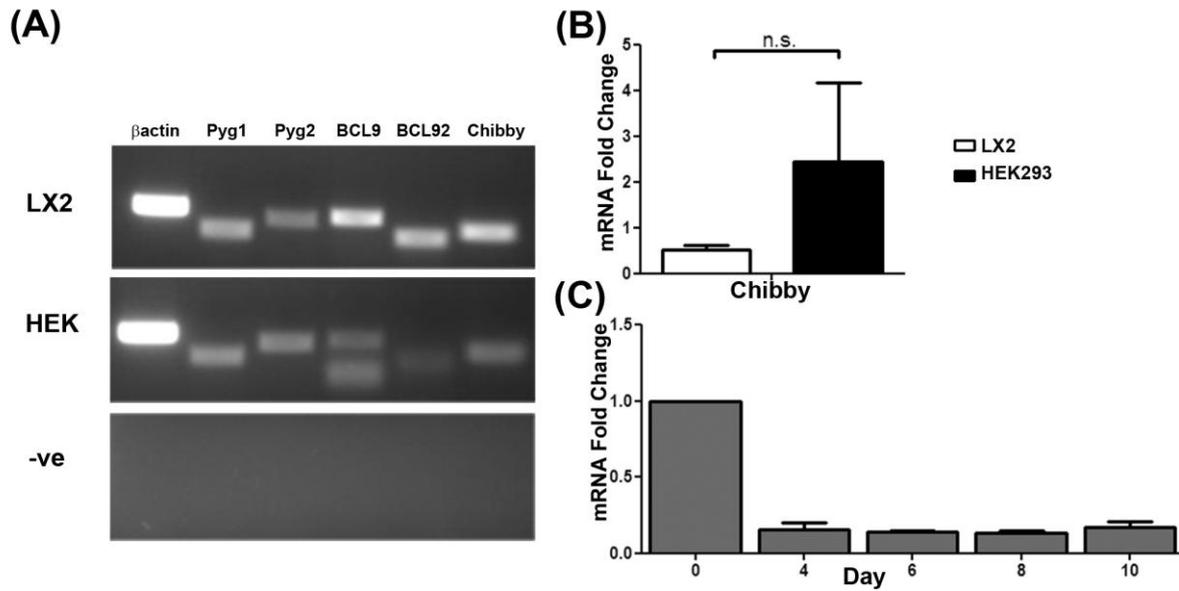


Figure 4.8 Expression of key β-Catenin interactors detectable in HSCs

(A) mRNA expression of key β-catenin interacting proteins detectable by RT-PCR in both LX-2 and HEK293 cells. (B) mRNA expression levels of β-catenin inhibitor Chibby compared between LX-2 and HEK293 cells (n=4). (C) mRNA expression of Chibby analysed across an in vitro activation time course of rat HSCs (n=3). qRT-PCR results are expressed as fold change normalised to control ± SEM.

4.6 HSCs appear deficient in TCF/LEF expression.

β -Catenin's direct targets are members of the TCF/LEF transcription factor family comprising TCF1,3,4 and LEF1. β -Catenin triggers their activation by displacement of the repressor Groucho/TLE, forming an activation complex, enabling binding to target genes. The literature is highly contradictory as to the exact roles of TCF/LEF members. TCF1, 4 and LEF1 are generally considered to activate β -Catenin target genes(73) whereas TCF3 is suggested to have a repressive role(155-157). I next investigated whether HSCs may have a deficiency in expression of TCF/LEF member. Firstly, I analysed expression of TCF1, TCF3,TCF4 and LEF1 in LX-2s by Western Blot using HEK293s as a control. LX-2s express substantially less TCF1, TCF4 and LEF1 compared with HEK293s along with similar levels of TCF3. (Figure 4.9)

I next aimed to increase the ability of LX-2 cells to respond to canonical stimulus by increasing expression of the TCF/LEF1 family. LEF1 appears to be substantially decreased in LX-2 cells and was overexpressed in LX-2 cells either singly or in conjunction with active β -Catenin. However, TOPFLASH activity was not stimulated instead appearing to decrease upon LEF1 overexpression (Figure 4.10). Furthermore, there was minimal effect on fibrogenic markers. This appears to be the first source of deficiency in the canonical pathway identified in HSCs. However, as simply increasing expression of TCF/LEF family members was ineffective there are likely more factors influencing canonical activity.

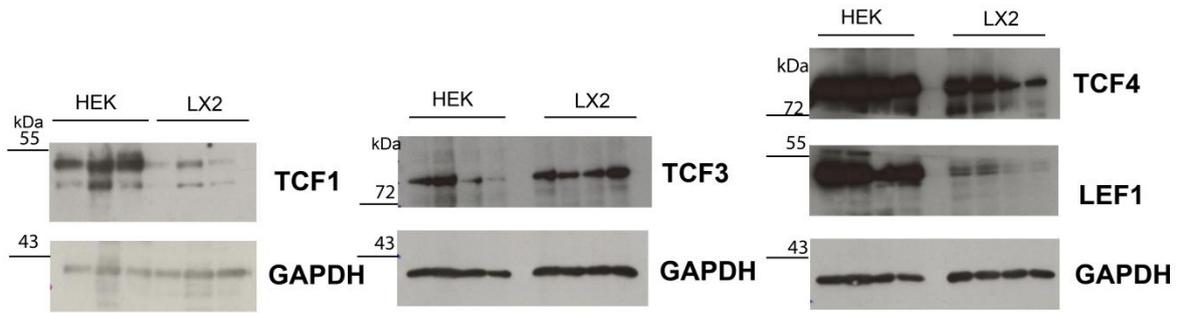


Figure 4. 9 Expression of TCF1, TCF4 and LEF1 decreased in LX-2 cells

Western Blot analysis of TCF1, TCF3, TCF4 and LEF1 expression compared between LX-2 and HEK293 cells. Decreased TCF1, TCF4 and LEF1 expression apparent in LX-2s. GAPDH serves as a loading control.

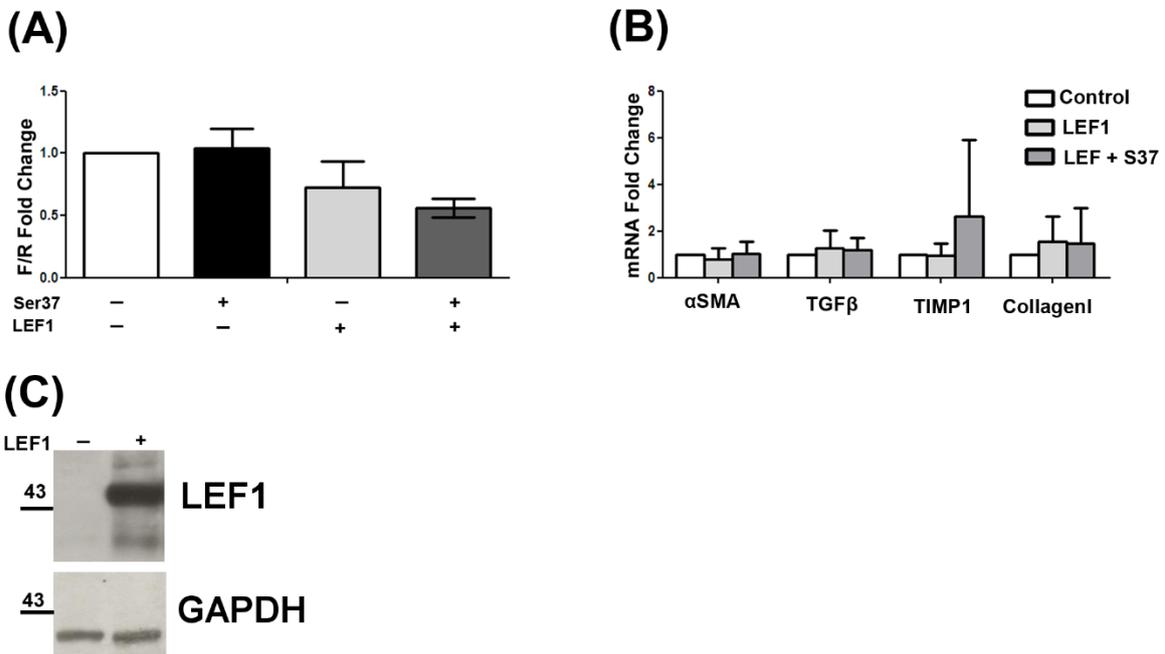


Figure 4. 10 LEF1 overexpression does not stimulate a canonical response

(A) LX-2 and HEK293 cells were transfected with a TOPFLASH luciferase expression vector and cotransfected with either constitutively active β -catenin (Ser37), LEF1 or empty control expression vector. Cotransfection of *Renilla* luciferase served as a control of transfection efficiency. Data presented as fold change of firefly to *renilla* ratio (F/R) \pm SEM. **(B)** mRNA expression of fibrogenic markers analysed in of LX-2s overexpressing LEF1 either singly or in conjunction with β -Catenin (Ser37) (n=3). qRT-PCR results are expressed as fold change normalised to control \pm SEM. **(C)** Western Blot analysis of LEF1 expression in control and LEF1 overexpressing LX2s. GAPDH serves as a loading control.

4.7 Wnt Interactors and β -Catenin

In Chapter 3 I demonstrated that aHSCs significantly upregulate expression of Wnt interactors such as sFRP members and Dkk3 however a specific function was not isolated. How they influence Wnt signalling has not yet been resolved and appears to be highly cell specific. Several sources suggest roles in inhibition of β -Catenin/TCF activity (158, 159) and the ability to inhibit canonical Wnt signalling even in the presence of activating β -Catenin mutations(160). Furthermore, epigenetic inactivation of sFRPs is a frequent occurrence in numerous cancers (146, 161, 162). I next explored the possibility that HSCs may inhibit canonical activity through production of these secreted inhibitors.

For this, I used HEK293 cells overexpressing active β -Catenin as a positive control for canonical activity. Medium taken from LX-2 cells and incubated with HEK293 cells for six hours appeared to decrease TOPFLASH activity approximately 30% in comparison to medium taken from HEK293 cells (Figure 4.11). This suggests the possibility that LX-2s may secrete factors able to negatively influence canonical signalling. I next tested whether sFRP overexpression could have a similar negative influence on canonical signalling. I co-transfected β -Catenin overexpressing HEK293 cells with a sFRP4 expression vector and again observed a similar reduction in TOPFLASH activity. This could be a preliminary suggestion as to how HSCs prevent canonical signalling, despite the presence of all necessary downstream components.

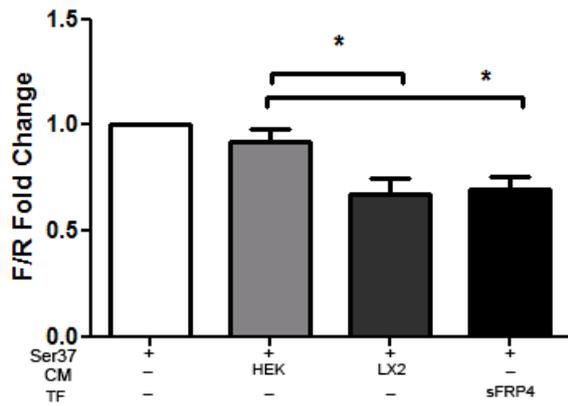


Figure 4.11 sFRP4 and canonical inhibition.

β -Catenin overexpressing HEK293 cells co-transfected with TOPFLASH expression vector were treated with HEK293 cell conditioned medium, LX-2 cell conditioned medium or co transfection with sFRP4. *Renilla* luciferase served as a positive control. Data presented as fold change of firefly to *renilla* ratio (F/R) \pm SEM. Significant decrease in TOPFLASH activity observed upon LX-2 conditioned medium treatment or sFRP4 co transfection. * $p < 0.05$ Student's *t* Test (unpaired)

4.8 Non Canonical Activity in HSCs

So far this chapter has demonstrated that HSCs show limited endogenous canonical activity, despite abundant expression of β -Catenin and canonical components. Furthermore, they appear resistant to canonical stimulus even under conditions that provoke a response in other cell types. Reasons for this remain unclear although I have been able to identify a decreased expression of β -Catenin target and an increased expression of potential Wnt inhibitors in HSCs.

As HSCs express a variety of non-canonical ligands I next turned to establishing the presence of non canonical signalling. Two major effectors of the non-canonical branches of Wnt signalling are JNK as part of the PCP pathway and CamKii as part of the Wnt/ Ca^{2+} pathway. JNK mediated activation of AP1 components is well established in contributing to HSC activation(163) and increased calcium signalling is important in mediating increasing contractility of aHSC(164). Non-canonical effectors therefore have high potential for regulating HSC behaviour. I tested response of these effects upon Wnt stimulation in HSCs.

Wnt conditioned medium stimulation in primary rat HSCs and overexpression of Wnt in LX-2 cells both stimulate a marked increase in JNK phosphorylation (Figure 4.12A and C respectively), This suggests the potential for Wnt to activate JNK signalling in HSCs. Expression of the Ca^{2+} pathway mediator CamKii was also elevated upon Wnt stimulus (Figures 4.12B and D). Response of non-canonical effectors and the predominantly non-canonical Wnt ligand expression profile suggest that non-canonical signalling is the main branch active in aHSCs.

As non-canonical Wnt activity has been shown to inhibit canonical/ β -Catenin activity(139, 165, 166) I again aimed to increase the ability of LX-2 cells to respond to canonical stimulus through non-canonical inhibition. LX-2 cells overexpressing active β -Catenin were treated with either SP600125 to inhibit JNK phosphorylation or Cyclosporin A, an inhibitor of calcineurin, a downstream effector of CamKii. However, again no upregulation in TOPFLASH activity was observed with inhibitor treatment (Figure 4.13)

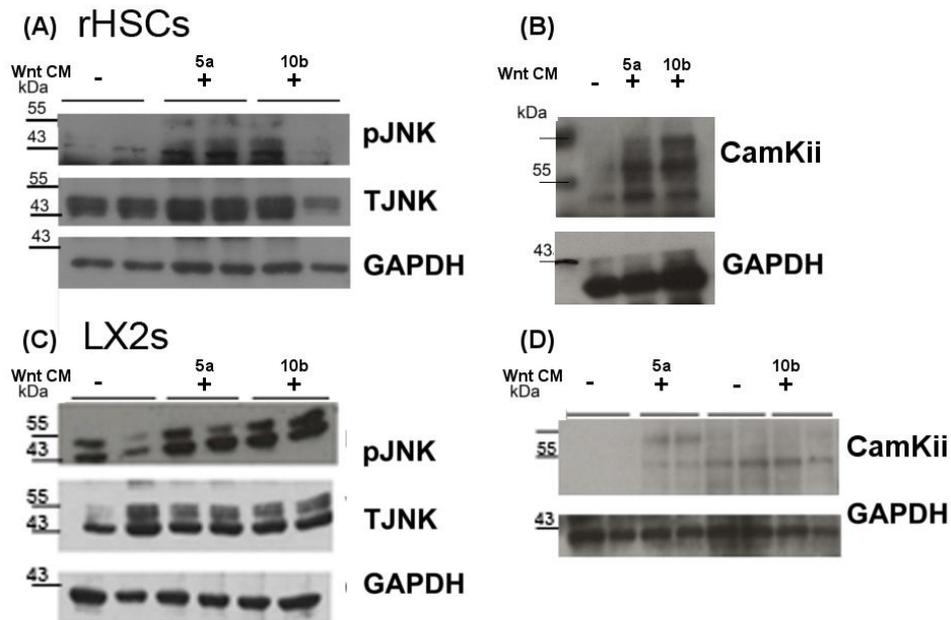


Figure 4.12 Non-Canonical Effectors in HSCs

rHSCs (A) and (B) or LX-2s (C) and (D) were stimulated by 24 hours Wnt conditioned medium (CM) or 48 hours Wnt Transfection respectively. Protein lysates were immunoblotted for expression of Phospho-JNK, (PJNK), total JNK (TJNK) and CamKii. GAPDH serves as a loading control.

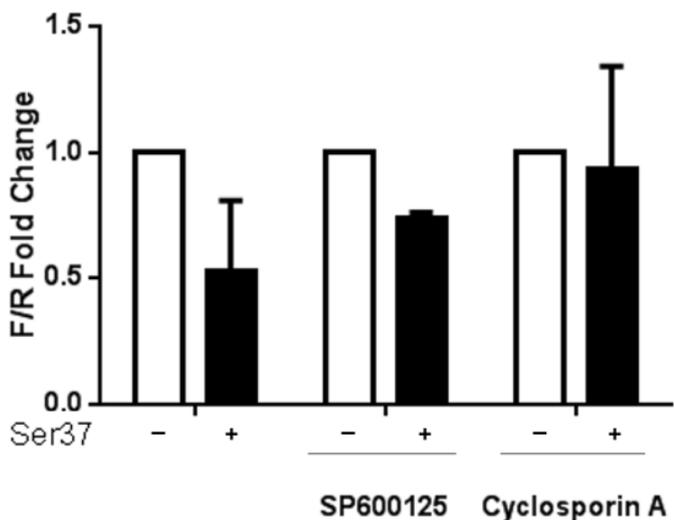


Figure 4.13 Non Canonical Inhibition in HSC

LX-2 cells were transfected with a TOPFLASH luciferase expression vector and cotransfected with either Ser37 β -Catenin mutant or empty control expression vectors. *Renilla* luciferase served as a control of transfection efficiency. Data presented as fold change of firefly to renilla ratio (F/R) \pm SEM. Cells were treated with SP600125 (50 μ M) or Cyclosporin A (50 μ M) for 24 hours.

The transcription factor family associated with calcium signalling is the NFAT family (NFAT 1 -4)(167). To establish whether activity of non-canonical associated transcription factors was present, rat HSCs and LX-2s were transfected with a total NFAT luciferase reporter. Endogenous NFAT activity was far greater in LX-2s and rat HSCs than TCF transcriptional activity (Figure 4.14A) RT-PCR analysis demonstrates expression of all four NFAT transcription factors in rat HSCs, with NFAT3 particularly abundant (Figure 4.14B). However, NFAT3 does not appear to be differentially expressed upon HSC activation (Figure 4.14C) and non-canonical stimulus through Wnt5a overexpression does not increase NFAT luciferase activity in LX-2s (Figure 4.14D). This suggests that high levels of NFAT activity in HSCs may not be attributed to increased Wnt signalling. One of the significant issues of working with the non-canonical pathway is that there is no functional read out specific to Wnt signalling, no non-canonical version of the TOPFLASH assay for example. Numerous factors have the potential to influence JNK and CamKii and attributing alterations in function directly to Wnt signalling can be difficult.

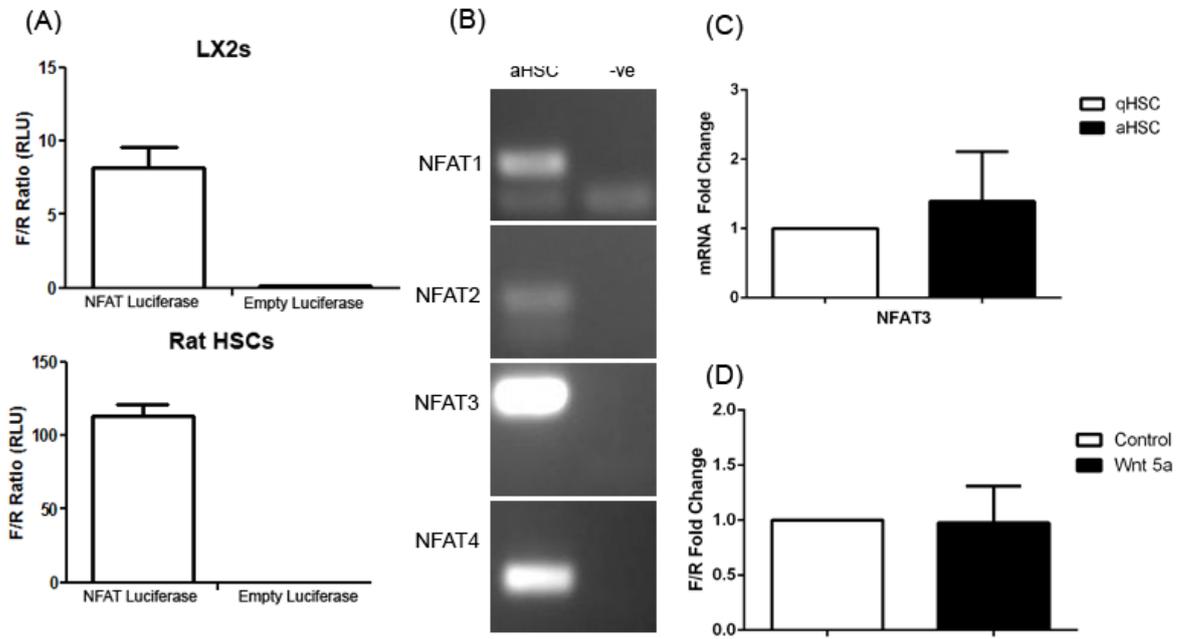


Figure 4.14 NFAT Activity in LX-2s and HSCs

(A) Rat HSCs and LX-2s were transfected with a total NFAT luciferase reporter or an control promoterless luciferase vector for 48 hours. Renilla luciferase served as a positive control. Data presented as firefly to renilla ratio (F/R) in relative light units (RLU). **(B)** mRNA Expression levels of NFAT family members detected in aHSCs by RT-PCR, with water only (-ve) serving as a negative control. **(C)** mRNA expression levels of NFAT3 compared between three separate preparations of quiescent (qHSC) and active (aHSC) rat HSCs. No significant difference observed. **(D)** LX-2s were transfected with a total NFAT luciferase reporter and co-transfected with either a Wnt5a or empty control expression vector. *Renilla* luciferase served as a control of transfection efficiency. Data presented as fold change of firefly to *renilla* ratio (F/R) \pm SEM.

4.8 Discussion

This Chapter aimed to explore the activity of canonical and non-canonical effectors in HSCs, attempting to determine which branch was functional. Canonical activity has been demonstrated as pro-fibrogenic in HSCs and in fibroblasts across numerous fibrosis models. The main finding from this Chapter contradicts this identifying an apparent defect in canonical β -Catenin/TCF dependent signalling.

HSCs express all necessary components of canonical pathway and correctly localise β -Catenin upon stimulus. If aHSCs are regulated by activity of Wnt3a and Wnt10b, high levels of canonical activity would be expected even without stimulus. Under endogenous conditions however, no transcriptional activity could be measured either through TOPFLASH activity or analysis of downstream transcriptional targets. Stimulus through either Wnt overexpression or active mutant β -Catenin overexpression successfully elicited response in the HEK293 cells used as a positive control. HSCs and LX-2 cells however remained unresponsive. As β -Catenin is correctly localised, the defect is likely to be at the nuclear level: either β -Catenin unable to associate with its TCF/LEF targets or TCF/LEF transcription factors unable to bind to their target genes.

From this Chapter, two possible causes of transcriptional defect are highlighted. Firstly, the reduced protein expression of canonical TCF/LEF targets observed in LX-2 cells in comparison to HEK293 controls. Target availability for β -Catenin would therefore be reduced, although this is more likely to decrease in canonical signalling rather than cause a total absence. Furthermore, overexpression of LEF1 did not promote TOPFLASH activity or expression of target genes, despite significant increases in protein expression. Therefore a simple defect in protein expression is unlikely to be the major cause.

LX-2 cells do not appear to uniformly express TCF/LEF members. This appears similar to problems encountered in Chapter 3, multiple TCF/LEF family members with interactions and functional redundancy levels not fully outlined. LEF1 and TCF1 expression is markedly reduced. In contrast TCF3 expression is marginally increased in LX-2s in comparison to HEK293s. TCF1, TCF4 and LEF1 members behave similarly upon β -Catenin binding with TLE/Groucho repressors displaced and transcriptional activating complexes formed(73). TCF3 however is considered a divergent member of the family, associated with a repressive role. Wu *et al* (157) demonstrate that endogenous levels of LEF1 activity are reduced upon transgenic overexpression of TCF3 and that β -Catenin promotes LEF1 activity through inhibition of TCF3 repression. β -Catenin is thought to relieve the repressive effect through binding and targeting TCF3 for degradation(156). In mouse models, early depletion of TCF3 disrupted gastrulation and caused defects which phenocopied models of canonical Wnt overactivation (168). Here, depletion of TCF3 in LX-2 and removing its possible repressive effect could be a method of stimulating canonical activity.

Establishing functional β -Catenin-TCF/LEF binding will be essential in clarifying the canonical defect. High TCF3 protein levels in LX-2s could indicate a lack of β -Catenin mediated degradation and therefore inefficient β -Catenin/TCF binding. However attempted Immunoprecipitation (IP) assays to determine binding between β -Catenin and TCF/LEFS as well as attempted Chromatin IP (ChIP) assay to determine binding between TCF transcription factors and target genes were unsuccessful. Further optimisation of conditions to provide conclusive data about β -Catenin, TCF/LEF and target gene binding interactions will be invaluable in continuing study.

Inhibition of β -catenin by sFRPs is another possibility for canonical defect as sFRPs are able to decrease canonical signalling even in the presence of activating β -Catenin mutations(160).

Additionally, in breast cancer cells, TCF/LEF activity was increased only when siRNA depletion of sFRP1 accompanied mutant β -catenin expression(162). Here, sFRP4 overexpression in HEK293s

stimulated with β -Catenin significantly decreased TOPFLASH activity by 30%. This could serve as an explanation for the greatly increased expression of sFRPs upon HSC activation: they are factors secreted to repress canonical signalling rather than directly influencing the fibrogenic phenotype. This is a very preliminary finding and will require extensive investigation into potential mechanisms.

The possibility that HSCs have a particular nuclear interactor involved in β -Catenin repression is an attractive one. However, β -Catenin has known ability to interact with over 80 different proteins in the nucleus. Expression profiles of nuclear β -Catenin interactors varies widely with cell type and their function as repressors or interactors can be highly context specific. Furthermore, interactions between different nuclear components are not well known. Identifying a HSC specific β -Catenin repressor is therefore likely to be an arduous task. One promising candidate is SOX9, a member of the *SRY*-related high-mobility-group box (SOX) family of transcription factors. SOX9 is able to compete with TCF/LEF targets for β -Catenin binding and promote its degradation(169, 170). SOX9 is highly expressed in aHSCs with its nuclear localisation demonstrated by immunofluorescence(171). In HSCs it is able to regulate collagen expression: induction of SOX9 by TGF β treatment led to increased collagen expression while siRNA depletion caused a reduction. Increased expression of SOX9 associated with HSC activation could therefore disrupt β -Catenin /TCF interaction in the nucleus of HSCs provoking defective canonical signalling. IP assays to demonstrate SOX9/ β -Catenin interactions followed by siRNA depletion of SOX9 to determine whether canonical signalling can be induced would be necessary to further explore this possibility.

This Chapter has revealed an absence of canonical/ TCF driven signalling in HSCs and proposes several mechanisms by which it may occur (Figure 4.15). This appears at first to contradict data from previous studies. Previously published data along with results here from section 4.1 clearly demonstrate increased expression of total and active β -Catenin in aHSCs and decreased fibrogenic activity upon its suppression. However, these studies do not conclusively show β -Catenin functioning

through TCF/LEF family members. Furthermore, downregulation of β -catenin is accompanied by decreased activity of effectors such as Smad3(123), PDGF, Akt (129) and TGF β (128) and not classic canonical Wnt effectors. The ability of the canonical inhibitor Dkk1 to promote reversion of aHSC to more quiescent phenotype *in vitro* as well as amelioration of a cholestatic liver injury model has been used as evidence of canonical Wnt activity in HSCs(110). Dkk1 functions through binding and inhibiting the canonical associated co-receptor Lrp6(93). However, in addition to Wnt stimulation, Lrp6 activity can also be independently stimulated by multiple profibrogenic pathways such as TGF β , CTGF and PDGF, all disrupted through Dkk1 treatment (172). Dkk1 treatment of HSCs could therefore be inhibiting activity of these pathways instead of Wnt to modulate HSCs behaviour.

β -Catenin is able to act as a transcriptional activator independent of Wnt/TCF stimulus, although examples are few in comparison to its role in regulating canonical Wnt signalling. Evidence so far supports a TCF independent role for β -Catenin in HSCs which requires much further exploration. TGF β is one alternative inducer of β -Catenin activity. TGF β treatment frequently is observed to stimulate β -Catenin nuclear translocation and complex formation with the Smad family of transcription factors in the absence of Wnt signalling(173-175). In renal tubular epithelial cells, TGF β promoted β -Catenin to act as a transcriptional co-activator of Smad3, upregulating Smad3 reporter activity but no concurrent upregulation in TOPFLASH activity(176). This demonstrates transcriptional activity of β -Catenin without reliance on its better known TCF binding partners. Another study demonstrated a complex of Smad3/ β -Catenin directly binding to α SMA promoter and regulating transcription, again in the absence of TCF activity(177). TGF β is a major profibrogenic cytokine and secreted in abundance by HSCs and other cells in the injured liver. TGF β mediated β -Catenin signalling could therefore be one potential role for β -Catenin in aHSCs. Several studies require exogenous TGF β stimulus before β -catenin function is observed. The β -catenin overexpression experiments in Figures 4.4 and 4.10 were conducted under standard culture conditions. Addition of recombinant TGF β stimulus may have enabled greater β -catenin activity.

Whether non-functional β -Catenin /TCF signalling is due to a defect in signalling machinery or through active repression cannot be determined from this data. Abnormal β -Catenin signalling is associated with numerous cancers and other pathologies: active β -Catenin mutations can be detected in 25% of HCCs for example(145). Minimal levels of canonical Wnt signalling may serve as a regulatory mechanism preventing the dysregulated proliferation and cell signalling associated with overactive β -Catenin mutations.

Canonical Wnt signalling through TCF/LEF mediated transcription therefore appears to be absent in HSCs. Instead HSCs and LX-2s upregulate non-canonical effectors such as Jnk and CamKii in response to Wnt stimulus as demonstrated by Figure 4.12. Based on this as well as the expression of non-canonical ligands and receptors in aHSCs, it can be proposed that non-canonical Wnt signalling is the active branch. Non-Canonical Wnt signalling has been shown to regulate proliferation, migration and contractility in numerous cell types. These are typical traits of the profibrogenic myofibroblast therefore non-canonical signalling has much possibility for regulating HSC behaviour.

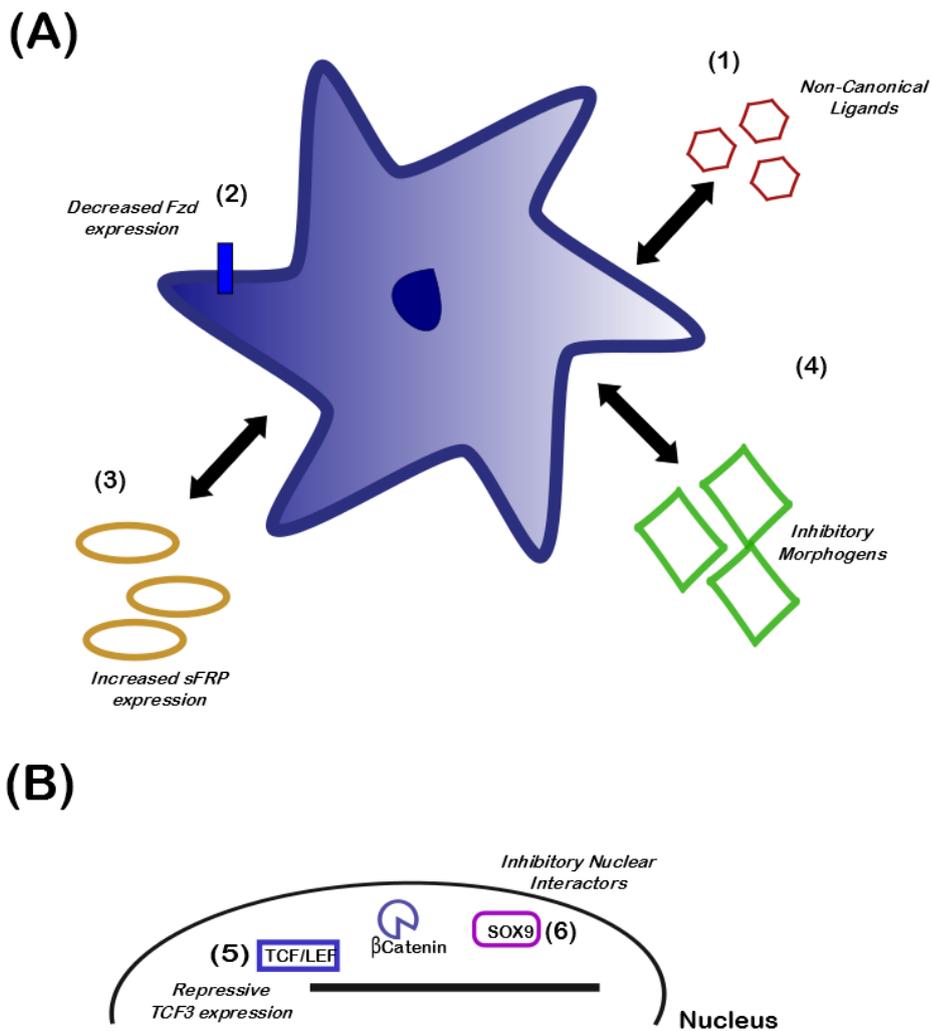


Figure 4.15 Points of inhibition of Canonical/TCF mediated Wnt signalling

This study has highlighted six possible points of canonical Wnt inhibition occurring in HSCs. (A) (1) Predominantly non-canonical ligand expression. Non canonical signalling is inhibitory to canonical signalling(139) (2) Decreased Fzd receptor expression, reducing response to canonical ligands secreted by other cells (3) Increased sFRP expression (4) Increased expression of other morphogens such as Notch, able to directly inhibit β -Catenin(178)

(B) In the nucleus, HSCs express higher levels of the repressive TCF3 in comparison to the other TCF/LEF family members associated with transcriptional activation(5) (6) Transdifferentiation is associated with upregulation of SOX9, direct inhibitor of β -Catenin activity (171)

4.9 Conclusions

In summary, this chapter demonstrates:

- Absence of canonical β -Catenin/TCF mediated transcriptional activity, even under effective canonical stimulus
- Decreased expression of TCF/LEF targets in
- Response to Wnt stimulus through non-canonical effectors

Chapter 5: Role of Wnt in HSC Behaviour

5.1 Introduction

Chapter 3 identified active Wnt signalling in HSCs. Chapter 4 explored which branch of Wnt could be operating, identifying flaws in the canonical pathway and highlighting non-canonical Wnt as the most likely mechanism. This Chapter focuses on how exactly non-canonical signalling contributes to HSC behaviour.

Previous studies have identified expression of non-canonical ligands in HSCs yet their function remains relatively unexplored. This Chapter intends to build on these studies and identify a specific role for Wnt ligands. Based on findings in previous chapters, Wnt5a was selected as a suitable candidate for further study.

5.2 Inhibition of Wnt signalling influences activation of HSCs

Firstly, as an initial method of determining the role of Wnts in HSCs, I attempted a simple depletion of Wnt activity via commercially available inhibitors. There are a large number of inhibitors selective for Wnt signalling, targeting numerous points in the pathway. However, as for many other reagents, most focus on the disruption of the canonical pathway. Reliable inhibitors targeting the non-canonical pathway only are not widely used. Three inhibitors were chosen, each targeting a different stage in the signalling cascade: IWP2, a Dvl-PDZ domain inhibitor (Dvl-PDZ) and ICG001.

IWP2 is an antagonist of porcupine (Porc), the upstream component responsible for the palmitoylation of Wnt ligands and essential for secretion (80, 179). Disruption of Porc function will therefore prevent release of Wnt ligands, halting signalling at the beginning of the pathway. The Dvl-

PDZ inhibitor targets the central PDZ domain of Dvl proteins, preventing Dvl association with Fzd receptors and transduction of Wnt signal(180). Both of these are inhibitors act upstream of the separation of canonical and non-canonical pathways and should function as 'pan-Wnt inhibitors', affecting both pathways equally. ICG001 is an inhibitor of β -Catenin, preventing association with one of its co-activators CBP(181). Active β -Catenin is clearly present in aHSCs as demonstrated by Figure 4.6 Disruption of its activity may provide further clues as to its function.

The efficacy of IWP2 treatment can be clearly demonstrated by decreased expression of hyperphosphorylated Dvl with increasing doses of IWP2 (Figure 5.2A). Dvl-PDZ inhibitor is able to significantly decrease common downstream Wnt targets such as Sox9 and Axin2 (Figure 5.2B) Finally, ICG001 can be shown functional as it significantly decreases TOPFLASH activity in active β -catenin overexpressing HEK293 cells (Figure 5.2C).

Initially, day 7 rat cells were treated for 24hrs with doses commonly used in the literature: IWP2 at 5 μ M, Dvl-PDZ inhibitor at 15 μ m and ICG001 at 5 μ M. Morphologically, IWP2 and Dvl-PDZ inhibitor treated HSCs appear identical to controls when assessed by bright field microscopy. In contrast, ICG001 treatment dramatically alters morphology with cells appearing smaller, rounder and fewer in number (Figure 5.3A). Canonical Wnt/ β -Catenin inhibition in HSCs has previously been alleged to promote reversion to a quiescent phenotype(110). ICG001 administration has also proved successful in reducing fibrosis with treatment of alveolar epithelia cells in a pulmonary fibrosis model able to suppress TGF- β induced α SMA and collagen expression(182, 183). Data here however do not correspond to this. Despite the dramatic change in appearance, no significant reduction occurs in expression of myofibroblast markers such as α SMA (Figure 5.3B).Furthermore, β -Catenin inhibition promotes no increase in expression of PPAR γ , the adipogenic master regulator and typical marker of quiescence (Figure 5.4A). The number of apoptotic cells increases approximately two fold with ICG001 treatment in comparison to over vehicle control. This offers an alternative explanation for the abnormal cell appearance (Figure 5.4B).

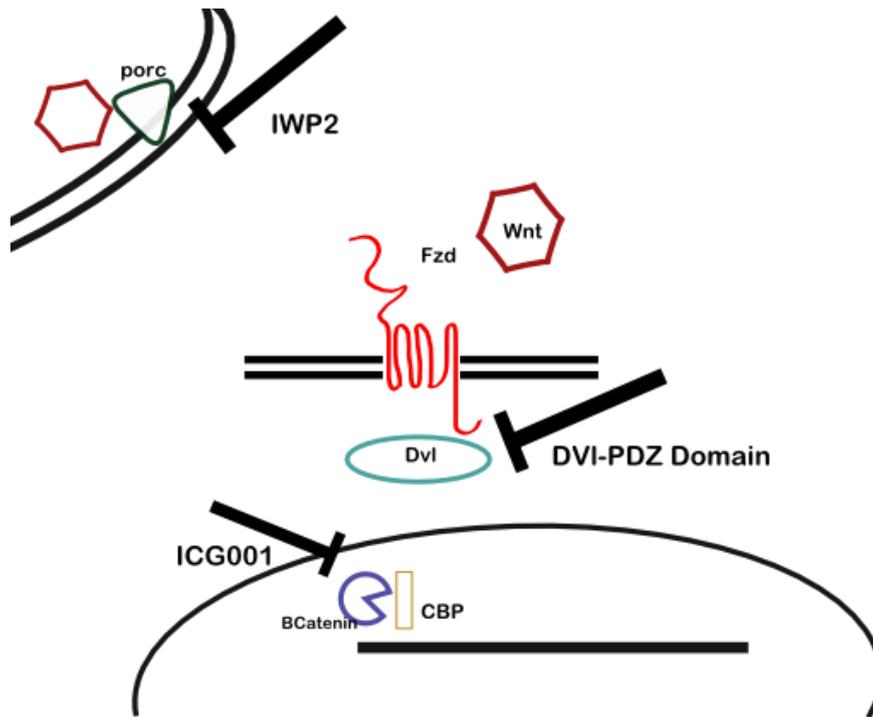


Figure 5.1 Wnt pathway in HSCs targeted at three different points of signalling pathway

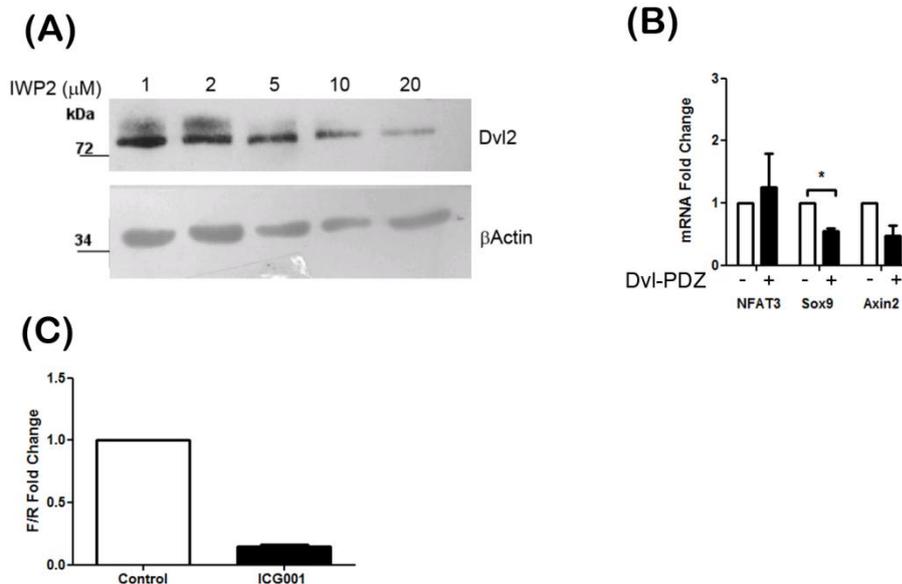


Figure 5.2 IWP2, Dvl-PDZ domain, and ICG001 act as effective Wnt inhibitors in HSCs

(A) Western Blot analysing Dvl2 expression upon 24 hours treatment with increasing doses of IWP2. β Actin serves as a loading control. **(B)** qRT-PCR analysis of Wnt target genes in rat aHSCs treated with either 15 μ m Dvl-PDZ domain inhibitor or vehicle control (n=3) **(C)** HEK293 cells overexpressing active β catenin (Ser37) were transfected with a TOPFLASH luciferase reporter (n=2). Renilla luciferase served as a control of transfection efficiency. Data presented as fold change of firefly to renilla ratio (F/R) \pm SEM. qRT-PCR results are expressed as fold change normalised to control \pm SEM. * p <0.05 Student's t Test (unpaired)

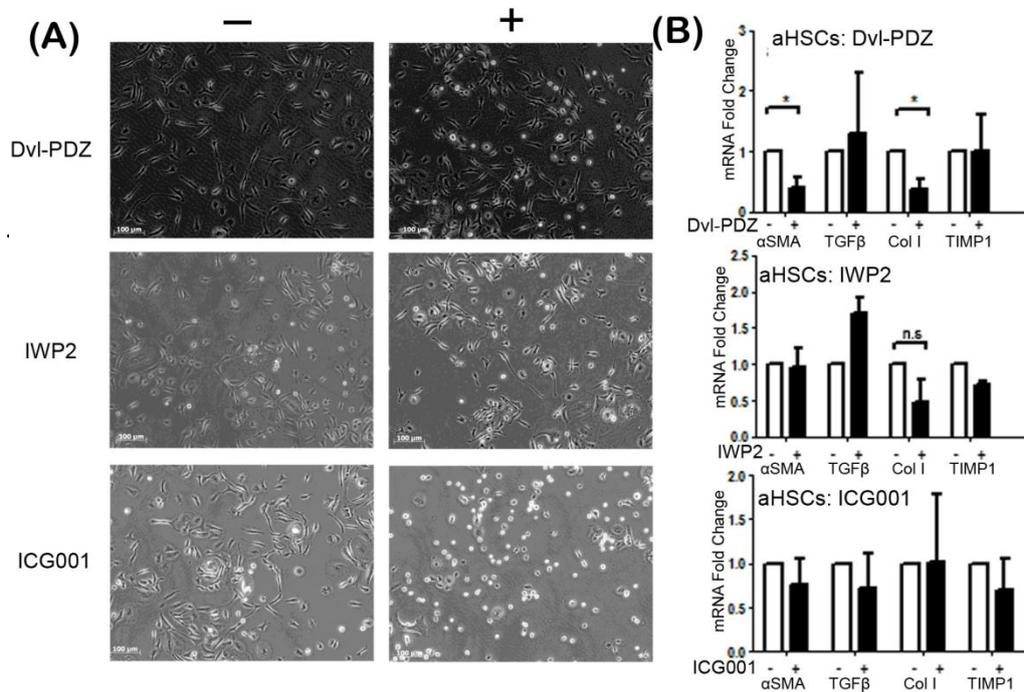


Figure 5.3 Wnt inhibitors and HSCs.

Rat aHSCs (7 days in culture) were treated with either 15μM Dvl-PDZ inhibitor, 5μM IWP2, 5μM ICG001 or vehicle control (DMSO). **(A)** Changes in cell morphology were assessed by bright field microscopy **(B)** mRNA expression of fibrogenic markers was analysed by qRT-PCR for each inhibitor treatment (+) compared with vehicle control (n=3). qRT-PCR results are expressed as fold change normalised to control ± SEM. *p<0.05

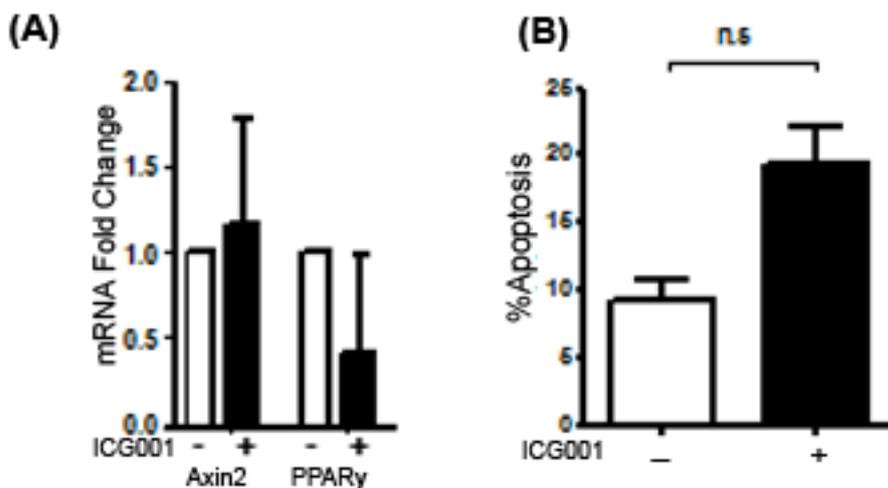


Figure 5.4 ICG001 does not appear to promote reversion of HSCs to quiescent phenotype.

Rat aHSCs (7 days in culture) were treated for 24 hours with 5μM ICG001. **(A)** qRT-PCR analysis of mRNA expression of β-Catenin target Axin2 or the quiescence marker PPARγ in ICG001 and vehicle control (DMSO) treated aHSCs (n=3). **(B)** Acridine Orange staining was used to measure apoptosis in ICG001 and vehicle control treated aHSCs (n=3) *p<0.05 Student's *t* Test (unpaired)

In contrast, treatment with IWP2 or the Dvl-PDZ inhibitor suppressed expression of fibrotic markers. Dvl-PDZ treatment significantly decreases mRNA expression of both α SMA and Collagen 1. IWP2 similarly decreased Collagen1 expression but not to a statistically significant level (Figure 5.3B). This further supports a pro-fibrogenic role for Wnt signalling independent of β -Catenin.

IWP2's striking decrease of Dvl phosphorylation was chosen as the more reliable marker for Wnt inhibition. From Figure 5.2, IWP2 appears effective across several doses from 5 μ M to 20 μ M, reducing expression of the higher weight, hyperphosphorylated form. At the 5 μ M dose used above, minor expression of hyperphosphorylated Dvl can still be detected. I next attempted treatment with a higher dose of 20 μ M and extended treatment time, administering two doses of IWP2 over a 48 hour period, attempting to further increase Wnt inhibitory potential. Two time points were chosen: quiescent HSCs (treated at days 1 and 2) and active HSCs (treated at days 7 and 8), allowing assessment of Wnt function during both initiation of HSC activation and perpetuation of the active phenotype. IWP2's suppression of Wnt signalling was assessed through analysis of downstream markers. Firstly, expression of Evi mRNA, the direct downstream component of Porc and another component necessary for Wnt secretion(184), is reduced by approximately 40%. This is further accompanied by significant reductions in commonly used markers of Wnt activity such as NFAT3 and SOX9(Figure 5.5A). SOX9 profibrogenic role is well known(171), and its decrease further supports a profibrogenic role of Wnt signalling in HSCs. Phosphorylated Dvl is also markedly decreased in treated cells at Days 7/8 compared to controls when analysed by Western Blot. (Figure 5.4B)

As predicted, a 48 hour treatment with IWP2 significantly decreased α SMA and Collagen1 expression in aHSCs (Figure 5.5C), supporting the hypothesis that Wnt signalling contributes to HSC activation. Surprisingly, the opposite results are apparent upon early treatment, marked increases TIMP1 and Collagen1 transcript levels observed (Figure 5.5C). This suggests temporal differences in Wnt activity: potentially inhibitory early in activation and stimulatory later on. As IWP2 treatment blocks both canonical and non-canonical signalling, discerning from this which Wnts may be active at an early time point is not possible.

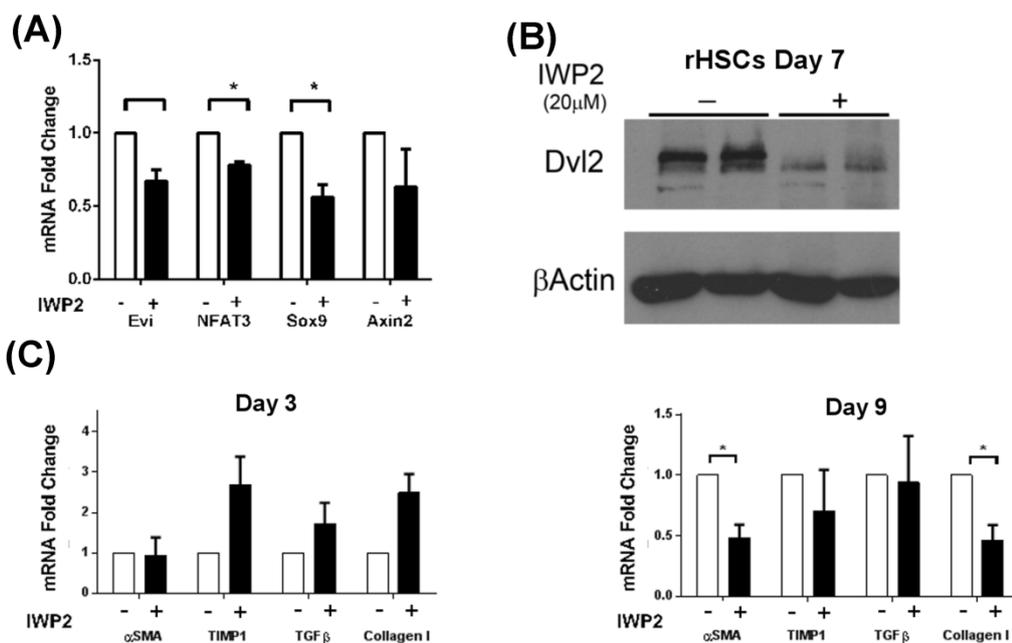


Figure 5.5 IWP2 successfully inhibits Wnt signalling and decreases expression of fibrogenic targets in aHSCs.

Primary rat HSCs were treated with two doses of 20µM IWP2 or vehicle control (DMSO) over a 48 hour period. **(A)** mRNA expression of Wnt pathway components and targets was analysed by qRT-PCR (n=4). **(B)** Western Blot analysing protein expression of Dvl2 after 48 hours in IWP2 or vehicle control (DMSO) treated aHSCs. βActin serves as a loading control. **(C)** mRNA of fibrogenic markers was analysed by qRT-PCR in aHSCs treated with IWP2 or vehicle control (DMSO) at Days 1 and 2 of culture or Days 7 and 8. (n=4). qRT-PCR results are expressed as fold change normalised to control ± SEM. *p<0.05 Student's *t* Test (unpaired)

5.3 Overexpression of Wnt

Pan-Wnt inhibition therefore significantly alters the fibrogenic potential of HSCs. I next aimed to identify function for specific Wnt ligands, primarily using non-canonical Wnt5a as a target.

Accomplishing specific depletion of Wnt5a is likely to be difficult. Commercial inhibitors selective for Wnt5a are not available and reliable siRNA knockdown would be complicated by the poor cross-reactivity of Wnt5a antibodies in rat. Instead, Wnt overexpression was first attempted in the easily transfectable LX-2 cell line. To begin, LX-2s were transfected with constructs for either Wnt10b or Wnt5a ligands, questioning whether there would be a difference in response to canonical and non-canonical Wnts. Overexpression was highly effective with mRNA increasing by approximately 160 fold for Wnt5a and 70 fold for Wnt10b (Figure 5.6A). Phosphorylation of Dvl is markedly increased in LX-2s cells upon Wnt overexpression, to a similar extent for both canonical (Wnt10b and Wnt3a) and non-canonical ligands (Wnt5a) (Figure 5.6B). This again demonstrates the initial robust response to both types of Wnt ligand. Wnt overexpression does not significantly alter viability of LX-2 cells as measured by MTT assay (Figure 5.6C) nor causes any overt differences in morphology (Figure 5.D).

Wnt inhibition in aHSCs in the previous section decreased transcript levels of fibrogenic markers.

Wnt overexpression here was expected to enhance expression of fibrogenic markers. Figure 4.4 demonstrated that Wnt10b overexpression did not stimulate increased expression of fibroblast markers in LX-2s. Here, a similar lack of response is observed in Wnt5a overexpressing cells (Figure 5.7)

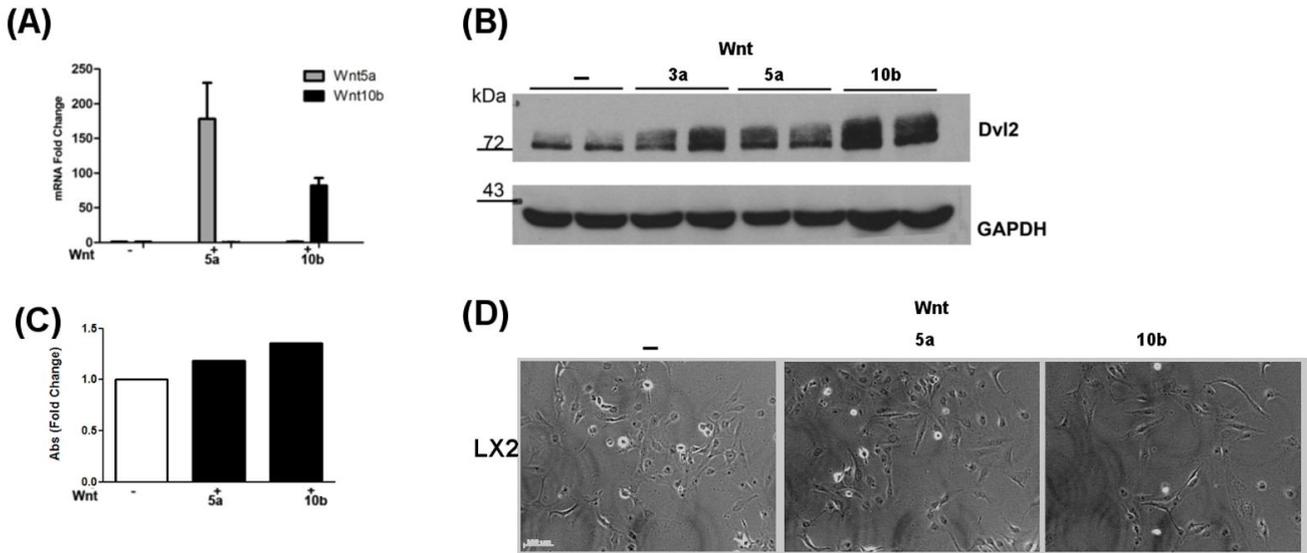


Figure 5.6 LX-2s readily respond to overexpression of canonical and non-canonical Wnts.

(A) LX-2 cells were transfected with a Wnt5a, Wnt10b or empty control expression vector. After 48 hours, mRNA expression of Wnt5a and Wnt10b was analysed by qRT-PCR (n=3) qRT-PCR results are expressed as fold change normalised to control \pm SEM. **(B)** Western Blot analysis of Dvl2 expression in LX-2 cells overexpressing Wnt3a, Wnt5a or Wnt10b compared to empty control vector. GAPDH serves as a loading control **(C)** Viability of Wnt5a or 10b overexpressing LX2 cells determined via MTT assay (n=2) **(D)** Cell morphology of Wnt overexpressing LX-2s assessed by bright field microscopy.

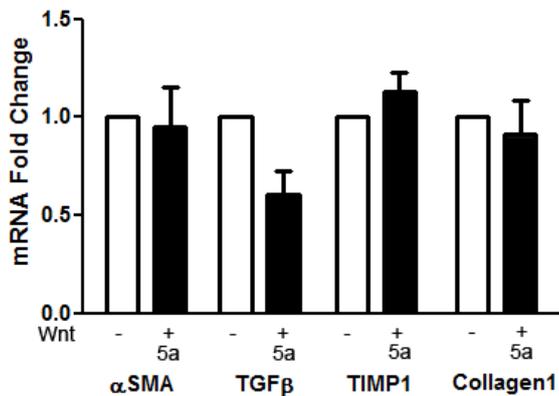


Figure 5.7 Overexpression of non-canonical Wnt5a does not affect expression of fibrogenic genes.

mRNA expression of fibrogenic genes analysed by qRT-PCR in LX-2 cells overexpressing Wnt5a for 48 hours (n=3) qRT-PCR results are expressed as fold change normalised to control \pm SEM

The majority of Fzd receptors were seen to decrease upon HSC activation (Figure 3.4)

Overexpression of Fzd receptors was next attempted in LX-2 cells, hypothesising that a deficiency in receptors may prevent a response to Wnts being secreted. Overexpression of the significantly decreased Fzd4, Fzd8 and ROR2, the classic non-canonical receptor, was successful in LX-2s, with mRNA expression increased by approximately 200, 800 and 600 fold respectively (Figure 5.8). Again however, overexpression of receptors did not stimulate transcriptional change in any of the fibroblast markers analysed.

From these overexpression experiments, LX-2s display an initial robust response to Wnt stimulus with phosphorylation of the signal mediator Dvl. However, no downstream transcriptional effects are apparent. LX-2s are considered representative of fully differentiated myofibroblasts. One possibility is that they may be 'too activated' in phenotype for increased Wnt signalling to provoke further effect. Wnt stimulation of primary cells is therefore likely to be more valuable in investigating function of HSC expressed Wnts.

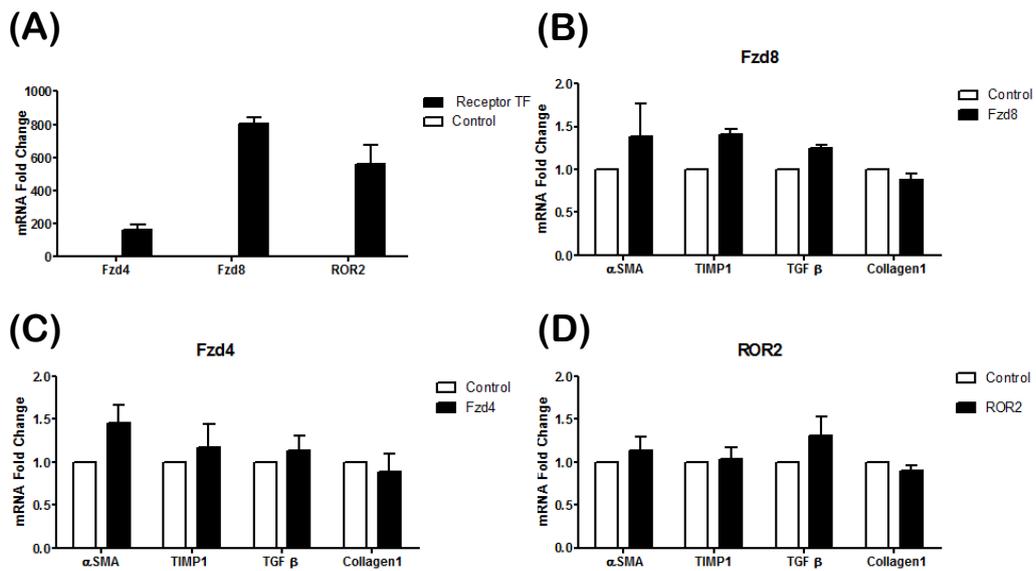


Figure 5.8 Overexpression of Wnt receptors does not alter fibrogenic gene expression.

(A) Wnt Receptors Fzd4, Fzd8 and ROR2 were overexpressed in LX-2 cells for 48 hours and receptor mRNA expression analysed by qRT-PCR (n=3). mRNA levels of fibrogenic markers were then analysed in Fzd8 (B), Fzd4 (C) and ROR2 (D) overexpressing LX-2 cells compared to controls. qRT-PCR results are expressed as fold change normalised to control \pm SEM

5.4 Wnt Stimulation of Primary Cells: Fibrogenic Markers

Overexpression of Wnt ligands in primary HSCs was considered unlikely to be an effective stimulus as they are typically a difficult cell type to transfect(185). Instead, Wnt overexpressing LX-2s were used to produce medium conditioned with Wnt ligands, similar to a method used previously(166) and summarised in Figure 5.9. LX-2s were transfected with 1 μ g of Wnt expression vector for the standard 48hour transfection period. Medium was then collected and used to treat primary rat HSCs. Medium from LX-2s transfected with an empty expression vector was used as a control. This method enables treatment of rat HSCs with cell produced Wnt ligands, having undergone the post-translational modifications such as palmitoylation necessary for secretion and function.

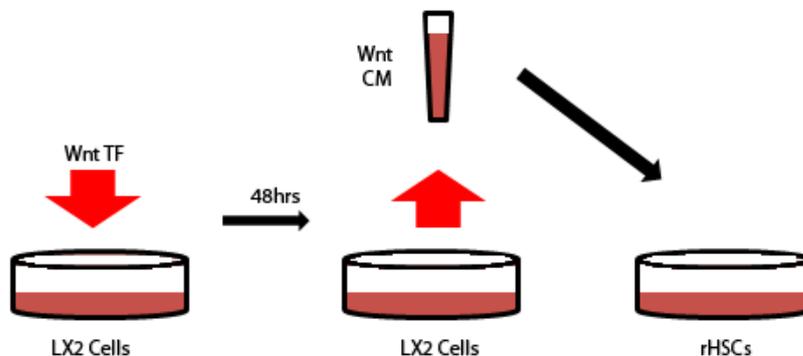


Figure 5.9 Method of Wnt conditioned medium treatment of primary HSCs

LX-2s were transfected with 1 μ g of either an empty control or Wnt expression vector in 16% medium (Wnt TF). Medium was collected after 48 hours and used in treatment of primary rat HSCs

Primary rat HSCs were incubated in medium conditioned with either canonical (Wnt10b) or non-canonical (Wnt5a) ligands for 24 hours. Dvl2 phosphorylation substantially increased in response to both canonical and non-canonical ligands (Figure 5.10A), comparable with the response of LX-2s to Wnt overexpression (Figure5.6B). This again demonstrates the ability of HSCs to initially respond to both canonical and non-canonical stimulus. Conditioned medium treatment brought about no overt change in cell morphology (Figure 5.10B) and no significant differences in viability as measured via MTT assay. (Figure 5.10C)

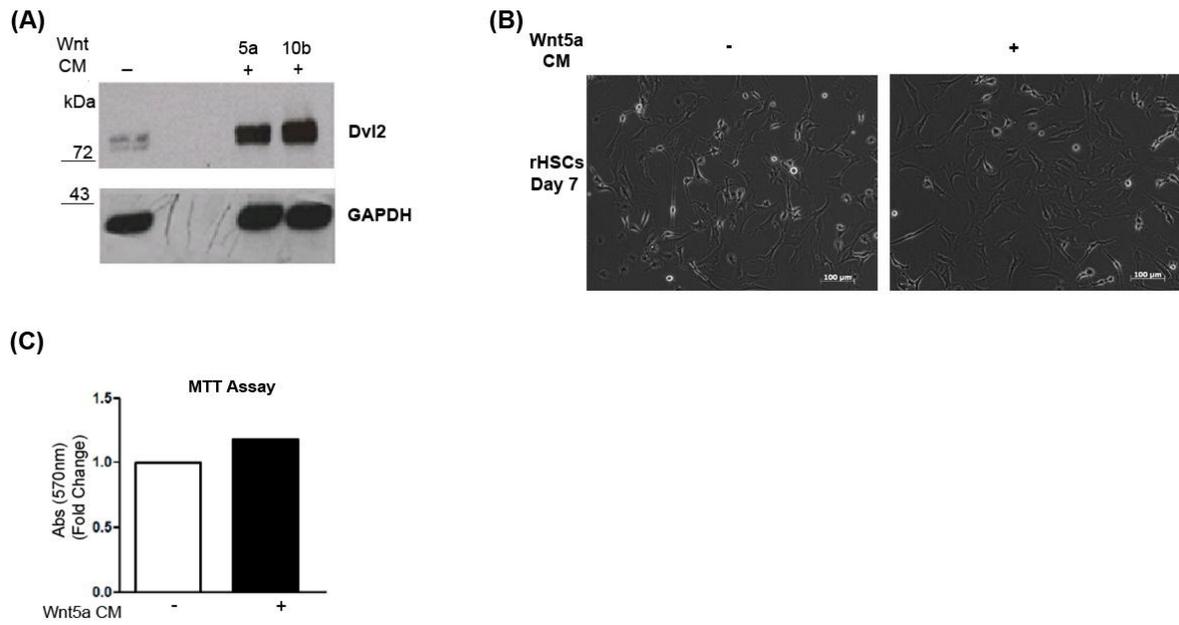


Figure 5.10 Primary HSCs respond to stimulus with both canonical and non canonical ligands.

(A) Western Blot analysis of Dvl2 expression in protein lysates from rat aHSCs treated for 24hours with conditioned medium (CM) from LX-2s over expressing Wnt5a, 10b or an empty control expression vector. GAPDH serves as a loading control. **(B)** Cell morphology of 24 hour Control or Wnt5a CM treated rat HSCs assessed by bright field microscopy **(C)** Viability of Control or Wnt5a CM treated rat HSCs determined via MTT assay (n=2)

After establishing the Wnt stimulating effect of conditioned medium, two treatment points were then chosen: Day 3 when cells are early in activation, before a significant increase in Wnt expression occurs and Day 7 when cells are approaching full activation and Wnt signalling is already established. These two time points allow either an early induction of Wnt signalling or enhancement of Wnt already present.

Day 3 cells stimulated for 24 hours did not respond as predicted. No significant effect on fibrogenic markers was observed, the typical myofibroblast marker α -SMA is increased by approximately 3 fold but with great variability (Figure 5.11A). Other key fibrogenic markers such as Collagen1, TGF β , TIMP1 and MMP9 remain unchanged. Day 7 treated HSCs modestly increased TGF β and α -SMA mRNA expression by approximately 2.5 fold, although not to a statistically significant extent (Figure 5.11A). Wnt5a and TGF β are well documented interactors and whether Wnt5a treatment influenced downstream TGF β signalling was next questioned. However, no increase in mRNA expression levels of TGF β receptors, the Smad transcription factor family or the downstream target CTGF was detected (Figure 5.11B). Furthermore, no increase in TGF β protein expression was observed when assessed by Western Blot or ELISA. (Figure 5.11C and D)

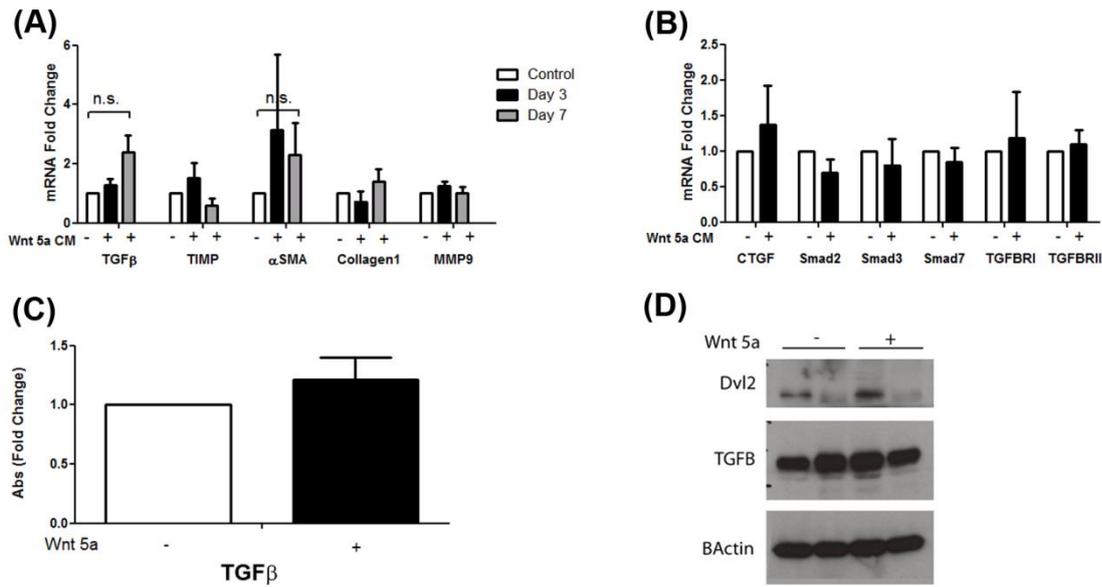


Figure 5.11 Wnt CM stimulus does not significantly alter fibrogenic gene expression

Primary rat HSCs were treated for 24 hours with control or Wnt5a conditioned medium (CM). **(A)** mRNA expression of fibrogenic markers was analysed by qRT-PCR in rat HSCs treated at Day 3 or Day 7 in culture (n=4) **(B)** Further analysis of mRNA expression of TGFβ signalling components in rat aHSCs (Day 7 in culture) (n=3) **(C)** TGFβ Protein levels were analysed by ELISA in medium harvested from control or Wnt5a CM treated aHSCs (n=2) **(D)** TGFβ Protein levels were analysed by Western Blot in protein lysates from control or Wnt5a CM treated aHSCs. βActin serves as a loading control. qRT-PCR results are expressed as fold change normalised to control ± SEM

These findings once more demonstrate HSCs responding strongly to Wnt stimulus but without downstream effects on fibrosis associated targets. Two possible conclusions can be drawn from this. Firstly, that Wnt signalling has limited effect on typical fibrogenic gene expression in HSCs and may influence behaviour through other mechanisms. Alternatively, HSCs may secrete Wnts for the benefit of neighbouring cells.

Wnt5a is a well-known modulator of macrophage behaviour(186-188). Based on this, I next attempted stimulus of rat Kupffer Cells, the resident liver macrophages, with conditioned medium produced by LX-2s. Here a more marked response was observed. Expression of TGF β and MCP1 mRNA expression increased significantly, accompanied by increased expression of TGF β protein when visualised by Western Blot (Figure 5.12A and B) This may support the theory that Wnts produced by HSCs do not function as autocrine signallingers but instead work in a paracrine manner, influencing behaviour of other cells.

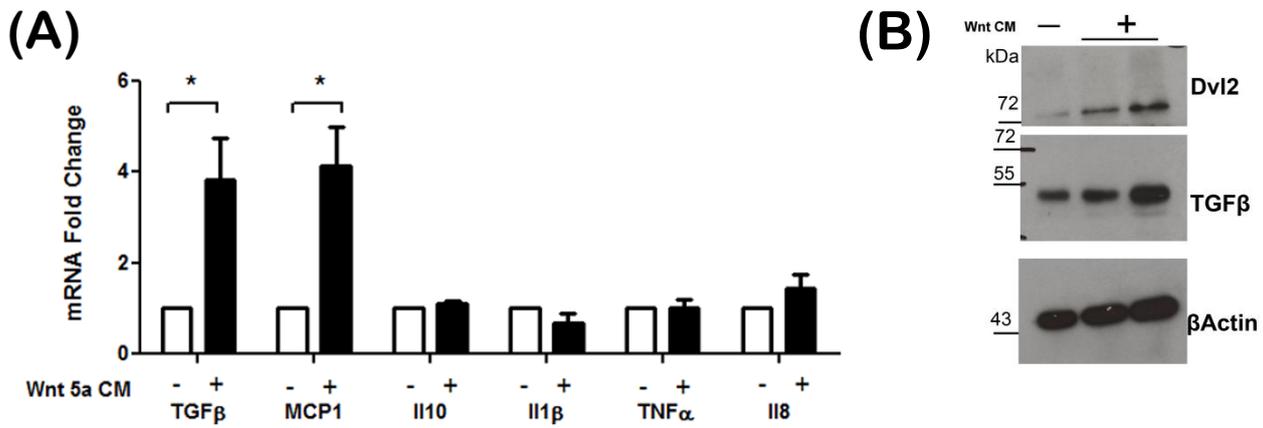


Figure 5.12 Wnt CM stimulus significantly increased TGFβ expression in Kupffer cells.

(A) mRNA expression of fibrogenic and inflammatory markers were analysed by qRT-PCR in rat Kupffer cells treated for 24 hours with control or Wnt5a CM (n=4). **(B)** Western Blot analysis of Dvl2 and TGFβ protein expression in control or Wnt5a CM treated rat Kupffer Cells. βActin serves as a loading control. qRT-PCR results are expressed as fold change normalised to control ± SEM. *p<0.05 Student's *t* Test (unpaired)

5.5 Wnt Stimulation of Primary Cells: Proliferation and Migration

Wnt5a stimulus therefore promotes limited effect on fibrogenic markers in HSCs. I next questioned its influence on other characteristic myofibroblast traits. Wnt3a treatment of several different pulmonary fibroblast lines in one study for example was sufficient to induce nuclear localisation of β -Catenin but had no downstream effect on targets of fibroblast activation. Instead increased proliferation of fibroblasts was observed(109). Wnt5a has also been implicated in regulation of proliferation in fibroblasts(148). MTT assay can be used as a crude measure of proliferation and as shown in Figures 5.6C and 5.10C, neither Wnt overexpression nor Wnt conditioned medium treatment resulted in significant change. I next briefly assessed expression of proliferation markers known to be Wnt targets such as Cyclin D and cMyc (189). Neither Wnt overexpressing LX-2s nor Wnt CM treated rat HSCs increase protein expression of these markers when analysed by Western Blot (Figure 5.13A). mRNA expression of proliferation markers was not significantly altered in primary rat HSCs treated with Wnt CM or 20 μ m IWP2. However, modest changes in CyclinD are suggested: a slight decrease upon IWP2 treatment and slight increase upon Wnt CM treatment. From this, opposite effects of Wnt overexpression and inhibition can be inferred (Figure5.13B).

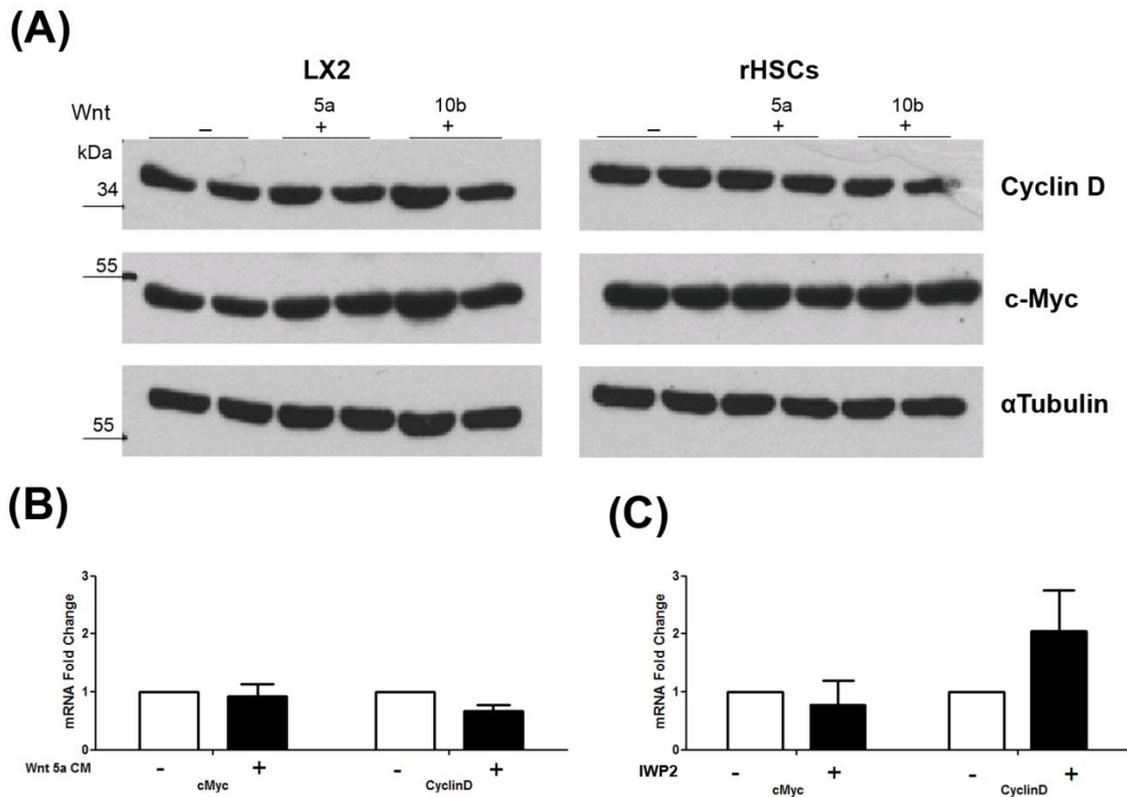


Figure 5.13 Wnt stimulus or inhibition does not alter markers of proliferation in HSCs.

(A) Western Blot analysis of Cyclin D1 and cMyc expression in protein lysates from Wnt overexpressing LX-2s or primary rat HSCs stimulated with control, Wnt 5a or Wnt 10b conditioned medium (CM) for 24 hours **(B)** mRNA expression of cMyc and Cyclin D was analysed by qRT-PCR in 24 hour control or Wnt5a CM treated primary rat aHSCs (7 days in culture) (n=3) **(C)** mRNA expression of cMyc and Cyclin D was analysed by qRT-PCR in primary rat aHSCs treated with 20 μ M IWP2 or vehicle control (DMSO) for 24 hours (n=2). qRT-PCR results are expressed as fold change normalised to control \pm SEM.

Transdifferentiation of HSCs is associated with increased migratory potential, induced by increased exposure to growth factors such as PDGF, EGF and TGF β in the injured environment(23). Wnt5a has been implicated in migration of fibroblasts, able to regulate the activity of Rho GTP and ROCK, key players in controlling convergent extension and cell contractility (115, 190, 191). Treatment of rat HSCs with inhibitors of ROCK is able to decrease migration and reduce activation as well as improving fibrosis in CCl₄ models (192, 193). Based on this, I hypothesised that increased expression of Wnt5a may enhance migration in HSCs. To test this, LX-2 cells were stimulated through overexpression of Wnt5a or treatment with conditioned medium and a simple scratch wound assay conducted. Wound width was measured at 6, 8 and 24hrs but no significant differences in wound closure were observed at any time point. (Figure 5.14). Next, cells were treated with 20 μ m IWP2, questioning whether inhibition of all Wnts rather than overexpression of a single ligand would be more effective, predicting a decrease in rate of wound closure in HSCs. However, again wound widths at 6, 8 and 24 hours were similar between control and IWP2 treated cells. (Figure 5.14)

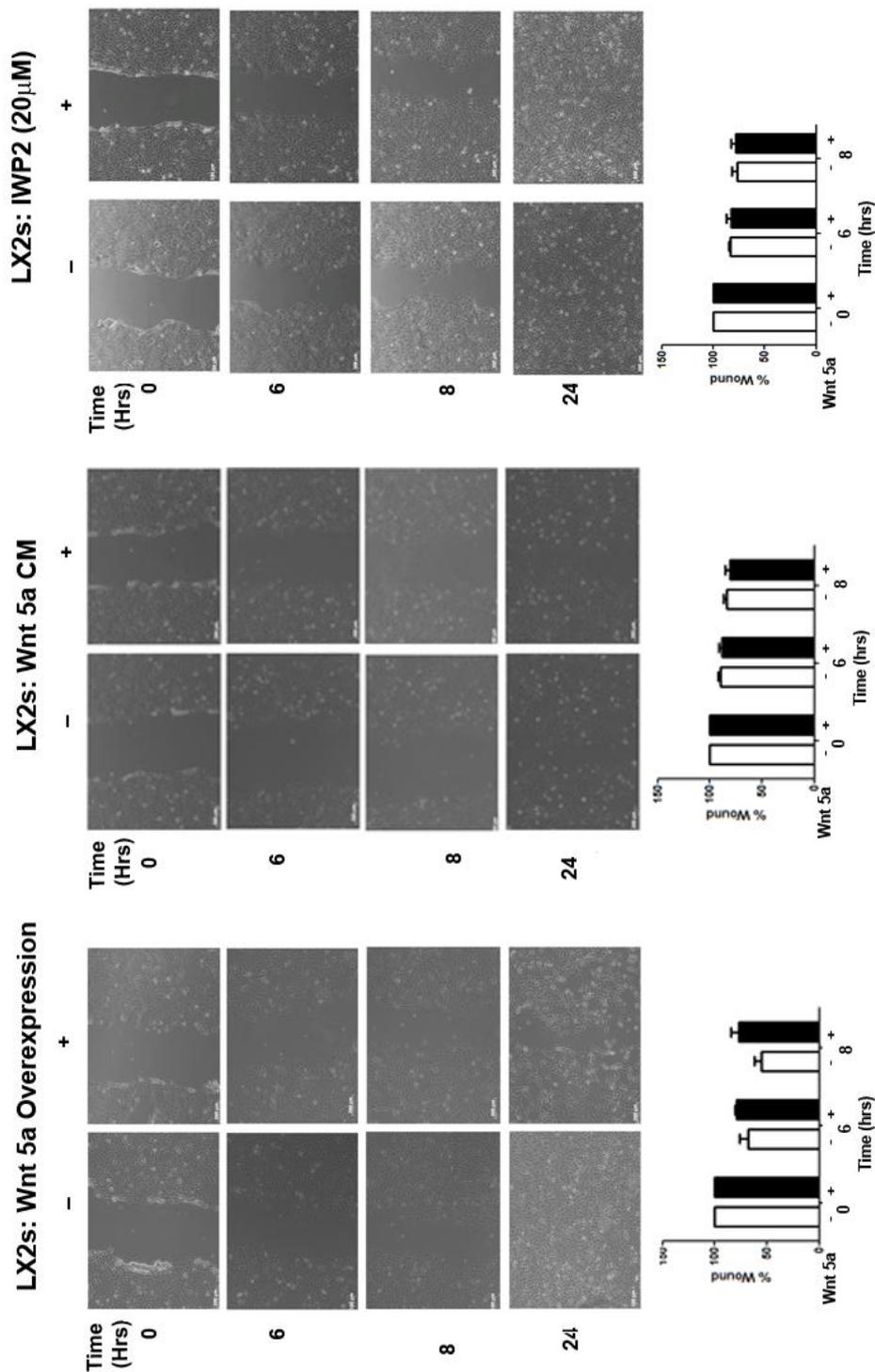


Figure 5.14 Wnt stimulus or inhibition has minimal effect on migration of HSCs.

Confluent LX-2s were stimulated by Wnt5a overexpression (48hour transfection), Wnt5a Conditioned medium treatment (24hours) or IWP2 treatment (20µm) (24hours). Cells were injured with a single scratch and wound width measured over a 6 8 and 24 hour period. IWP2 treatment and Wnt5a CM treatment was simultaneous with injury.

5.6 Wnt Stimulation of Primary Cells: Apoptosis

Wnt5a expression is also able to modulate apoptosis resistance, as demonstrated in lung fibroblasts(148) and its effect in HSCs was next explored. Under standard culture conditions i.e. medium containing 10% FBS, Wnt5a overexpression in LX-2s did not alter numbers of apoptotic cells, as measured by Acridine Orange assay (Figure 5.15A). I next tested whether it may play a protective role in cells undergoing stress. Apoptosis was induced in LX-2s overexpressing Wnt 5a either by incubation in serum free medium alone or serum free medium combined with hydrogen peroxide (H₂O₂) treatment. A sixteen hour serum free medium incubation appeared sufficient to induce a mild apoptotic response, control cells increasing apoptosis percentage from six to approximately twenty percent (Figure 5.15A). Wnt5a overexpression significantly decreased this induction of apoptosis with levels remaining at approximately nine percent. This protective effect was not maintained under the greater stress of H₂O₂ treatment however with number of apoptotic cells increased in both control and Wnt5a overexpressing cells to 40-50%.

Next it was tested whether this mild protective effect was specific to Wnt5a or occurred with overexpression of all Wnts. Wnt10b overexpression however stimulated the opposite effect: serum free conditions inducing a significant upregulation of apoptosis, from approximately twenty to forty seven percent. This is the first example described here of LX-2 cells responding differentially to canonical versus non-canonical ligands. Specificity of this protective effect to Wnt signalling was confirmed using IWP2. Wnt antagonism via IWP2 treatment abolishes Wnt5a's protective effect: Wnt5a overexpressing cells under serum free conditions treated with IWP2 display a marginally increased apoptosis percentage when compared to control cells (Figure 5.15B

Work in primary cells appears to further confirm these findings. Day 7 rat HSCs pre-treated with Wnt5a conditioned medium showed a similar decrease in apoptotic cell percentage under serum free conditions when compared to control treated cells albeit to a less significant extent (Figure 5.15C). This may be attributed to conditioned medium stimulus being more transitory than overexpression in LX-2s.

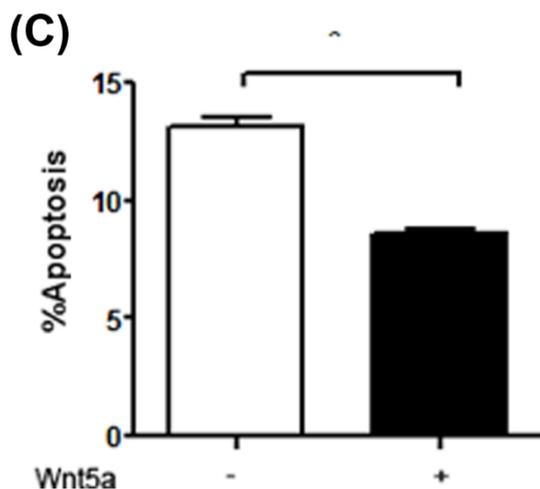
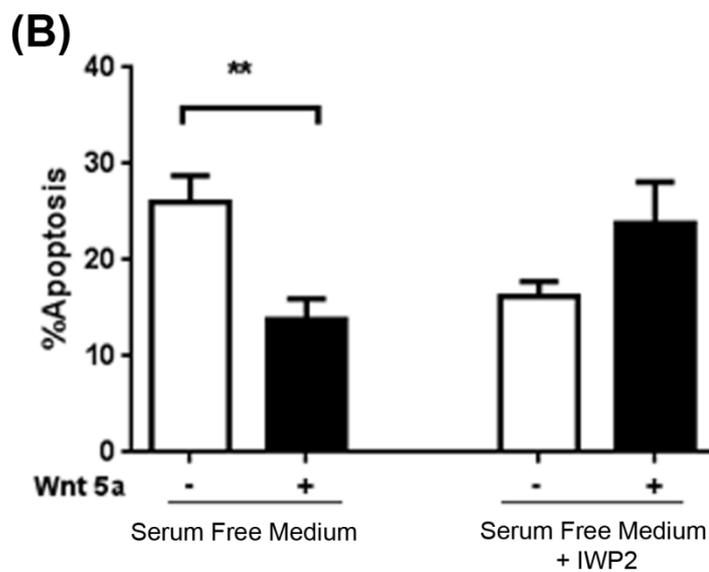
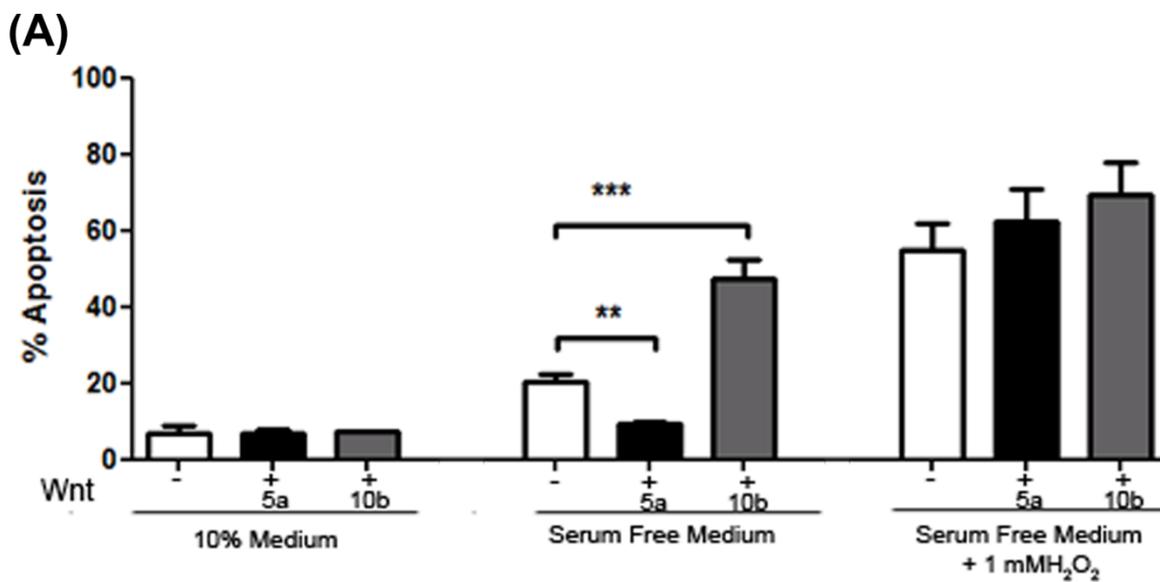


Figure 5.15 Wnt stimulus may modulate resistance to apoptosis in HSCs

(A) LX-2 cells were transfected with either a Wnt5a, Wnt10b or empty control expression vector. 24 hours after transfection cells were cultured in standard 10% medium or stimulated for 16 hours with either serum withdrawal (Serum Free Medium) or serum withdrawal with 1mM H₂O₂ treatment. Percentage apoptosis was analysed by Acridine Orange assay (n=4) **(B)** LX-2 cells were transfected with either a Wnt5a or an empty control expression vector. 24 hours after transfection cells were stimulated by serum withdrawal and treatment with either vehicle control (DMSO) or 20µm IWP2. Apoptosis percentage was analysed 16 hours after stimulus by Acridine Orange assay (n=3). **(C)** Primary rat HSCs were pretreated with either control or Wnt 5a conditioned medium for 24 hours before stimulus with 16 hours serum withdrawal (n=2) * p <0.05 ** p <0.01 Students t Test (unpaired).

How this protective effect might be regulated was next assessed. NFAT activity has been linked with Wnt5a in regulating apoptosis in pancreatic cancer cells(194) and Chapter 4 demonstrates high levels of endogenous NFAT activity in LX-2s along with abundant expression of NFAT family members in primary HSCs (Figure 4.11). Based on this, I investigated whether NFAT activity may be regulating the protective effect of Wnt5a overexpression. I co-expressed Wnt5a and the total NFAT luciferase reporter in LX-2 cells either in standard serum containing medium (10% FBS) or serum free conditions. Wnt5a expression did not alter NFAT luciferase activity under either condition (Figure 5.16). I next expressed Wnt10b in conjunction with the NFAT luciferase reporter, hypothesising that Wnt10b may reduce NFAT activity, disrupting its protective effect and leading to the increase in apoptosis percentage observed upon serum free medium treatment. Surprisingly, expression of Wnt10b under standard conditions appears to increase NFAT luciferase activity by approximately two fold. Furthermore, serum free medium treatment significantly reduced NFAT luciferase activity in Wnt10b overexpressing cells (Figure 5.16). Limited NFAT response to Wnt5a stimulus therefore suggests against an NFAT mediated protective effect. There is little evidence in the literature for Wnt 10b regulating NFAT activity and the response of Wnt10b overexpressing LX-2s here is surprising.

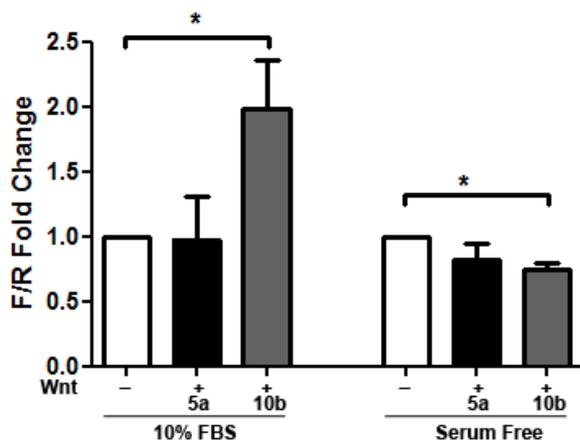


Figure 5.16 Protective effect of Wnt5a is not mediated through NFAT.

LX-2s were transfected with a total NFAT luciferase reporter and co-transfected with either a Wnt5a, Wnt10b or empty control expression vector (n=3). NFAT activity was measured under standard culture conditions (10% FBS) or after 16hours serum starvation (Serum Free). Renilla luciferase served as a control of transfection efficiency. Data presented as fold change of firefly to renilla ratio (F/R) \pm SEM. * p < 0.05 Students *t* Test (unpaired).

5.7 Discussion

This Chapter aimed to pinpoint the mechanism by which increased Wnt signalling affected HSCs.

Wnt signalling has been attributed to promote differentiation of myofibroblasts from precursor cells during injury in numerous organs such as skin, lung and kidney and shown able to promote aspects of the fibrogenic phenotype such as increased contractility, increased collagen production and increased migration. Functional studies concerning how Wnt regulates HSC behaviour during liver injury are limited for both canonical and non-canonical branches. Inhibition of canonical Wnt signalling in primary aHSCs through Dkk1 treatment is shown to promote reversion to a more quiescent like phenotype(110) while siRNA knockdown of Wnt5a in LX-2s decreases proliferation, supporting the importance of non-canonical signalling(130).

Here, I attempted different methods of Wnt inhibition than used previously in HSCs. From Figures 5.2 and 5.5, IWP2 appears an effective Wnt inhibitor decreasing phosphorylation of Dvl and suppressing expression of Wnt target genes. IWP2 treatment of Day 7 aHSCs supports the hypothesis of Wnts playing a profibrogenic role. Expression of α SMA, the commonly used marker of myofibroblast differentiation significantly decreases by approximately 50%. This is accompanied by a similar decrease in Collagen 1 expression, suggesting that active Wnt signalling promotes the fibrogenic phenotype. Surprisingly, treatment of HSCs during the initial stages of activation, at Days 1 and 2 of an *in vitro* time course appears to have the opposite effect. This is an intriguing finding, suggesting that there may be Wnt signalling present early in HSC activation which may be anti- rather than pro-fibrogenic.

Kordes *et al* (122) suggest a model of Wnt activity whereby canonical Wnt signalling is active in qHSCs and maintains the quiescent phenotype. Transdifferentiation induces a shift in Wnt expression profile, from canonical to non-canonical ligands. IWP2 data here corresponds with this

model whereby early treatment inhibits secretion of canonical, inhibitory Wnts and later treatment inhibits secretion of non-canonical, pro-fibrogenic Wnts. Commonly known Wnts such as Wnts 3a, 4, 5a, 6, 7b,9b, 10b or 11 could not be detected by RT-PCR in qHSCs as demonstrated by Chapter 3. Furthermore, protein expression of Dvl could not be detected until Day 3, suggesting an absence of active Wnt signalling before this time point. In order to further verify this model, expression of lesser known Wnts could be investigated in quiescent HSCs and during early activation. Wnt2 for example has recently been suggested as important in liver function with evidence *in vitro* using sinusoidal endothelial cells and *in vivo* using zebrafish as a model organism(195, 196). Regarding the lack of Dvl protein expression, here protein expression of Dvl2 has been used as an effective marker of Dvl activity. Although three members of the Dvl family exist in mammals, there is no evidence suggesting that different Wnts have different Dvl specificities and in HSCs all three Dvl members exhibited similar behaviour at the mRNA level (Figure 3.1). However, investigating protein expression of all three Dvl members in early HSCs would be necessary in order to fully rule out presence of Wnt signalling.

Pan Wnt inhibition in the first part of this Chapter therefore supports the hypothesis that Wnt signalling actively promotes the fibrogenic phenotype. β -Catenin activity alone was also targeted in an attempt to decipher its function in aHSCs. The ICG001 inhibitor selectively disrupts interaction between β -Catenin and the transcriptional co-activator CBP(181). CBP/ β -Catenin activity regulates downstream targets associated with cell survival and proliferation whereas β -Catenin/p300, the other major nuclear transcriptional co-activator, regulates targets associated with cellular differentiation(197). β -Catenin/CBP interaction is clearly important for HSCs as there is major change in morphology and increased apoptosis. However, results here do not recapitulate those obtained using ICG001 in a bleomycin injury model of pulmonary fibrosis where reduced expression of α SMA and Collagen1 were observed upon inhibitor treatment(183). From this inhibition of β -Catenin in HSCs cannot be suggested to promote reversion to a quiescent phenotype. Instead, β -Catenin's role

may be in governing cell survival. ICG001 treatment of murine hepatoblasts supports a role for β -Catenin/CBP activity in regulating cell survival with inhibition resulting in decreased proliferation and survival (198). Furthermore, this effect was shown to be Wnt independent, mediated instead by Akt phosphorylation of β -Catenin and not dependent on the TCF transcriptional activity shown deficient in Chapter 4.

Pan Wnt inhibition via IWP2 clearly suggests that Wnt signalling plays a role in HSC behaviour. Attempts at identifying the specific role of Wnt5a in HSCs however were less successful. Although both LX-2s and primary HSCs responded initially to Wnt5a stimulus α SMA expression levels were unaffected and there was no increase in profibrogenic mediators such as TIMP1 and MMP9. There are several possible explanations for this lack of effect. Firstly, Wnt5a may not directly regulate expression of fibrogenic mediators in HSCs. Instead, Wnt5a regulates other aspects of the myofibroblast phenotype such as the increased resistance to apoptosis demonstrated in section 5.6 and discussed further below

Secondly, in Kupffer cells, the Wnt stimulus provided by conditioned medium appeared sufficient to provoke a significant response. Kupffer Cell stimulation for 24 hours with LX-2 produced Wnts significantly increased TGF β and MCP1 expression, key regulators in the initial response to injury. Two populations of macrophages function during liver injury with Kupffer cells the resident liver macrophages and typically associated with healthy liver(199). They are critical in the initiation of the inflammatory response, activated by release of damage associated molecular patterns (DAMPs) from damaged hepatocytes and cholangiocytes and stimulated to produce pro-inflammatory mediators(200). Increased expression of TGF β by Kupffer cells will promote the transdifferentiation of quiescent HSCs to profibrogenic myofibroblasts. Increased expression of MCP1 (Monocyte Chemoattractant Protein (MCP1) also known as CCL2) will promote recruitment of circulating monocytes(201). As fibrosis progresses, Kupffer cell numbers decrease, replaced by macrophages

derived from monocytes positive for LY6C, a typical marker of inflammatory monocytes. These are the major pro-fibrotic macrophages, secreting proinflammatory mediators such as $IL1\beta$ and $TNF\alpha$ as well as driving activation of myofibroblasts through production of $TGF\beta$ and PDGF(202). The importance of MCP1/CCl2 can be demonstrated by the suppressed development of fibrosis upon its ablation(201). Here, effect of Wnt5a on Kupffer cells suggest a role in initiation of inflammatory response. Whether Wnt5a has a similar effect on the $LY6C^{hi}$ macrophages driving fibrogenesis next needs to be addressed.

This supports an alternative possibility that HSCs may secrete Wnts as part of a paracrine signalling network . HSCs are highly secretory capable of producing a diverse repertoire of cytokines, functioning in proliferation, regeneration, chemotaxis and promotion of fibrogenesis(26). It has been reported that macrophages are a major source of canonical Wnts in the liver: macrophage produced Wnt3a playing a significant role in the differentiation of hepatocytes from hepatic progenitor cells after injury. Data here could suggest that HSCs may be a source of non-canonical Wnts. Due to having encountered difficulties with hepatocyte culture, effects of Wnt5a conditioned medium treatment could not be assessed. Canonical Wnt signalling and β -Catenin activation is known to play a role in liver zonation and regeneration as well as the differentiation of hepatocytes from precursors mentioned above. Evidence of non-canonical Wnts influence on hepatocytes is extremely limited therefore optimising culture conditions to further pursue this may yield novel findings.

The majority of cytokines produced by HSCs are thought to function in an autocrine manner. In a potential model of HSCs producing Wnts to function in paracrine signalling, the increased sFRP expression and decreased Fzd receptor expression described in Chapter 3 may be a protective mechanism to prevent HSCs from responding to their own ligands.

This Chapter intended to assess the function of a single Wnt ligand. However, stimulation of HSCs with Wnt5a alone was less successful than pan Wnt inhibition via IWP2 treatment. Both Wnt5a overexpression and conditioned medium treatment upregulated Dvl phosphorylation, therefore proving successful in initiating Wnt signalling. However, *in vitro* conditions here may not have been optimal for full function of Wnt signalling.

LX-2s are considered representative of fully differentiated myofibroblasts and may therefore be 'too activated' in phenotype for Wnt stimulus to provoke further transcriptional activity. Regarding the lack of transcriptional response in primary HSCs, Wnts function as morphogens with cellular response heavily dictated by concentration. Morphogens function through establishment of concentration gradients, cellular response dependent on amount of stimulus received, with 'low threshold' and 'high threshold' target genes. Furthermore, receptor expression profiles specific to receiving cell will further modify response. Stimulus of primary cells with Wnt conditioned medium was clearly sufficient to instigate Wnt signalling in both HSCs and KCs. However, the fibrogenic targets analysed may have had lower thresholds of activation in KCs compared with HSCs. One of the caveats of working with conditioned medium as a stimulus is that Wnt concentrations cannot be easily modulated. A tightly regulated dose titration with recombinant Wnt proteins would be one method to test this. However, complications with this may involve the issues with purity and activity associated with use of recombinant Wnts(203)

Furthermore, HSCs express multiple ligands and single ligand stimulus may not be sufficient. Overexpression or stimulus with multiple ligands may be required before a function can be determined. This is yet again a similar problem as witnessed in Chapters 3 and 4, encountering a multiplicity of Wnt ligands and interactors. A further complication is the increased expression of sFRPs accompanying activation. If they are indeed a protective mechanism to prevent HSCs responding to their own Wnt ligands, they are likely to interfere with exogenous Wnts also. Chapter 3 described upregulation of four secreted interactors of Wnts: sFRP1, sFRP2, sFRP4 and Dkk3. There

is little evidence for interaction between Wnt5a and sFRP4 or Dkk3. Interactions between Wnt5a and sFRP1 and sFRP2 on the other hand are well documented but the functional outcome appears to be highly cell context specific. Numerous references support sFRP1 and sFRP2 as inhibitory factors. Evidence demonstrates sFRP2 able to reduce Wnt5a mediated Dvl2 phosphorylation and impair migration in breast cancer cells for example or able to bind to and antagonise Wnt5a's promotion of prochondrogenic differentiation. sFRP1 has similarly been shown able to disrupt Wnt5a mediated PCP signalling in the developing gut(204). However, there are several examples of sFRP1 selectively associating with canonical ligands over non-canonical Wnt5a or sFRP2 recombinant treatment having no influence on Wnt5a/Fzd interaction. One study demonstrates co-upregulation of Wnt5a and sFRP1 and sFRP2 in migrating immortalised keratinocytes, a similar situation as observed in aHSCs. Here, sFRP1 and sFRP2 do not appear to interact with Wnt5a but instead impair canonical signalling.

Functional outcome of sFRP/Wnt interaction therefore appears to be highly cell context specific. The use of sFRPs as Wnt inhibitors should therefore be undertaken with extreme caution: concentrations strictly controlled and interactions in cell type of choice clearly defined. One group advocates use of a 'cocktail' of sFRPs, combining multiple sFRPs to achieve effective Wnt inhibition. Multiple sFRPs upregulation may be a method of inhibiting all putative Wnt interactions in HSCs. From the literature described above it appears that where physical binding of sFRP1 and sFRP2 occurs, Wnt5a activity is inhibited. Wnt CM treatment here lacks a thorough characterisation of how sFRPs behave upon stimulus. To further clarify role of these proteins in HSCs, stimulus of HSCs could be repeated and the resulting protein interactions of Wnts and sFRPs examined.

Characteristic traits of myofibroblasts include increased migration, contractility, proliferation and resistance to apoptosis. Of the traits analysed, manipulation of Wnt signalling in HSCs appeared to influence apoptosis resistance only. Contractility was not examined. However as Wnt5a is a known

promoter of stress fibre formation mediated by RhoA this could be a promising target. Undertaking a simple F-actin staining assay using Wnt CM treated rat HSCs would allow further investigation of this.

Wnt5a overexpression in LX-2 cells appeared to promote resistance to mild apoptotic stimuli. Clearance of aHSCs by apoptosis is a critical during fibrosis resolution, selectively targeting α SMA positive cells for apoptosis significantly improves fibrosis resolution(60). During injury, HSCs are maintained by anti-apoptotic signals derived from injured environment or autocrine production of TIMP1. Upon cessation of injury and a change towards normal liver architecture, these survival signals are removed and HSCs undergo apoptosis(23). Data here suggests possibility that increased Wnt5a may act as one of those survival signals. Rather than directly enhancing fibrogenic activity, Wnt5a may play a role in modulating HSC apoptotic response.

How exactly Wnt5a is mediating this protective effect cannot be determined from this data. From Figure 5.16 NFAT appears an unlikely contender as total NFAT luciferase reporter activity appeared unresponsive to Wnt5a treatment. Interaction between Wnt5a and NF κ B is one possibility. Upregulation of NF κ B activity mediated by Wnt5a has been described in several studies using macrophages and lymphocytes(205, 206) with decreased expression of NF κ B regulated prosurvival genes such as Bax and Bcl2 observed upon Wnt5a siRNA knockdown(206). NF κ B plays a critical role in mediating survival of HSCs(207) therefore linking these two pathways in HSCs is an intriguing possibility.

Another attractive candidate is Wnt5a/ PKC signalling with protein kinase C (PKC) is a known promoter of apoptosis resistance. Upon irradiation induced apoptosis in fibroblasts, phosphorylation of PKC mediates a protective effect through upregulation of pro-survival signals such as CREB phosphorylation along with anti-apoptotic markers such as BAD phosphorylation(208). Wnt5a is a

well-established activator of PKC in governing cell motility and recent evidence shows Wnt/PKC signalling enhancing survival of murine mesenchymal stem cells upon exposure to H₂O₂ (209, 210). Further work exploring Wnt5a's influence on apoptotic mechanisms in HSCs is now required. Investigating its potential effect on PKC downstream effectors and potential role in apoptosis with further aid establishing a mechanism of Wnt5a function and survival signals it produces

5.8 Conclusions

This chapter aimed to identify the exact role Wnt ligands are playing in HSC activation and behaviour and has demonstrated:

- Inhibition of Wnt signalling in aHSCs decreased fibrogenic markers
- Wnt5a stimulus did not increase fibrogenic markers in aHSCs instead promoting profibrogenic and proinflammatory cytokine expression in Kupffer Cells
- Wnt5a treatment of LX-2s and primary HSCs appears to confer resistance to mild apoptotic stimuli

Chapter 6: H2A.Z's Role in Influencing DNA Methylation in Early Zebrafish Embryos

6.1. Introduction

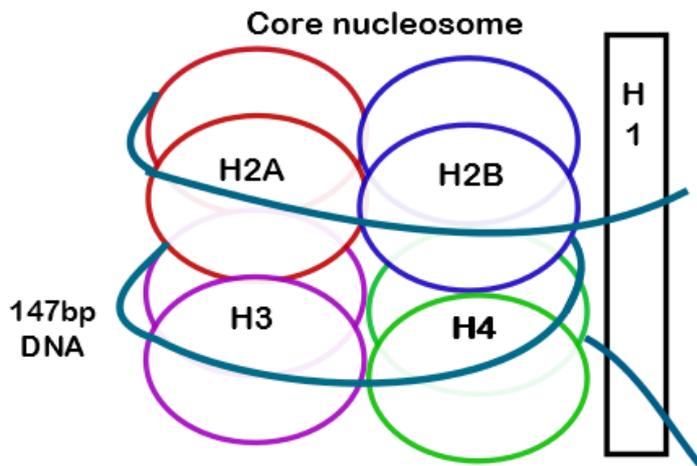
H2A.Z's role in influencing DNA Methylation in early zebrafish embryos

This chapter serves as an initial exploration of how changes early in development may impact injury response in the adult, mediated through epigenetic mechanisms. As mentioned in the introduction, a recent study has explored multigenerational adaptation to liver fibrosis where ancestral injury conveyed a protective effect upon subsequent generations(211). A reduced fibrotic response was associated with differential methylation levels of key fibrotic markers: high methylation of profibrotic TGF β and low methylation of antifibrotic PPAR γ . How this adaptation is transmitted between generations is not clear. Chromatin modifications in sperm were investigated as a potential mechanism and CHIP analysis demonstrated enrichment of the histone variant H2A.Z at the PPAR γ promoter in sperm of injured rats.

6.1.1 Canonical and Variant Histones

Chromatin is the complex of DNA and packaging proteins, with twice the protein mass of DNA alone(72). Chromatin exists in two states: heterochromatin, a highly condensed form, associated with low transcriptional activity or euchromatin, a more open conformation, allowing access of transcriptional machinery to DNA(37). Histones are the protein family responsible for packaging DNA and consist of four core members: H2A, H2B, H3 and H4 and the linker histone H1. Core histones dimerise to form an octamer around which 147bp of DNA is wrapped, this is termed a nucleosome and is the basic unit of chromatin (Figure 6.1A).

(A)



(B)

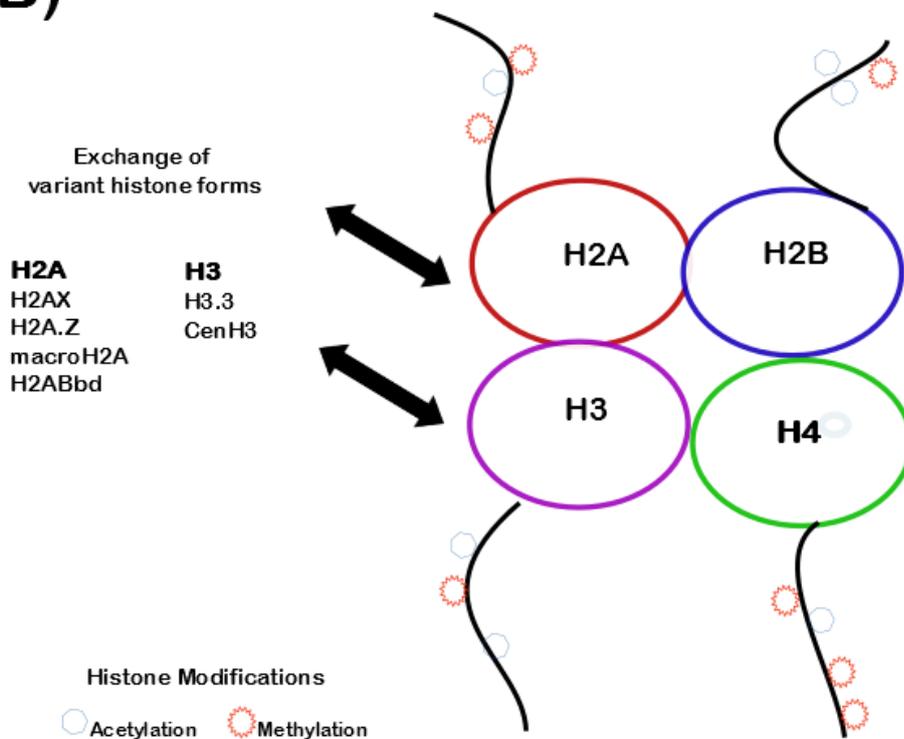


Figure 6.1 Summary of Nucleosome Structure (A) Histones are the protein family responsible for packaging DNA and consist of four core members: H2A, H2B, H3 and H4 and the linker histone H1. Core histones dimerise to form an octamer around which 147bp of DNA is wrapped. This is termed a nucleosome and is the basic unit of chromatin (B) Modifications to histones result in alterations in chromatin structure and alterations in transcription. Modifications include methylation, acetylation and phosphorylation of histone tails or exchange of variant histone forms.

The N terminal tails of histones are significant sites for modifications. Classic histone modifications include methylation, acetylation and phosphorylation(212). These changes can result in either altered contacts between nucleosomes, thus altering chromatin state or promoting recruitment of non-histone proteins which can influence transcription of genes(212). Different histone modifications are therefore associated with either transcriptional promotion or repression.

Chromatin structure can also be changed through incorporation of variant histone forms. Canonical histones (H2A, H2B, H3 and H4) are solely involved in DNA packaging and their deposition is tightly coupled with DNA replication. Variant histone expression is less strictly regulated and their deposition can occur independent of the cell cycle(72). Furthermore, structural differences of variant histones in comparison to their canonical forms enable function beyond DNA packaging(213).

Histone H2 has the largest number of variants including H2A.Z, macroH2A, H2A-Bbd, H2AvD, and H2A.X (214) Some variants such as H2A.Z and H2A.X are conserved throughout evolution while others such as macroH2A are restricted to mammals. H2A.Z has the highest level of conservation, more similar across species than even the canonical H2A itself(215). H2A.Z is termed a replacement histone and exchanges with H2A at specific points during development and differentiation with its deposition driven by the Swr1 chromatin remodelling complex(216)

Structurally, H2A.Z is relatively similar to H2A. One key divergence is the presence of an extended acidic patch on the nucleosome surface. Mutations of this patch lead to disruption of H2A.Z function in developing *Xenopus* embryos, demonstrating its functional importance(72). One hypothesis is this acidic patch changes interaction with neighbouring nucleosomes, altering chromatin architecture(217).

H2A.Z is implicated in a diverse range of functions. It is shown to localize to transcriptional start sites and is associated with promotion of gene activation and initiation of transcription. In mammals, H2A.Z is predominantly associated with actively expressing genes(218) and studies in embryonic

stem cells suggest H2A.Z is enriched at genes associated with developmental regulation(219). The necessity of H2A.Z is clear from depletion studies. RNAi mediated knockdown of H2A.Z leads to missegregation of chromosomes(220) and failure to differentiate while knockout in mice causes failed gastrulation and early embryonic lethality(215).

Another potential role for H2A.Z recently identified is in the antagonism of DNA methylation(222, 223). Enrichment of H2A.Z at the PPAR γ promoter in the sperm of injured rats suggests it might play a role in transmission of hereditary information, influence methylation levels of genes in offspring.

6.1.2 DNA Methylation dynamics during development

Changes in methylation patterns are a key contributing factor to embryogenesis. During development, pluripotent cells become differentiated, requiring tightly controlled regulation of transcription factors(211). Epigenetic changes during development are likely to be one of these regulatory factors. DNA methylation has been shown to have roles in genomic imprinting, transposon silencing, X-inactivation and gene repression(221)

During embryogenesis, methylation patterns are highly dynamic. Global demethylation occurs upon fertilisation, followed by a gradual reannotation of methylation patterns mediated by Dnmt3a(40). The paternal genome is rapidly and actively demethylated, whereas maternal demethylation occurs passively during cleavage, dependent on replication (222) This reprogramming of methylation is thought to aid developmental transitions. A key developmental event is the midblastula transition (MBT), the point at which the zygotic genome transcription becomes active. This is a period of significant chromatin remodelling and dynamic DNA methylation patterns are crucial at this point. Genes involved in regulation of processes such as metabolism and protein synthesis are hypomethylated whereas genes involved in later developmental processes or terminal

differentiation are hypermethylated(223). Disruption of methylation dynamics at this early stage leads to dysregulated transcription and severe consequences for development (224, 225)

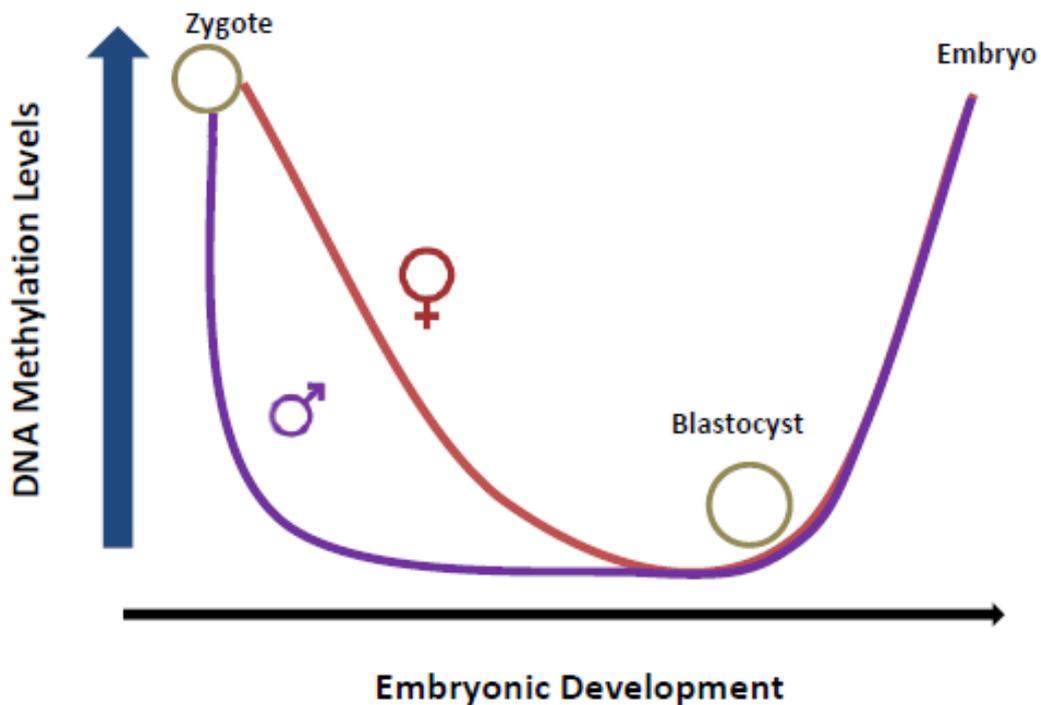


Figure 6.2 Methylation dynamics during embryogenesis

Schematic illustrating dynamic nature of methylation during embryogenesis. Demethylation of gametes occurs upon fertilisation with active demethylation of paternal genome and passive demethylation of maternal genome. There is a gradual reannotation of methylation patterns as development proceeds.

6.1.3 H2A.Z and DNA Methylation

H2A.Z and DNA Methylation therefore appear to have opposing roles: H2A.Z associated with active transcription and DNA methylation associated with repression. Whether a functional relationship exists between H2A.Z and methylation was first explored in the model plant organism *Arabidopsis thaliana*(226). Whole genome mapping of DNA methylation patterns and H2A.Z location revealed an anti-correlative relationship with H2A.Z excluded from regions of DNA methylation. Loss of function mutations in *Met1*, the *Arabidopsis* DNA methyltransferase, dysregulated methylation patterns across the genome. In these plants, distribution of H2A.Z was also altered: H2A.Z was decreased in regions of increased methylation while increased H2A.Z enrichment accompanied decreased methylation. Further experiments using loss of function mutations in *PIE1*, a component of the complex involved in deposition of H2A.Z, resulted in genome wide hypermethylation and decreased H2A.Z distribution. From this, the authors proposed a mechanism where methylated regions exclude H2A.Z, further ensuring repression of transcription. Conversely, regions of H2A.Z enrichment prevent methylation and further promote gene activation.

Based on these findings we wanted to explore the possibility that H2A.Z enrichment in sperm could modulate gene expression in the adult through influencing methylation. The first step was to investigate the role of H2A.Z in governing methylation during early embryogenesis. We aimed to deplete H2A.Z and investigate subsequent effect on genome wide methylation levels. The rodent model used previously presents significant obstacles as H2A.Z is essential for embryonic development. As mentioned, H2A.Z knockdown is early embryonic lethal in mouse models and H2A.Z's presence is essential for the differentiation of embryonic stem cells(215, 227). In order to circumvent this, the zebrafish was chosen as a model organism.

6.1.3 Zebrafish as a model organism

The use of zebrafish in medical research is significantly increasing. Homology between zebrafish and human is high with at least one zebrafish ortholog existing for 71% of human genes. Moreover, 82% of disease related genes in humans have at least one zebrafish ortholog (39). Using zebrafish as a model organism has a great number of benefits. Genetic manipulation in zebrafish is relatively simple with diverse methods such as targeted mutagenesis, transient knockdown of expression and conditional gene expression facilitating identification of gene function. Furthermore their small size and low associated husbandry costs make large scale screens attractive and affordable(228).

Existing disease models in zebrafish include models for myopathies, muscle degeneration, neurodegeneration, carcinogenesis and metabolic disorders to highlight a few(40, 229, 230).

Focusing on liver disease, the zebrafish liver is fully mature by four days post fertilisation (dpf) and can be used as a model of hepatic disease(233). Treatment of zebrafish with ethanol is a robust model of alcoholic liver disease, with treated embryos displaying metabolism of alcohol, oxidative stress and the development of steatosis and hepatomegaly(55, 231).

One of the major advantages of zebrafish as a model organism is their rapid embryogenesis, with major organs fully formed by 5dpf. Furthermore, zebrafish develop externally and early embryos are transparent, allowing for significant developmental milestones such as axis polarity specification and gastrulation to be observed without need for specialist equipment or invasive imaging(27). Early stages of development are summarised by Figure 6.4, with the embryonic period lasting until approximately 72 hours post fertilisation, after hatching of larvae (232)

The first cleavage occurs approximately 40 minutes after fertilisation. Embryos pass rapidly through cleavage and blastula stages. Epiboly, the first major cell movement involving the thinning and spreading of the blastodisc over the yolk cell begins at approximately and is commonly used to stage early embryos(233). Embryos complete gastrulation, the formation of the primary germ layers of

ectoderm, mesoderm and endoderm by approximately 10 hours post fertilisation (hpf). The period of segmentation (10 – 24 hpf) is the period during which primary organogenesis occurs and can be staged by the number of developing somites, paired blocks of mesoderm along the anterior posterior axis, from which skeletal muscle, cartilage and bones of the spine and ribcage develop. The period between 24 and 48 hpf is termed the Pharyngula period. During this time there is further development of the nervous system with the brain differentiating into distinct lobes as well as the circulatory system as the heart begins to beat. Embryos normally hatch independently after approximately 72hpf. At this point organogenesis of most systems is complete and animals display tactile sensitivity and independent movement. Hatching marks the end of the embryonic period. During development, zebrafish undergo similar wave of demethylation and remethylation as observed in mammals(234). The genome is hypomethylated until the early blastula stage, the wave of de novo methylation beginning approximately at the mid blastula stage and continuing until 6hpf or the beginnings of gastrulation.

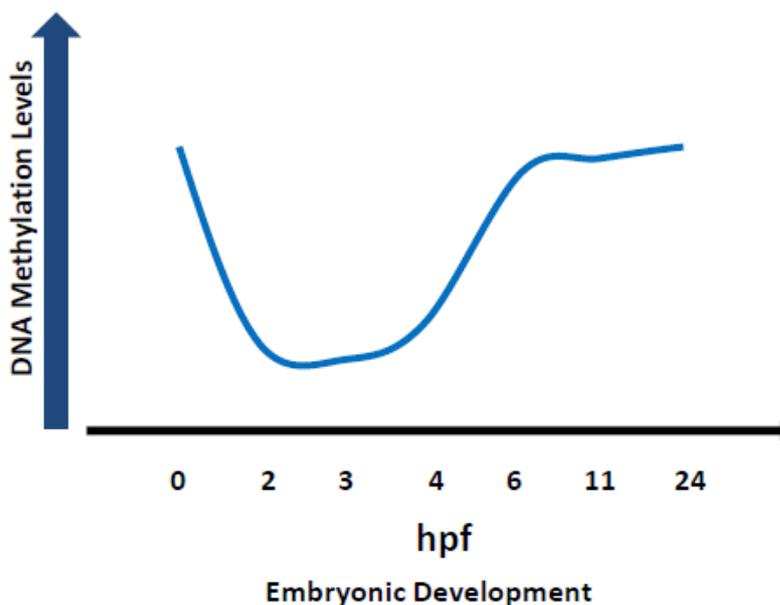


Figure 6.3 Methylation Dynamics during zebrafish embryogenesis

As in mammals, DNA methylation levels during zebrafish embryogenesis are also highly dynamic. Demethylation occurs upon fertilisation, de novo methylation begins approximately at the mid blastula stage (3hpf) and continues until 6hpf (the beginnings of gastrulation)



Period	Stage	Time (hpf)	Description
Zygote	1 cell	0	
Cleavage	2 cell	0.75	Rapid Cell Division
	64 cell		
Blastula	128 Cell	2.25	Point of Mid-Blastula Transition/ Zygotic Gene Activation Beginnings of Epiboly
	High		
	Sphere		
	Dome		
Gastrula	30% Epiboly	5.25	Body axis formation Germ Layer Formation Rudiments of brain and notochord formation
	50% Epiboly		
	Germ Ring		
	Shield		
	75% Epiboly		
Segmentation	90% Epiboly	10	Somite Formation Development of neuromeres Primary organogenesis
	Bud		
	3 Somite		
Pharyngula	26 Somite	24	First movements apparent
	Prim 5		

Figure 6.4 Summary of early zebrafish development

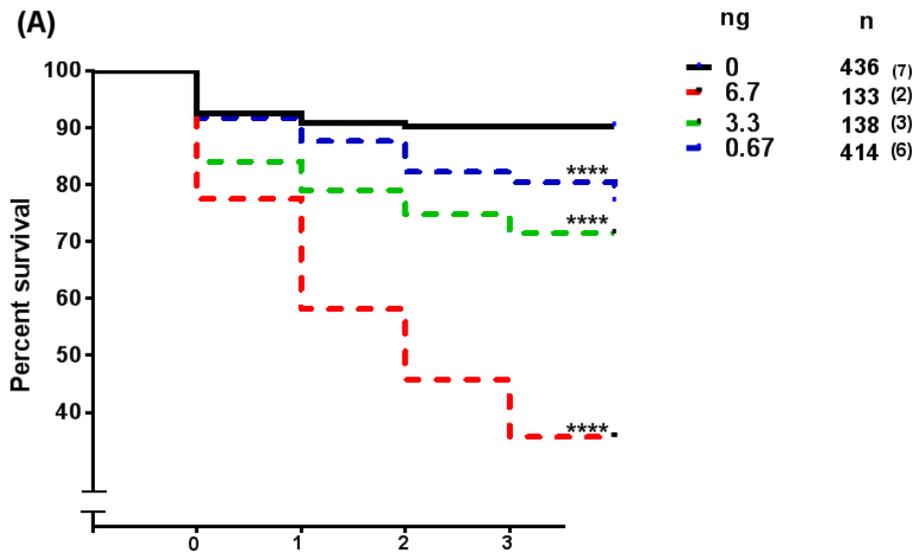
The ortholog of H2A.Z in zebrafish is H2afv. One previous study mutated H2afv during a mutation screen, with mutants characterised by significant curvature of the trunk and defects in pharyngeal arch development. Total mortality was observed by 13 days after fertilisation(235). Although this mutant is an efficient method of H2afv depletion it would not be suitable for the purposes here. As we intended to observe the effects of H2A.Z depletion early in embryonic development, H2afv depletion through morpholino knockdown was decided upon.

Morpholino oligonucleotides (MOs) are the most common form of anti-sense knockdown deployed in zebrafish(236). Morpholinos are approximately 25 base oligomers which recognise and pair with target RNA. Two types of morpholino exist: splice blocking which disrupt pre-mRNA processing through inhibition of spliceosome components or translation blocking which prevent ribosome function(237). Titration of morpholino dose should enable partial knockdown of H2afv protein expression, preventing lethality of null phenotypes observed in mice and drosophila yet allowing investigation of this histone variants role on embryonic development and methylation status.

6.3 Initial morpholino titration

The first step was to identify an effective dose of morpholino (MO), the ideal dose being one to provide a characteristic phenotype yet not so severe to impede correct development. I initially injected a titration of three doses: 6.7, 3.3 and 0.67ng and followed survival and development over the first five days after fertilisation (Figure 6.5A). Representative images at 4dpf of each dose compared with standard control injected fish are illustrated in Figure 6.5B. Morpholino knockdown was effective at all 3 doses, clearly demonstrated by Western Blot (Figure 6.5C). All three concentrations cause distinct phenotypic differences with severity increasing with increased dose. 6.7ng of morpholino causes substantial deformities with only a 40% survival rate and was therefore considered too strong. 3.3 and 0.67ng result in similar survival rate of 75-85%.

I went on to further characterise phenotypic abnormalities. Again fish were injected with either 0.67 or 3.3ng of *h2afv* MO or a standard control MO. Morphant morphology was assessed at two and four days post fertilisation and morphants were categorised as normal, mild or severe in phenotype. Representative images of each category are shown in Figure 6.6A along with phenotype percentage scores (Figure 6.6B). Each dose was injected into a minimum of three clutches. Although injection with 3.3ng of morpholino results in a similar survival rate to that of 0.67ng, severe phenotype percentage is increased. This results in a large number of highly deformed fish, making the study and characterisation of phenotype much more difficult. 0.67ng was selected as a suitable dose and used for all subsequent experiments.



(B)

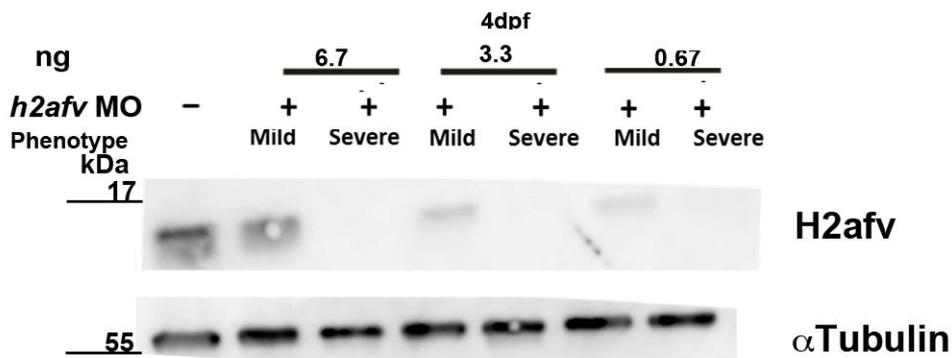


Figure 6. 5 Morpholino injection successfully suppresses H2afv expression.

(A) Kaplan-Meier curve demonstrating survival of *h2afv* morphants across the first 4 days of development compared to Std control MO injected fish. Three different doses of *h2afv* MO is used (0.67, 3.3 or 6.7ng). (B) Bright field images of fish injected with 0.67, 3.3 or 6.7ng *h2afv* MO compared to controls (C) Western Blot analysis of H2afv protein levels in lysates isolated from fish injected 0.67, 3.3 or 6.7ng *h2afv* MO compared to controls

6.4 Morphant phenotype

Morphants in Figure 6.6 can be characterised by distinct phenotypic abnormalities in comparison to standard control injected fish. Morphants present with ventral curvature of trunk, abnormalities in pigment, smaller eyes and a reduction in head length. Severe morphants are more exaggerated in these traits. Figure 6.7 further highlights their smaller size in comparison to control fish as well as the presence of down curved heads (a), under utilisation of yolk (b) and abnormal tail yolk extension(c). At later time points (4 to 5dpf) morphants show poor swim bladder development as well as moderate deformities in organ development. LiPan transgenic fish express dsRed and GFP under liver specific (*fabp10*) and pancreas specific (*ela3l*) markers respectively. Injecting into this line therefore allows visualisation of liver (Figure 6.8A) and pancreas development (Figure 6.8B). Both morphant livers and pancreases appear reduced in size and misshapen in comparison to control fish.

Another distinct characteristic displayed by morphants is reduced swimming ability and abnormal response to tactile stimulus. Touch response was quantified by a simple motility assay demonstrated by Figure 6.9A. 2dpf fish were used as it is the first timepoint at which they are sensitive to touch stimulus. Fish were placed into the centre of a dish with concentric rings labelled Zone 1, Zone 2 and Zone 3. They were lightly touched once on the head and scored based on the zone they swam into. Only *h2afv* morphants categorised as mild in phenotype were assessed as extreme ventral curvature of severe morphants prevented sufficient movement for measurement. *h2afv* morphants responded poorly in comparison to controls, with 40% unable to move beyond Zone 1 (Figure 6.9B).

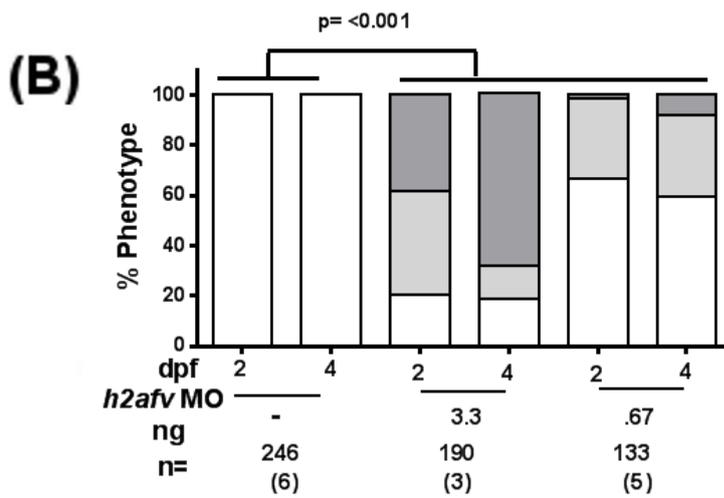
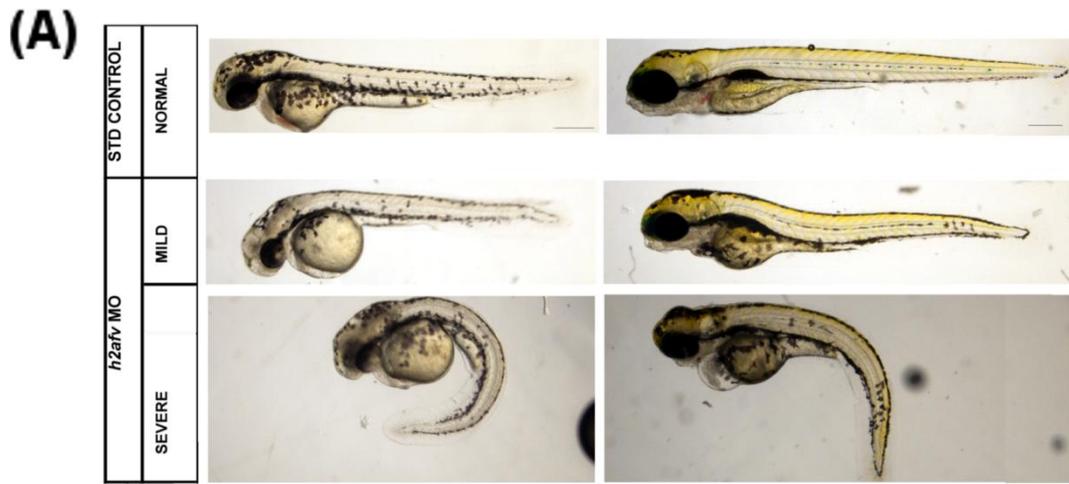


Figure 6. 6 Morphants display distinct phenotypic abnormality when scored at 2 and 4dpf.

(A) Bright field images comparing Std control and *h2afv* MO injected fish at 2 and 4dpf. *h2afv* morphants classified as mild or severely affected. **(B)** Percentage phenotypes of Std control and 3.3 or 0.67ng *h2afv* MO injected fish

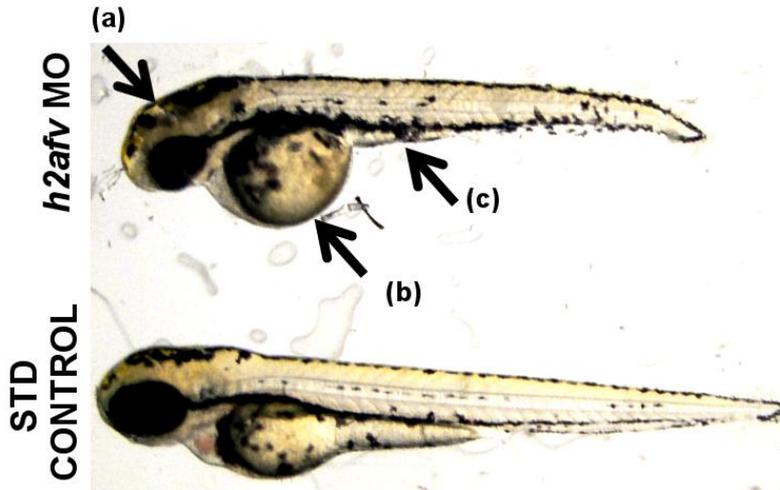


Figure 6.7 Further phenotypic characterisation of *h2afv* morphants at 3 dpf
h2afv morphants are noticeably decreased in size in comparison to Std Control fish. In addition, they present with down curved heads (a), under utilisation of yolk (b) and abnormal tail yolk extension(c)

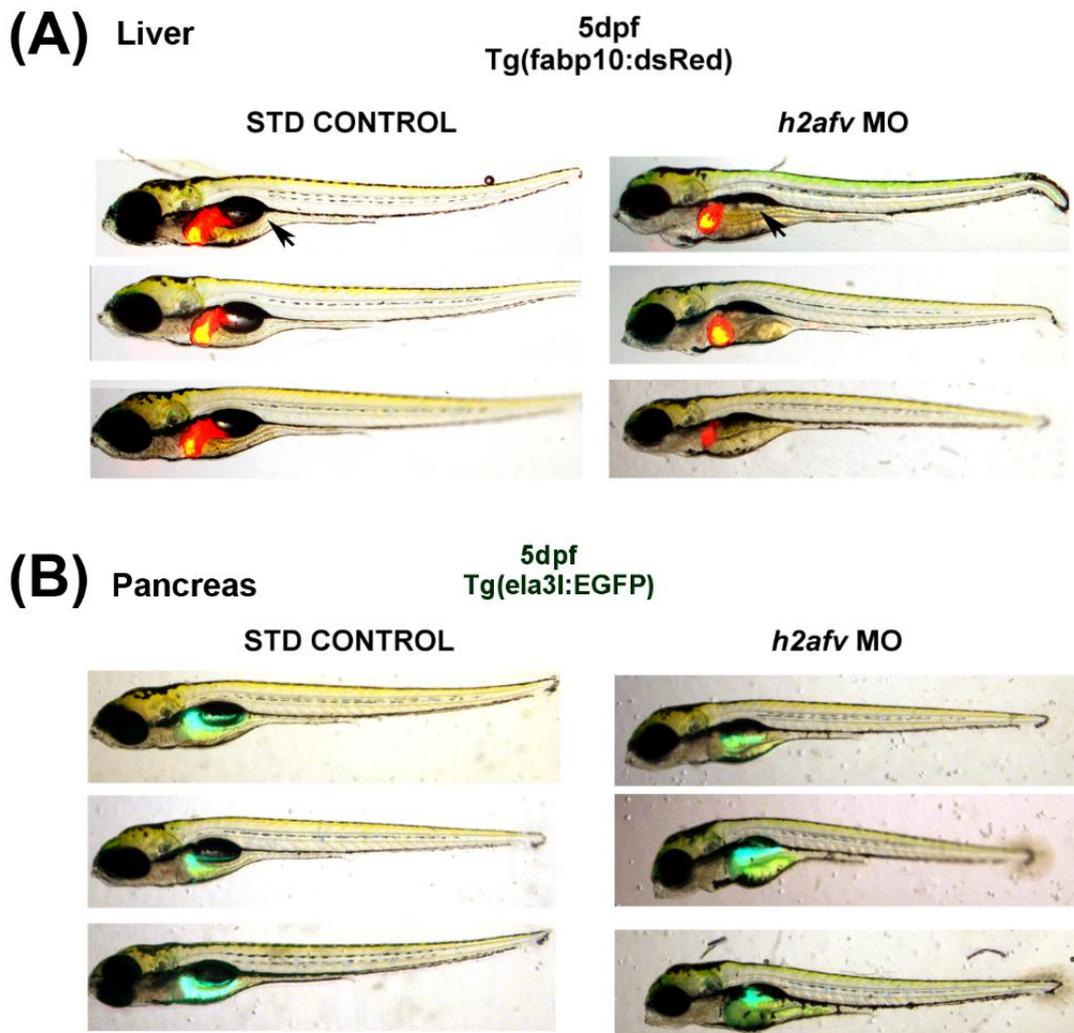


Figure 6.8 Organ defects present at later developmental stages.
 5 dpf morphants fail to develop swim bladders (black arrows). Reduced size and irregular shape of morphant livers (A) and pancreases (B) can be visualised using fish transgenic for liver specific (*fabp10*) and pancreas specific (*ela3l*) markers.

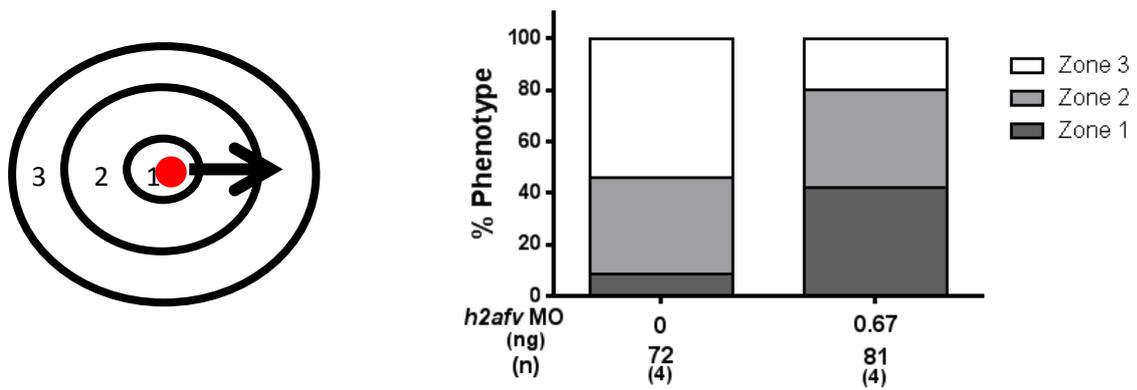


Figure 6. 9 Morphants respond abnormally to touch

(A) Diagram illustrating set-up of touch response assay. Fish were placed into the centre of a dish with concentric rings labelled Zone 1, Zone 2 and Zone 3 and their destination recorded upon stimulus **(B)** Percentages of *h2afv* morphants or control fish reaching each zone.

One caveat of working with morpholinos is the risk of non specific effects. Activation of the tumour suppressor p53 is one of the most common observed off target effects, leading to excessive apoptosis(238) To investigate the potential of off target cell death, apoptosis was investigated by acridine orange staining . At 24hpf, no significant differences between morphant and controls can be observed (Figure 6.10). To further control for off target p53 activation, I coinjected *h2afv* and *p53* targeting MOs in two separate clutches of fish and scored for phenotype at 2dpf. Co-knockdown of p53 and H2afv produced similar levels of phenotypic abnormality when compared to H2afv knockdown alone, with morphants still presenting with misshapen heads, curvature and reduced size. (Figure 6.11 A and B)

To further establish specificity of knockdown, rescue of phenotype was attempted by injection of *h2afv* mRNA. Fish were co-injected with when mCherry tagged *h2afv* mRNA and *h2afv* MO and morphants scored at 2dpf. Visually mRNA and MO co-injected fish appear similar to controls (Figure 6.12A). When scored across four separate clutches, phenotypic abnormality was reduced in co-injected fish from approximately 60% affected to 40% affected. (Figure 6.12B). Furthermore, Western blot confirmed that the exogenous H2afv-mCherry was well expressed but this did not restore expression of the endogenous H2afv. Phenotype was apparent in approximately 30% of mRNA only injected fish. However, developmental defects associated with *h2afv* mRNA injection have previously been reported in *Xenopus*(239).



Figure 6.10 *h2afv* MO injection does not cause excessive apoptosis at 24hpf
 24hpf Std Control and *h2afv* MO injected embryos were stained with Acridine Orange and visualised under fluorescence. No significant difference in number of apoptotic cells can be observed.

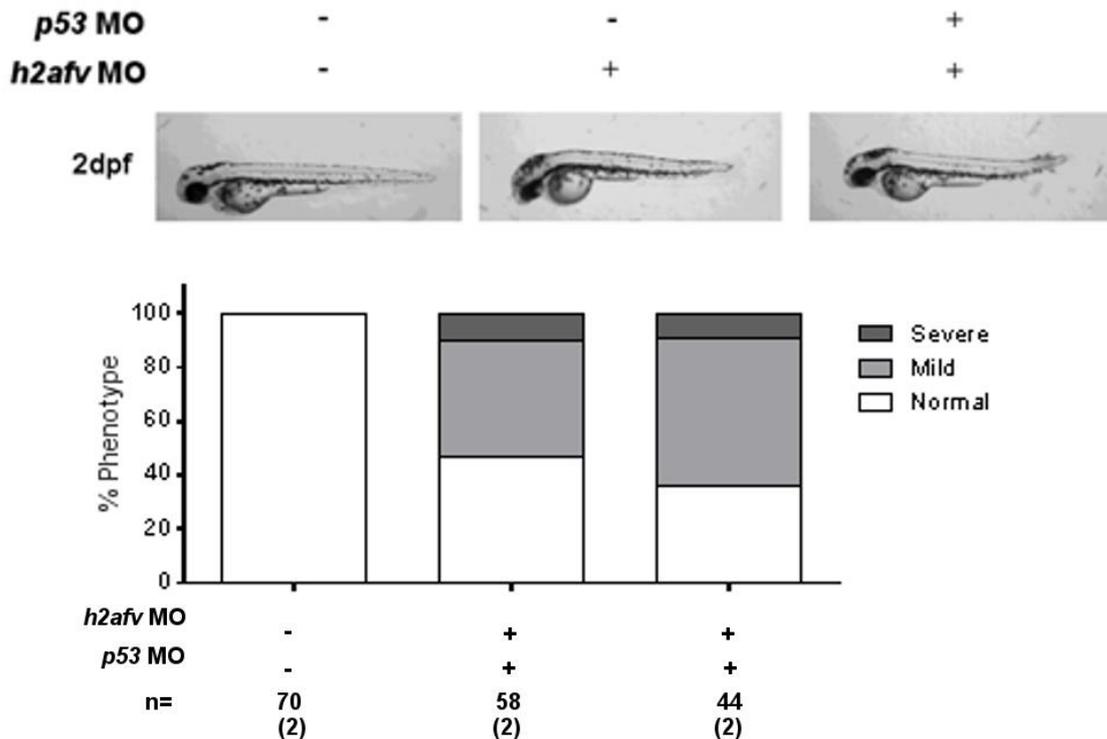
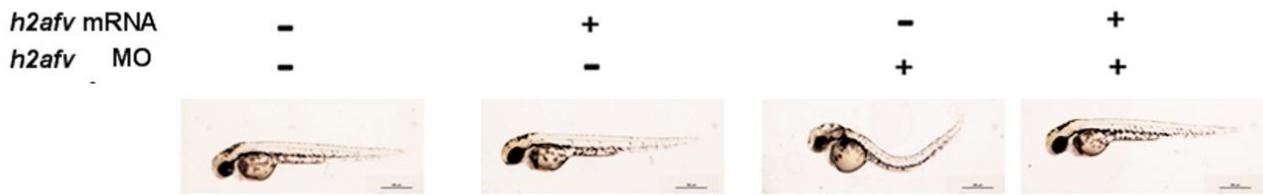
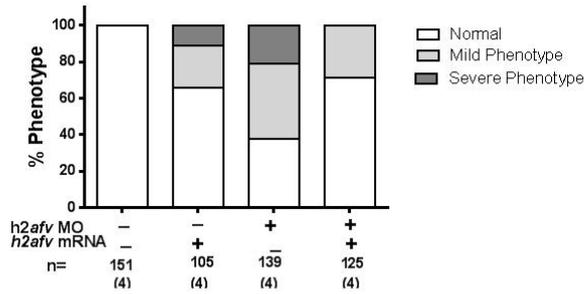


Figure 6.11 Co-knockdown of H2afv and P53 does not reduce phenotypic abnormality at 2dpf
 Embryos were injected with Std control, *h2afv* MO or *h2afv* MO and *p53* MO and scored at 2dpf. Bright field images demonstrate phenotypic abnormalities in *h2afv* MO and *h2afv* + *p53* MO injected fish. Similar % of phenotypic abnormality observed in both *h2afv* MO injected and co-injected groups.

(A)



(B)



(C)

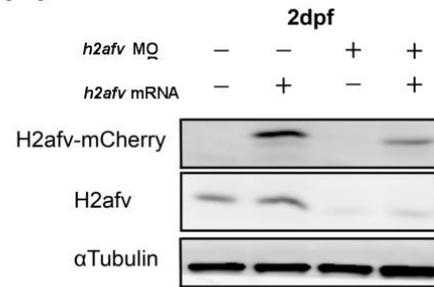


Figure 6.12 Injection of *h2afv* mRNA rescues phenotypic abnormality at 2dpf

Fish were injected with *h2afv* mRNA either singly or coinjected with *h2afv* MO and scored at 2dpf. **(A)** Bright field images demonstrated phenotypic rescue of coinjected fish. **(B)** Phenotype percentage scores at 2dpf **(C)** Western Blot analysis of H2afv protein levels at 2dpf in Control, *h2afv* mRNA, *h2afv* MO and co injected groups

6.5 H2afv at Early Developmental Stages

These data clearly demonstrate the importance of H2afv for early development in zebrafish as even injection of such a low dose of morpholino causes multi-system defects. I next aimed to determine when phenotypic differences were first noticeable. Std Control and *h2afv* MO injected fish were observed over the first 24 hours of development and scored at Shield, Bud, 15-17 Somite and Prim 5 developmental stages, with a minimum of three clutches observed at each stage (Figure 6.13). No significant differences in phenotype or mortality are observable at Shield or Bud stage. By mid-somatogenesis (15 – 17 somite stage) morphants can be categorised as mild or severely affected, again appearing reduced in size and with abnormalities in head development. By Prim5 a significant difference in mortality is apparent. Further deficiencies in head development are noticeable, with an apparent delay in formation of the midbrain-hindbrain boundary . Again traits are exaggerated in severely affected morphants, with significant disruption of head development. These data demonstrate that defects arising from *h2afv* deficiency can be observed at an early developmental time point.

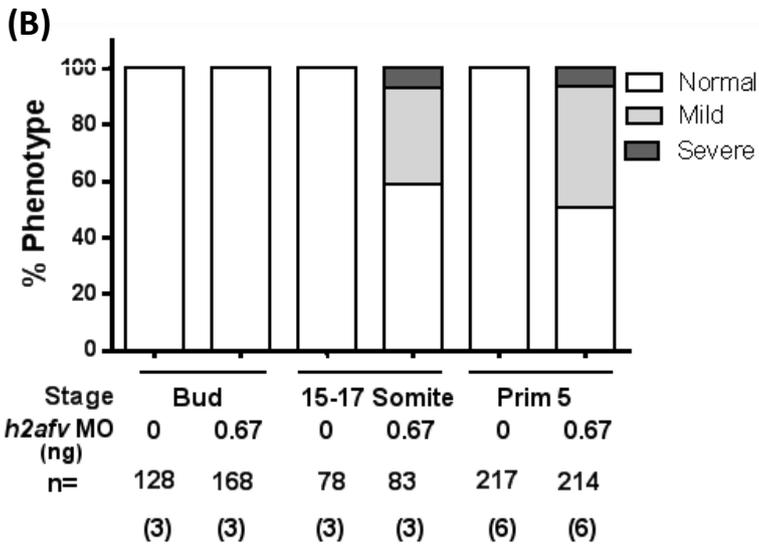
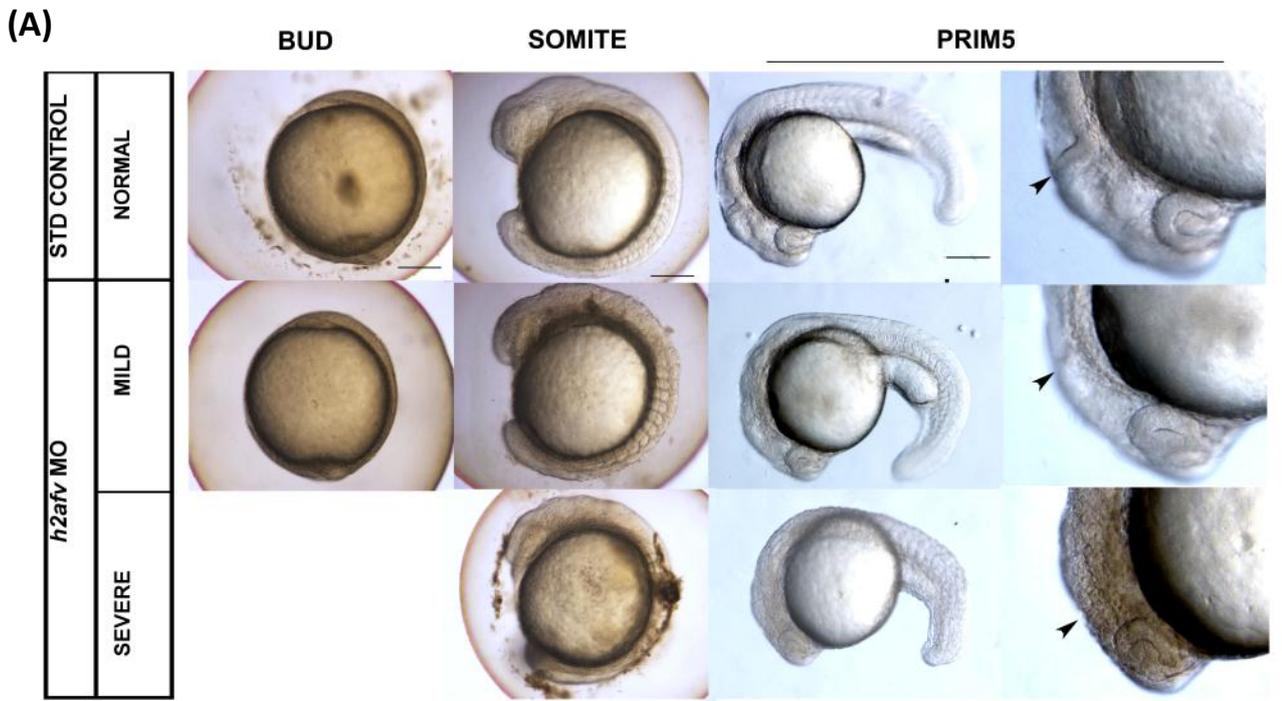


Figure 6. 13 Developmental abnormality first observed at the 15 – 17 somite stage
(A) Bright field images comparing Std control and *h2afv* MO injected fish at Bud, 15-17 somite and Prim5 stages. *h2afv* morphants classified as mild or severely affected. **(B)** Percentage phenotypes of Std control and *h2afv* MO injected fish at Bud, 15-17 somite and Prim5 stages

Further exploration of transcriptional disruption due to H2afv deficiency was next required. As H2A.Z depletion has previously been associated with gastrulation defects in mice and *Xenopus* (73, 216), I first asked whether a deficiency in a specific germ layer could be detected. For example, later morphants display moderate defects in endoderm derived organs such as pancreas and liver therefore disruption of early endodermal markers could precede this. RNA was isolated from whole embryos at the 15-17 somite stage, post-gastrulation and at the developmental stage where phenotype differences are first apparent.

qRT-PCR analysis markers specific for endo-,exo- and mesoderm was conducted in a minimum of three clutches (Figure 6.14). Expression changes were modest and no pattern specific germ layer disruption is apparent. Small but significant differences occur for several markers: *sox9a*, *pitx3*, *snail*, *sox17*, *ntla*. *sox9a*, *pitx3* and *snail* are noteworthy as their expression is significantly associated with neural regions while *ntla* is one marker particularly sensitive to methylation.

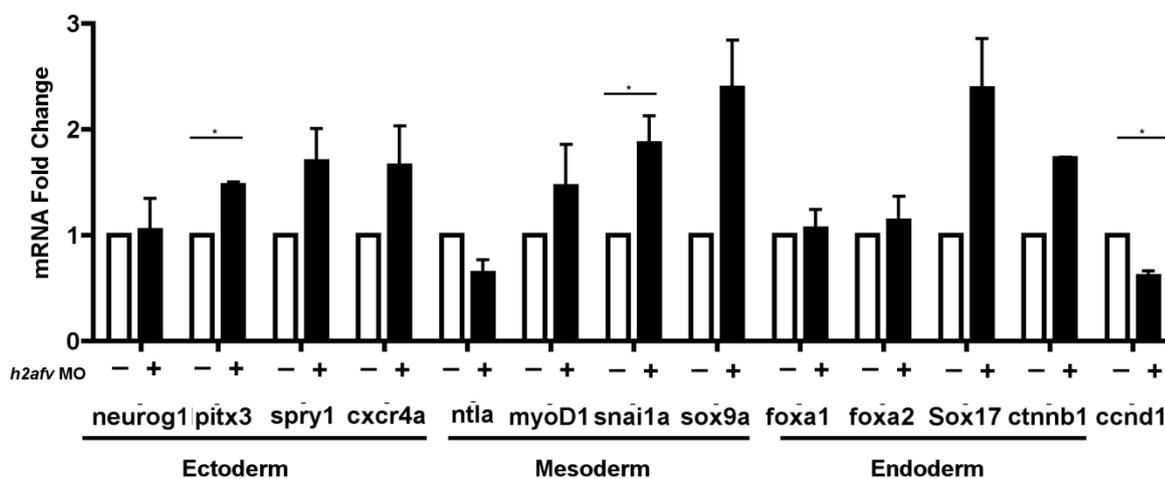


Figure 6. 14 Limited disruption of endoderm, mesoderm and ectoderm specific markers. mRNA levels of endoderm, mesoderm and ectoderm specific markers were analysed at the 15-17 somite stage by qRT-PCR in Std Control and *h2afv* MO injected fish (n=4). qRT-PCR results are expressed as fold change normalised to control \pm SEM. *p<0.05 Student's *t* Test (unpaired)

6.5 Characterisation of nervous system defects

It was next hypothesised that *h2afv* morphants may present with nervous system defects.

Morphologically, morphants display visibly misshapen heads, smaller eyes and poor motility along with moderate differential expression of neurally expressed genes at 15-17 somite stage. H2afv expression is predominantly localised to the CNS when mCherry tagged *h2afv* mRNA is overexpressed by injection at the 1 – 4 cell stage (Figure 6.15). Enrichment of H2A.Z at promoters of genes regulating neural development has been reported in human ESCs and interestingly, analysis of the zebrafish sperm genome identified enrichment of H2afv at genes associated with hindbrain formation (225, 227). Based on this, I hypothesised that genes involved in neural development and differentiation may be particularly susceptible to H2afv depletion. To explore this possibility of neural development defects, H2afv expression was first knocked down in the *ngn1*GFP transgenic line.

Ngn1 is a transcription factor which acts as a proneural marker. Expression of *ngn1* begins at gastrulation and remains until adulthood. It initially marks broad regions of cells with potential to adopt neuronal fate while later expression marks sensory and dorsal root ganglia(240). *Ngn1*:GFP transgenics were injected with Std Control or *h2afv* MO and GFP expression pattern observed across a time course of development. No differences in patterning in brain or somites were obvious at 19-21 somite stage or later at 1dpf (Figure 6.16A and B). This suggests that no differences in proneural differentiation driven by *ngn1* occur. However at later time points of 2 and 3dpf, ectopic dorsal root ganglia neurons can be observed in the trunks of morphants (Figure 6.14C).

At later time points, *ngn1* serves as a maker of sensory neurons, not motor neurons(241). Due to poor motility and movement of morphants, I hypothesised that a defect in motor neuron development could be occurring. To investigate this, I used a different transgenic line where GFP

expression is driven by *isl1*. *Isl1* is a LIM/Homeobox gene family member and expressed by primary motor neurons early in development(242). Again, early expression of *isl1* was not disrupted in *h2afv* morphants in comparison to controls. By 36hpf however, there were significant differences in patterning of expression, particularly in faciobranial motor neurons (Figure 6.17). This may suggest disruption of motor neuron development arising from *H2afv* deficiency.

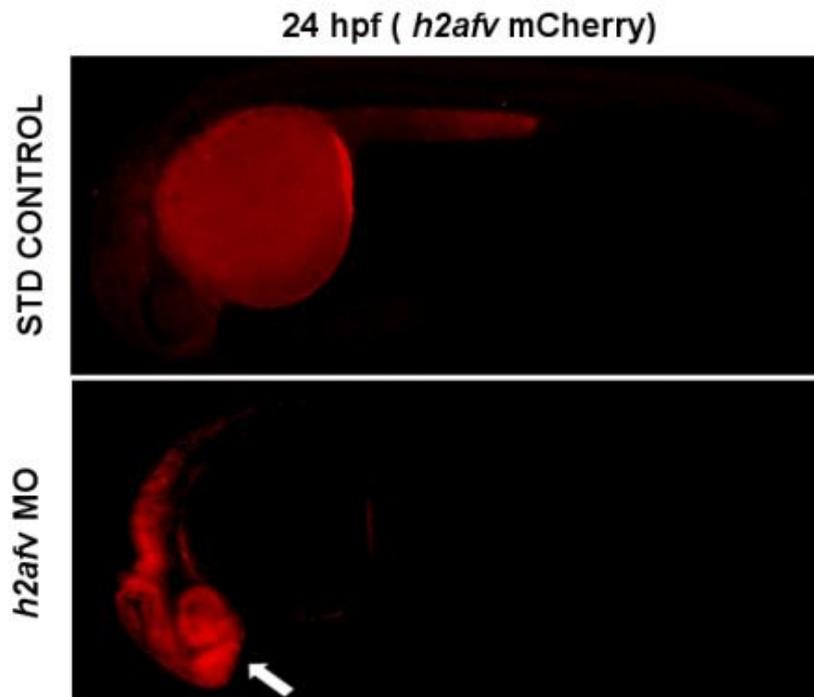


Figure 6. 15H2afv is localised to the CNS

Fluorescent microscopy visualisation at 24hpf of mCherry tagged *h2afv* mRNA. Robust expression can be observed in the CNS. No fluorescent signal is observed in control injected fish.

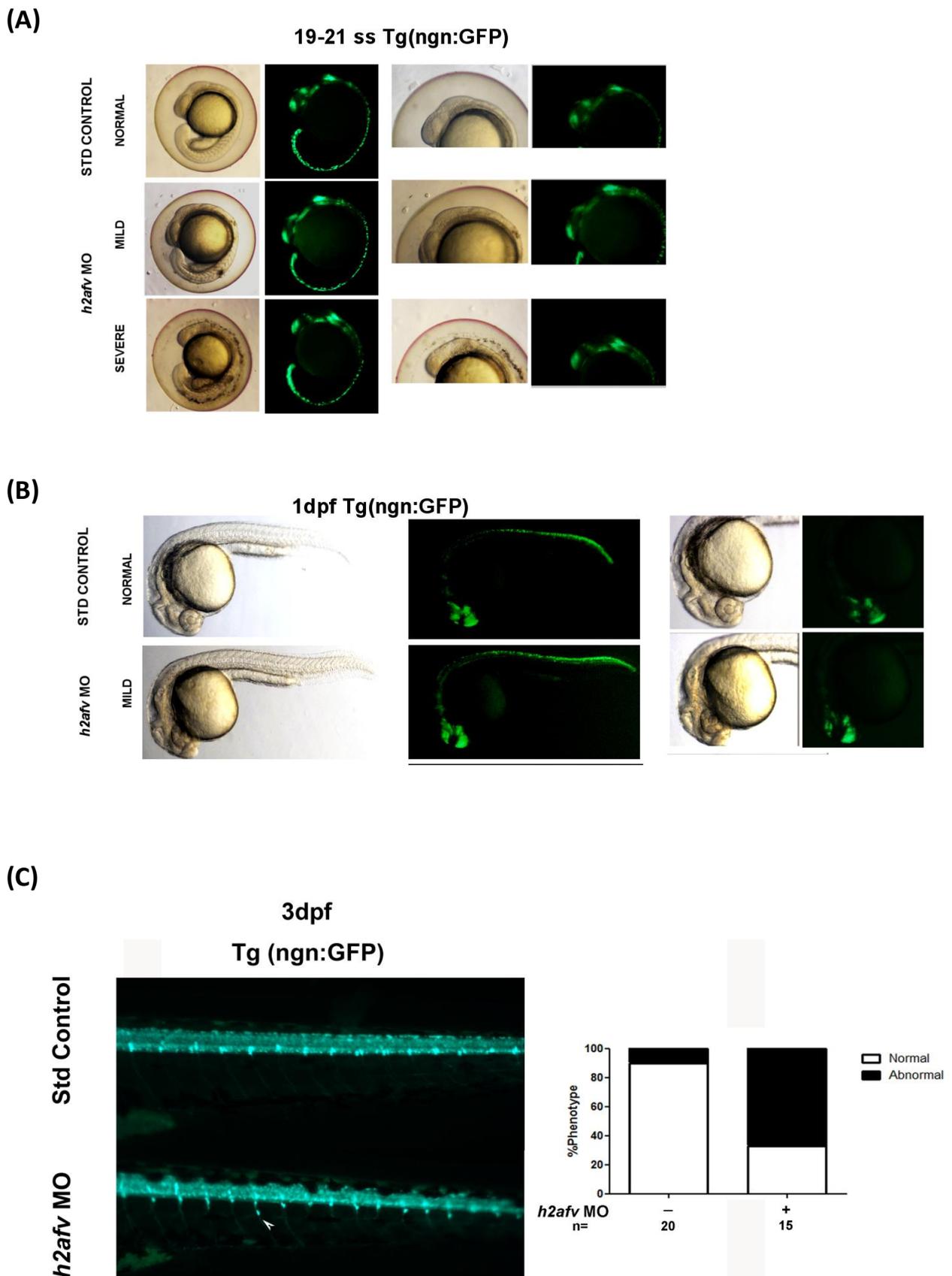


Figure 6. 16 *ngn1* expression in morphants at 19-21 somites, 1 dpf and 3dpf. *ngn1*GFP transgenics were injected with *h2afv* MO or standard control. **(A)** and **(B)** Fluorescent and Bright field images of fish at 19-21 somites or 1 dpf show no detectable differences in patterning **(C)** By 3dpf, ectopic expression of trunk *ngn1* positive neurons can be visualised to approximately 70% of morphants (white arrows)

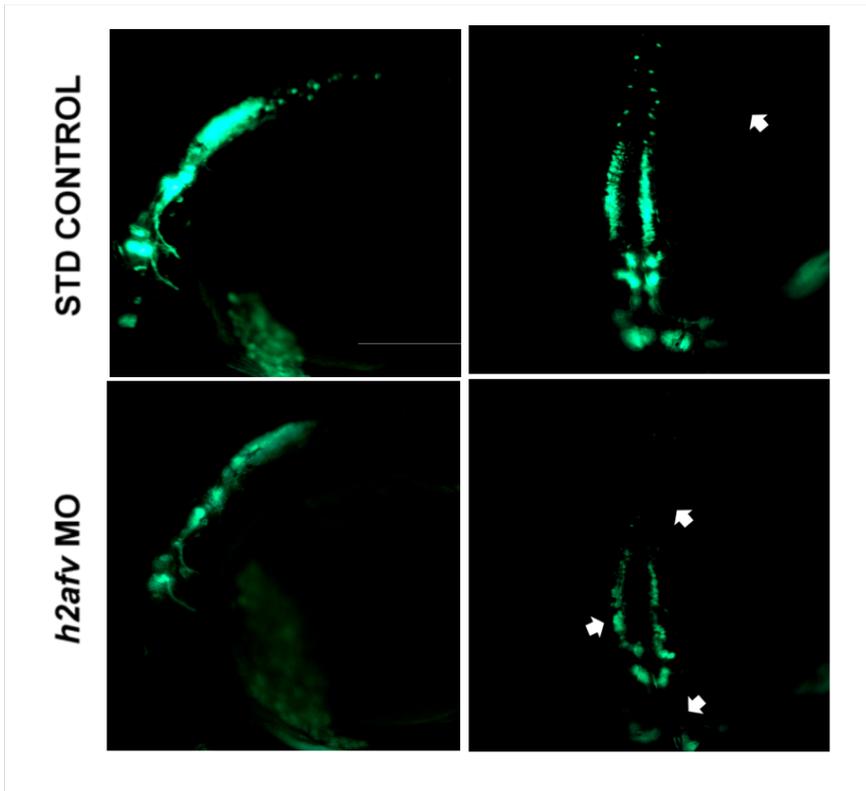


Figure 6. 17 Disrupted *islet1* expression visible in morphants.

Std Control and *h2afv* MO injected *islet1*:GFP transgenics were visualised at 36hpf. Disrupted *islet1* patterning can be observed in *h2afv* MOs (white arrows).

6.6 Assessment of methylation in early h2afv morphants

I have so far been able to demonstrate effective knockdown of h2afv resulting in characteristic phenotypes, detectable from an early developmental stage. Having established the importance of h2afv for correct development, I next wanted to investigate its potential effects on methylation. As mentioned previously, h2afv is thought to be anti-correlative to methylation, shown using the model plant organism *Arabidopsis*(226). From this I predicted increased levels of DNA methylation would be detectable in H2afv deficient fish in comparison to controls.

I investigated global levels of DNA methylation by quantifying levels of methylated cytosine (5 MeC) Genomic DNA was isolated from whole embryos at early developmental stages (Shield, Bud, 15-17 Somite and Prim5) and immunoblotted using a 5MeC specific antibody. Separate samples blotted in parallel were stained with 0.2% Methylene blue in 0.3M NaOAc to detect total DNA. 5MeC levels were determined by normalising to total DNA. A minimum of three clutches was used for each developmental stage. Morphants appear consistently hypermethylated across early development, difference reaching statistical significance at 15- 17 somite stage (Figures 6.18A, B and C). This correlates with hypothesis and confirms differences observed previously in plant models. Hypermethylation can only be observed during these early developmental time points. No differences in 5MeC levels are apparent at later stages such as 1dpf and 4dpf (Figure 6.18D and E).

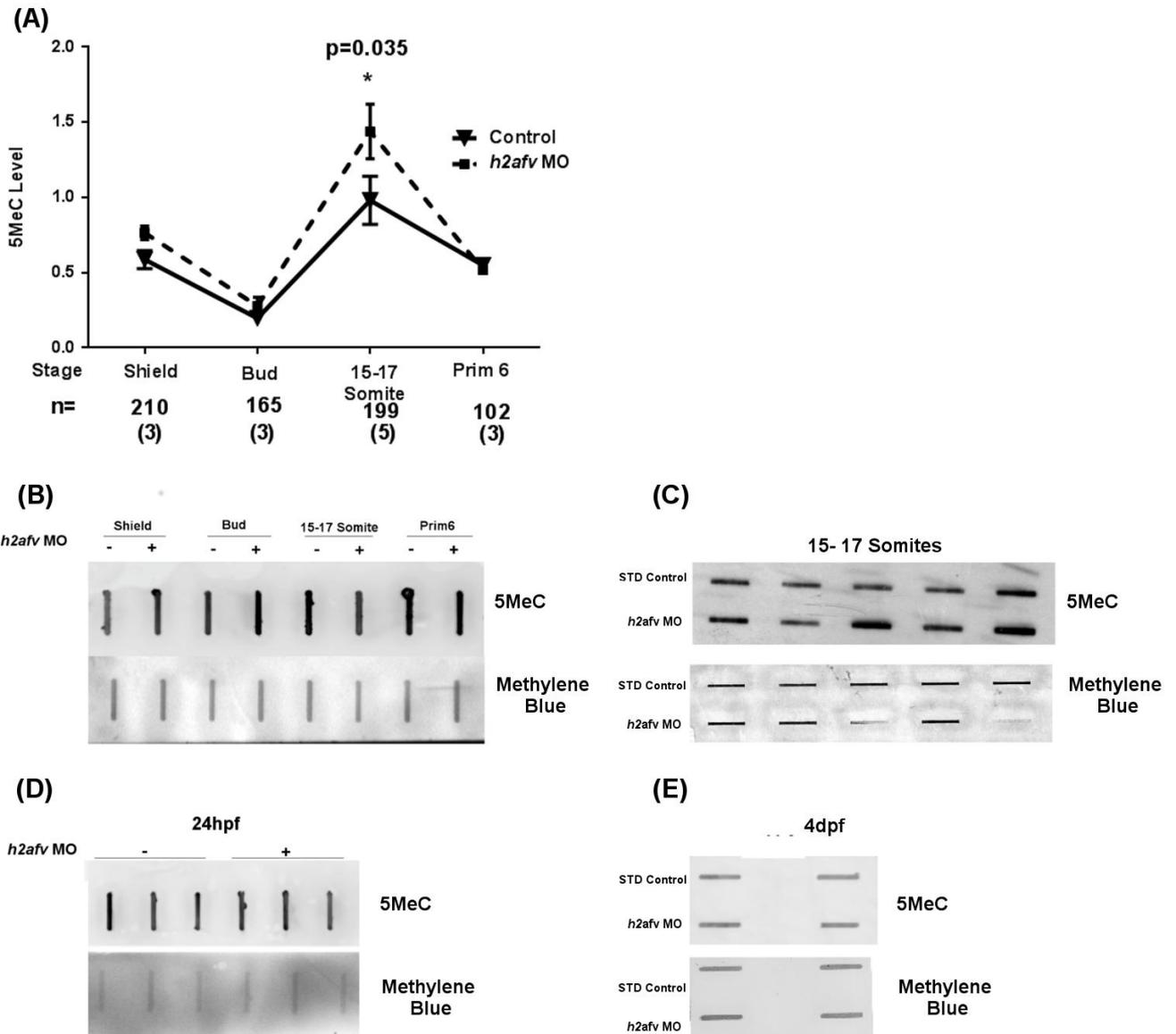


Figure 6.18 *h2afv* morphants are hypermethylated at early stages of development.

(A) Genomic DNA was isolated from whole embryos at the Shield, Bud, 15-17 Somite and Prim 6 developmental stages and levels of 5MeC analysed by immunoblot. Morphants are consistently hypermethylated at early developmental timepoints, reaching statistical significance at the 15-17 somite stage (B) and (C) Representative immunoblots. At later time points of 24hpf (D) and 4 dpf (E) no increased methylation could be detected. * $p < 0.05$ Students *t* Test (paired).

I next asked how this hypermethylation may be mediated. In Zilberman et al, MET1 the Arabidopsis homolog of dnmt1 was shown able to regulate DNA methylation patterns and subsequently alter H2A.Z distribution(226). Here, behaviour of the zebrafish homolog dnmt1 was investigated upon H2afv knockdown. Protein expression of Dnmt1 was unchanged upon H2afv knockdown at 1dpf (Figure 6.19A). Next it was questioned whether knockdown of Dnmt1 could reduce hypermethylation in morphants and therefore reduce the phenotype. Co-injection of *h2afv* and *dnmt1* MOs successfully knocked down dnmt1 protein levels (Figure 6.19B) and reduced 5 MeC levels in three clutches of morphants (Figure 6.19C and D). However, there was no improvement in phenotype of co-injected morphants over the first 4 days of development. Instead, phenotypes of co-knockdown appeared additive, resulting in a higher percentage of severely affected animals (Figure 6.19E). This suggests that hypermethylation and morphant phenotype is not driven by over activity of dnmt1.

Finally, a brief analysis of histone methylation was undertaken. Methylation of histone 3 at common sites such as Lysine 9 (H3k9me3) and Lysine 27 (H3k27me3), associated with transcriptional silencing was investigated by western blot. Again no differences were observable. This suggests disruption of DNA methylation only occurs upon H2afv knockdown (Figure 6.20).

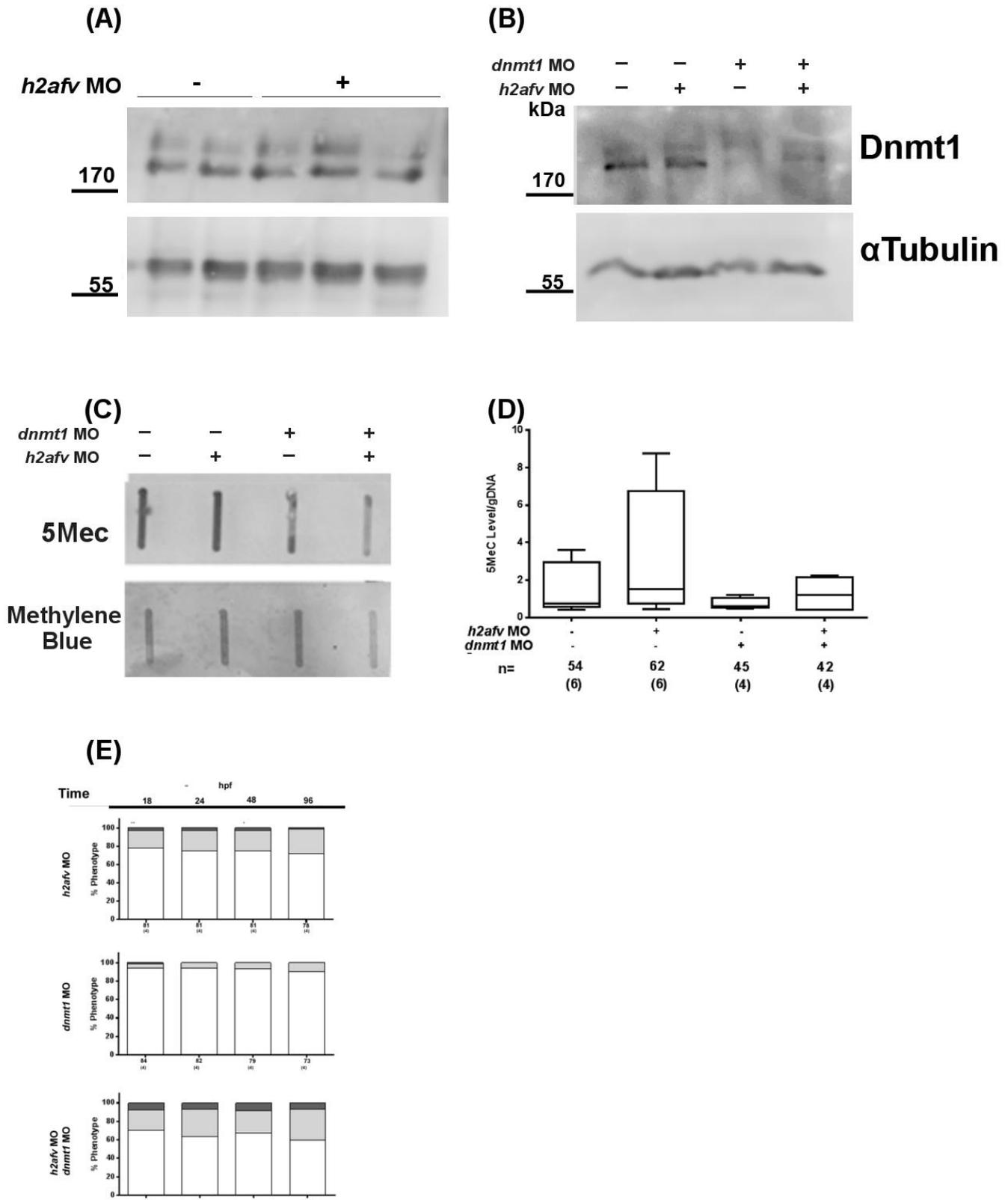


Figure 6. 19Dnmt1 does not appear to mediate hypermethylation

(A) Dnmt1 protein expression analysed in lysates from *h2afv* MO and Std control injected fish. **(B)** Dnmt1 protein expression reduced in protein lysates from fish injected with *dnmt1* MO and coinjected with *h2afv* and *dnmt1* MO. **(C)** and **(D)** Analysis of 5MeC levels in genomic DNA from Std control or *dnmt1* and *h2afv* MO injected fish. **(E)** Phenotypes of single MO or coinjected fish were scored until 96hpf. Coinjection did not reduce phenotype of *h2afv* morphants

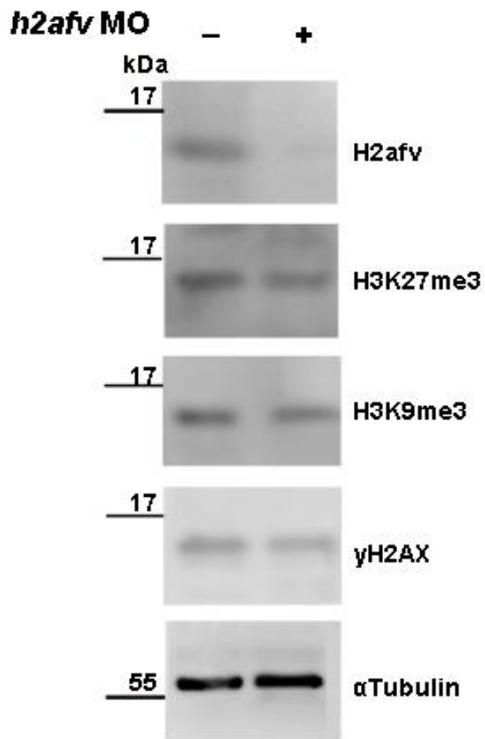


Figure 6.20 *h2afv* MO does not alter expression of histone marks at 24hpf

Western blot analysis of H2afv, H3K27me3, H3K9me3 and γH2AX protein expression in lysates from 24hpf control and *h2afv* morphant fish. αTubulin serves as a loading control.

6.7 Discussion

H2A.Z has previously been suggested as a factor involved in passage of non-mendelian hereditary information between generations: its incorporation in particular loci in sperm leading to decreased methylation of promoters in offspring(48). This chapter aimed to explore whether H2A.Z may be able to influence early methylation patterns using the embryonic zebrafish as a model organism. The anti-correlative relationship between H2A.Z and DNA methylation has been previously explored in the model plant organism *Arabidopsis*(226) and *in vitro* culture studies(243) however data from *in vivo* animal models remain lacking. Data from this chapter has served as a first step in establishing this.

Here I have been able to effectively knockdown the zebrafish homolog of H2A.Z (H2afv) during the first five days of embryonic development. Similar to previous reports, this chapter demonstrates the importance of H2A.Z for embryonic development with morpholino injected animals displaying significant mortality and physiological defects. The hypothesis of H2afv influencing DNA methylation is supported by simple analysis of global methylation levels. Immunoblotting for total levels of methylated cytosine in whole embryos suggests increased levels of DNA methylation during the early stages of development. DNA methylation levels are highly dynamic during early development(222). Genome wide demethylation occurs upon fertilisation, with gradual remethylation occurring as development precedes. In zebrafish, reannotation of the genome begins at approximately 4hpf(244). Here we demonstrate hypermethylation of morphants throughout this reannotation period and beyond until 24hpf.

Future work comparing methylomes of control and morphant samples through next generation sequencing will enable further insight into patterning of methylation differences. Another step will be to determine how this hypermethylation is mediated. The effects of H2afv depletion on Dnmt1, the maintenance methyltransferase, were investigated here. No differences in Dnmt1 expression could be observed upon H2afv depletion and no rescue of phenotype was observed upon co-knockdown. This may suggest that hypermethylation is not mediated by Dnmt1. As a maintenance

methyltransferase, Dnmt1 is limited to copying patterns of hemimethylated DNA. A next step would therefore be to look at the *de novo* methyltransferases functional during the period of reannotation(222). Multiple homologs exist in zebrafish, with dnmt3 most closely resembling mammalian dnmt3b(245).

Morphological defects observed such as malformed heads, smaller eyes, delayed brain development and poor motility particularly suggest defects in nervous system development. This is further suggested by preliminary work using fish lines transgenic for markers of motor (*isl1*) and sensory neurons (*ngn1*). The developing nervous system is acutely sensitive to changes in DNA methylation(246). Neurons undergo highly specified cycles of specification, differentiation and maturation, dependent on specific methylation profiles. Developmental disorders such as fetal alcohol syndrome have been strongly associated with disruption of DNA methylation in the CNS(247) and altered methylation patterns have been linked with neurodegenerative disorders such as Alzheimer's disease(248, 249). Furthermore, other models inducing hypermethylation at early developmental stages have reported nervous system abnormalities (250, 251). This coupled with the localisation of H2afv expression to the nervous system makes the possibility of nervous system defects highly likely. The observations using *ngn1*GFP transgenic fish suggest there is no abnormality in neural specification as patterning is similar between morphants and controls at the early stages of 19 – 21 somites and 1 dpf. Early observations using *isl1*GFP transgenics also show no difference between morphants and controls. It is only at later time points: 36hpf for *isl1*:GFPs and 2 and 3dpf for *ngn1*:GFP that differences are observable. This suggests that there may be a defect in the maturation or activity of post-mitotic neurons rather than neuron specification. A critical next step will be to identify the effect of H2afv depletion on the signalling pathways critical in regulating this time point.

6.8 Conclusions

- H2afv is necessary for correct development for zebrafish
- H2afv depletion leads to hypermethylation during early developmental stages, consistent with previous results in plant and *in vitro* models
- Phenotypic defects upon H2afv depletion suggest a role in CNS development and differentiation.

Chapter 7: Final Discussion

The process of wound repair after injury is reminiscent of developmental events such as cell migration, redifferentiation and proliferation(9). Events modulating this in the early embryo may therefore be important regulators in adult tissue repair. This thesis combined two projects exploring different aspects of this. Firstly, how reactivation of developmental signalling pathways might be contributing to regulation of myofibroblast transdifferentiation and behaviour in liver fibrosis. Secondly, how altering early DNA methylation pattern through inheritance of epigenetic marks might affect response to injury in adult, using zebrafish as a model organism for study.

7.1 Wnt Signalling in HSCs

Chapters 3 through 5 focused on reactivation of the Wnt morphogen family in hepatic myofibroblasts and its potential effect on fibrosis progression. Wnt signalling had previously been suggested as a promoter of HSC transdifferentiation. However, information regarding the exact ligands involved and the mechanisms they regulate was unclear. This suggested that further in depth characterisation of how Wnts influence HSC biology was required

Chapter 3 succeeded in identifying activation of Wnt activity accompanying transdifferentiation of quiescent HSCs to fibrogenic myofibroblasts. I demonstrated upregulation of predominantly non-canonical ligands in aHSCs, suggesting that non-canonical signalling is the active branch. Chapter 4 demonstrated a lack of β -Catenin/TCF mediated response to Wnt stimulus. Instead, HSCs responded to stimulus through non-canonical effectors, further implying activity of non-canonical signalling. Inhibition of Wnt signalling in Chapter 5 through pan-Wnt inhibition successfully decreased markers of activated fibroblasts. These results suggest active Wnt signalling present in HSCs and playing a

profibrogenic role. Single ligand stimulus of HSCs with non-canonical Wnt5a appeared to govern survival response of mildly stressed aHSCs as well as paracrine stimulus of Kupffer cells (KCs).

7.1.1 Wnt Environment in HSCs

Several issues raised by this study need to be addressed. Firstly, single ligand stimulus with Wnt5a did not appear to affect HSCs as potently as KCs, with limited changes in gene and protein expression of fibrogenic markers. This lack of response could be due to several limitations. Issues with strength of stimulus and ligand multiplicity are already discussed in Section 5.7. Another consideration is that the microenvironment in a monoculture of HSCs is not truly representative of an *in vivo*, injured environment. Single Wnt ligand stimulus in a monoculture of HSCs may have been too simplistic a system, missing a critical component for effective stimulus. Exogenous TGF β treatment for example was shown necessary to promote β -Catenin/Smad activity in several studies. Co-culture of *in vitro* activated HSCs with Kupffer cells or exposure to LPS is able to shift gene expression pattern of cultured cells to one more representative of those activated *in vivo* (252). From this, it could be hypothesised that more complex cellular interactions may have been necessary for optimal Wnt signalling. Future work to explore this could involve Wnt treatment of multicellular systems such as that of precision cut liver slices, allowing study of an environment more representative of that *in vivo*. Study of Wnt in co-culture environments, HSCs with KCs or HSCs with hepatocytes may have been more informative.

Leading on from this, another limitation of this study is that the influences of other morphogen pathways have not been considered. During development, morphogens co-operate as part of a signalling network with strict spatial and temporal regulation of expression(10). Activity in adult tissue is subject to the same regulations and altering activity of one pathway is likely to have significant effects on other pathways, for example the Hh/Notch cross talk shown previously important in HSCs(66).

Morphogen pathways should therefore not be regarded in isolation and the idea of a wider network kept in mind when considering cellular response. Hh activity in HSCs is one morphogen pathway already well described. Interaction with and promotion of Wnt signalling by Hh is one possibility and has been reported in cancer models(253). However, Hh is better described as an antagonist of Wnt, with opposing effects on cellular differentiation in numerous instances during embryonic development and adult tissue homeostasis(254, 255). *In vitro*, Hh has also been implicated in Wnt activity suppression through upregulation of sFRP1(256, 257). Notch1 is also able to mediate canonical Wnt signalling through direct post-translation modification, binding the active form of β -Catenin and targeting it for degradation(178). Canonical Wnt/ Notch antagonism also regulates fate specification of hepatic progenitor cells: high levels of β -Catenin activity promoting hepatocyte fate while high Notch activity specifies cholangiocyte fate(258).

These data suggest that the aHSC may be a hostile environment for Wnt to function with decreased Fzd receptors expression, increased sFRP expression and elevated levels of other potentially antagonistic morphogen pathways (Figure 4.15). Single ligand stimulus of Wnt in overexpression/ conditioned medium experiments may therefore not have been sufficient to overcome this inhibition.

7.1.2 β -Catenin and HSCs

The second issue requiring consideration is β -Catenin's role in HSCs. The limited effect of β -Catenin overexpression here is at odds with the pro-fibrogenic canonical Wnt emphasis in current literature(104). However, several of these studies infer functional canonical TCF mediated signalling from upregulated β -Catenin expression and nuclear localisation. Here, I have also observed significant upregulation of β -Catenin protein expression in aHSCs, both total and active non-phosphorylated form. HSC β -Catenin responds initially to Wnt stimulus, correctly localising to the nucleus. It is TCF dependent downstream transcriptional activity here that appears to be lacking.

As summarised in the introduction, β -Catenin activity is well established in models of fibrosis in the lung, kidney and skin. Particularly in models of lung and kidney fibrosis, β -Catenin is considered pro-fibrogenic through promotion of epithelial to mesenchymal transition (EMT)(106, 177). EMT is the process by which epithelial cells transition towards a mesenchymal phenotype and involves loss of epithelial markers such as E-cadherin and a gain of mesenchymal markers such as vimentin(259). This β -Catenin/ EMT association may offer explanation for the numerous reports in other models of β -Catenin's profibrogenic activity, but the lack of effect observed here.

EMT is alleged to be one source of myofibroblasts during fibrosis, particularly EMT driven by TGF β /Smad signaling(259). Active β -Catenin mediating TGF β /Smad induced EMT is implicated in numerous reports of idiopathic pulmonary fibrosis and renal fibrosis. In the case of liver fibrosis, HSCs are suggested to be mesenchymal in origin, therefore transdifferentiation to active myofibroblasts will not require EMT. *In vitro* evidence has suggested that cultured hepatocytes exposed to TGF β are able to transdifferentiate into myofibroblast like, collagen producing cells(260). Taura *et al* (261) have since demonstrated *in vivo* that hepatocytes and their derived cells do not express α -SMA or other mesenchymal markers and are unable to produce collagen, suggesting that EMT in the case of liver fibrosis is an artefact of culture. β -Catenin in HSCs therefore is unlikely to be pro-fibrogenic through this mechanism.

Despite a lack of pro-fibrogenic stimulus, the presence of β -Catenin is clearly important for HSCs. siRNA knockdown in previous studies significantly decreases proliferation for example and I have demonstrated here that inhibition of β -Catenin/CBP activity impairs survival of HSCs. Similar to Wnt5a above, it is possible that β -Catenin signalling may not directly mediate fibrogenesis in HSCs but regulate alternative pathways such as cell survival. As TCF/LEF1 activity in HSCs has been shown defective this is likely to be mediated through TCF independent activity. Although β -Catenin /TCF signalling is the default model for Wnt function, β -Catenin is still able to operate independent of Wnt stimulus and downstream TCF function. Rather than trying to force TCF dependent signalling in an

unsuitable environment, it may be more beneficial to focus future work on the possibilities of TCF independent signalling in HSCs.

Firstly, this study was limited in analysis of β -Catenin protein expression, focusing solely on its total form or that of its active form, dephosphorylated at the Ser37 or Thr41 position. However, β -Catenin can be phosphorylated at several positions. Phosphorylation at Y654 suggests TGF β induced activity (174, 262) with pY654- β -Catenin not promoting TCF dependent transcriptional activity(262).

Phosphorylation at the Ser552 position suggests activity stimulated by Akt phosphorylation and promotion of cell survival, corresponding with the data obtained with β -Catenin /CBP inhibitor (198, 263). Determining phosphorylation patterns of β -Catenin in aHSCs may give clues as to its function.

TGF β /Smad signalling is one possible route of Wnt independent β -Catenin function, with detailed examples given in the discussion section of Chapter 4. TGF β is a critical regulator of myofibroblast behaviour(49). Future work exploring the interaction of β -Catenin and TGF β in aHSCs could identify another means for TGF β mediated regulation of HSC behaviour. A first step would be to establish how HSC β -Catenin behaves under TGF β stimulus, whether overexpression is more effective under these conditions. Multiple studies demonstrate binding of β -Catenin/ Smad transcription factors to initiate transcription(175, 176, 182). Immunoprecipitation assays to identify a β -Catenin/Smad transcriptional activating complex rather than a β -catenin/TCF complex would be solid evidence implying TCF independent activity of β -Catenin. Compared with the wealth of knowledge regarding β -Catenin /TCF activity, this area of β -Catenin function is relatively unexplored. Positive evidence of β -catenin regulating HSC behaviour through TCF independent mechanisms would therefore be an exciting finding.

Section 4.6 investigated several key β -Catenin nuclear interactors however was severely limited in scope. A more thorough investigation of transcription factors present in the aHSC nucleus and able to interact with β -catenin is required. One candidate family worth exploring is the FOXO family of transcription factors. FOXO transcription factors competitively bind β -Catenin, FOXO3 and FOXO4 in

particular(264). They have a higher β -Catenin binding affinity in comparison to TCF/LEFs and prevent β -Catenin/TCF complex formation. Foxo1 is one transcription factor upregulated upon HSC transdifferentiation and Foxo3 is shown to be involved in alcohol induced autophagy and hepatotoxicity (although the study focused only on hepatocytes)(265, 266). One possibility could therefore be an upregulation of Foxo transcription factors upon HSC transdifferentiation which compete with TCFs for β -Catenin binding. Further exploring this transcription family would enable study of another route of TCF independent signalling in HSCs.

7.1.3 Wnt as a therapeutic target?

Increasing understanding of how HSC behaviour is regulated is hoped to identify points of therapeutic intervention. The final part of this discussion explores whether the Wnt pathway in HSCs yields attractive targets for potential inhibition.

Fibroproliferative disorders are a significant modern health issue yet anti-fibrotic therapy is currently not widely available. In the case of hepatic fibrosis and cirrhosis liver transplant is the only effective treatment(20). Early stages of liver fibrosis are asymptomatic with clinical complications arising from development of advanced fibrosis and cirrhosis, when reversion of fibrosis not possible. There is therefore a crucial need for pharmacological agents able to halt progression and promote fibrosis reversal. The activated myofibroblast, major source of excessive ECM production upon injury is an ideal target for this. Preclinical research has explored numerous potential targets with early phase clinical studies beginning. However, research is in early phases and much work needs to be done(8).

Regarding liver fibrosis, it is essential to fully understand the signalling events which either promote the transdifferentiation of HSCs from quiescent pericytes to profibrogenic myofibroblasts or maintain this activated phenotype. This will facilitate identification of possible intervention points, such as preventing activation of HSCs and secretion of ECM , enhancing degradation of deposited matrix or promoting apoptosis and clearance of aHSCs.

Wnt signalling was first discovered over 30 years ago with the mapping of the canonical/ β -Catenin pathway and its role in promoting numerous disorders, most significantly cancer, is well described(12). Despite this, no therapeutic anti-Wnt targeting compounds to date are available. Several are currently in development but these remain in early preclinical stages(267). Disrupting Wnt signalling for therapeutic gain endangers its role in tissue homeostasis and areas with a high rate of self renewal such as the intestinal epithelium will be at risk of toxicity(268). Overactive Wnt signalling is indeed reported in numerous models of fibrosis. Despite this, Wnt signalling has an undeniable role in cell regeneration and its blockade may have severe consequences for the repair process after injury(68).

Inhibitors targeting specific Wnts are not available. Pharmacological inhibitors can be broadly grouped into those which prevent ligand secretion or binding, those which prevent signal transmission and those which directly disrupt β -Catenin activity. Inhibitors of Wnt secretion include the Porc inhibitors such as IWP2 used in Section 5.2 or LGK974, currently in Phase I trials(179, 267, 269). Inhibitors of ligand binding include antagonists of Fzd receptors such as OMP-18R5 or soluble, sFRP like molecules such as OMP-54F28(270, 271). Signal transmission can be prevented through Dvl blockade such as the PDZ domain inhibitor used in Section 5.2 or stabilisation of the β -Catenin destruction complex through tankyrase inhibitors(180, 272). As the β -Catenin /Canonical signalling remains the best studied of the pathways, numerous compounds also exist to antagonise nuclear β -Catenin function such as ICG001, also used in Section 5.2(181)

In vitro, pan Wnt inhibition in aHSCs mediated by IWP2 appeared successful, significantly reducing expression of the myofibroblast marker α -SMA and Type 1 collagen. However *in vivo* administration of IWP2 is likely to have significant off target toxicity with trials of Wnt antagonists reporting detrimental effects on bone formation and turnover, haematopoiesis and disruption of intestinal stem cell turnover. Even targeting IWP2 specifically to HSCs is likely to have complications. Firstly, IWP2 would prevent only secretion of Wnt from HSCs and not response to paracrine Wnts. Secondly,

temporal differences in Wnt expression are suggested by Chapter 3 and differences in Wnt response suggested by Chapter 5 where early inhibition appears to increase profibrogenic targets. Inhibition of Wnt would have to be finely timed to reach specific targets. Finally, impaired Wnt secretion in HSCs prevents their potentially beneficial effect on other cell types. Wnts secreted by macrophages are important in influencing differentiation of hepatocytes from progenitors after injury, determining a cholangiocyte or hepatocyte cell fate(258). Only macrophage derived Wnts were assessed but HSC derived Wnts may also be likely to play a role. Blanket inhibition of Wnt signalling in HSCs may therefore have detrimental effects on repair and regeneration after injury.

Treatment with Fzd antagonists or sFRP like molecules can reduce response to Wnt ligands (270, 271). However, decreased response to Wnt appears the default state in aHSCs with significantly decreased receptor expression and increased sFRP expression. Therefore, treatment with these antagonists may have little effect on profibrogenic myofibroblasts but greater, potentially detrimental effects on neighbouring cells.

Upregulation of sFRPs in aHSCs is an intriguing finding. sFRPs themselves have been used to target Wnt signalling as putative antifibrotics, recombinant sFRP treatment promoting improvement in instances of cardiac and kidney fibrosis(273). However, Chapter 3 suggests reasons for careful use of sFRPs as Wnt inhibitors, with effects appearing highly dependent on cell types and Wnt ligands present. As well as functioning in Wnt signalling modulation, they also possess a netrin like domain, implicated in matrix remodelling. More interestingly, recent studies have shown effective reduction in fibrosis by treating sFRPs themselves, therapeutic antibody mediated blockade of sFRP2 improving a cardiac fibrosis model(274). Function of sFRPs was not resolved here, KO studies unsuccessful and overexpression inconclusive. As previously mentioned, careful study of sFRP/Wnt and sFRP/sFRP interactions as well as the possibility of functional redundancy would need to be undertaken before they could be considered as targets for therapeutic intervention. Similar problems are encountered

throughout this study: complexity of pathway, multiplicity of ligands and poorly defined interactions between interactors. Therapeutic intervention at the level of Wnt ligands or extracellular modulators is therefore unlikely to be an attractive target as the chance for interference from other components is great. Intervention at points further down the pathway with less chance for interference, such as through targeted inhibition of Dvl function, may be more effective.

Several points exist at which Wnt pathway could be targeted in HSCs. The presence of multiple Wnt ligands and secreted interactors however significantly complicate the environment, potentially making it difficult to judge effectiveness of inhibitor. Furthermore, neither Wnt stimulation nor β -Catenin upregulation appeared to have a direct profibrogenic effect although effects on survival of HSCs remain a possibility. From this, it could be considered that blockade of Wnt signalling in the liver may not be worth the potential risks to repair and homeostasis mechanisms, as significant reduction in fibrosis may not be achieved.

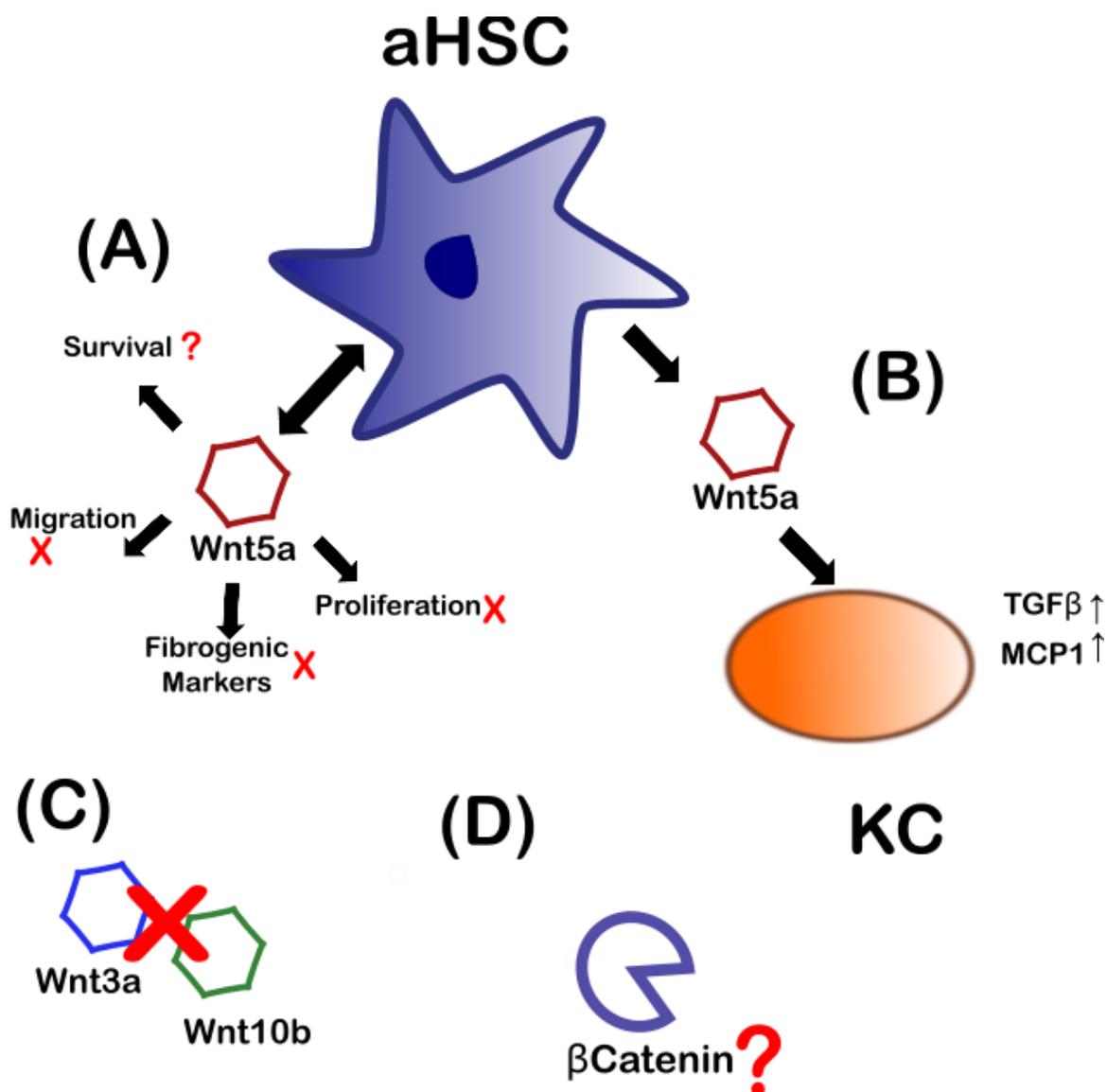


Figure 7.1 Summary of Summary of Wnt signalling in HSCs

Diagram illustrating aspects of the Wnt pathway investigated in this study **(A)** Influence of autocrine Wnt5a signalling on aspects of HSC behaviour such as survival, migration, proliferation and expression of fibrogenic markers was investigated. Only a potential influence on HSC survival was identified **(B)** Potential for paracrine Wnt signalling between HSCs and Kupffer cells (KC) was next explored. Wnt5a stimulus increased expression of inflammatory markers such as TGFβ and MCP1 in KCs **(C)** Expression of canonical Wnts 10b and 3a could not be detected in HSCs, implying HSCs function through non canonical signalling pathways **(D)** HSCs express abundant levels of β-Catenin despite lack of canonical Wnts detected and downstream TCF mediated transcriptional activity. Its function in HSCs therefore requires further exploration.

7.2 H2A.Z and DNA Methylation

Chapter 6 focused on how early developmental changes may influence the wound healing response in adults. This chapter questioned whether altering H2afv (the zebrafish ortholog of H2A.Z) could alter methylation levels in early embryo. I have succeeded in showing that H2afv is critical for embryonic development. Its absence increases mortality, causes phenotypic abnormality, particularly of the nervous system and results in hypermethylation of morphants at early stages.

These are preliminary results and many issues from this have yet to be addressed. Firstly, which signalling pathways does loss of H2A.Z disrupt to cause this phenotype? Analysis of the zebrafish sperm genome described enrichment of H2afv at loci particularly involved in development of the endocrine system and hindbrain as well as regulation of protein metabolism and gene transcription(225). Enriched genes included those of the *hox*, *pax*, *pitx*, *dlx* and *nkx families*, key regulators of embryogenesis, again supporting H2afv's essential role in development. The handful of markers analysed in section 6.5 was extremely limited. Analysis of *h2afv* morphants by next generation sequencing would significantly aid identification of pathways disrupted either directly through H2A.Z loss or through hypermethylation.

Here, I have been able to demonstrate hypermethylation in morphants during early stages of development through immunoblotting for 5MeC levels. However this is a rudimentary method of analysing global methylation levels. Again, next generation sequencing would allow genome wide methylation profiles to be examined at the single nucleotide level. One possibility could be reduced representation bisulfite sequencing (RRBS), a method of sequencing that biases towards areas of the genome high in CpG content such as promoters and avoids the high cost and sequence depth required for whole genome sequencing(70). This would enable identification of which specific sites are hypermethylated and paired with the transcriptome analysis, would give clues as to what may be mediating the phenotype.

The translation blocking ability of a MO is usually limited to the first 5 days of development, after which protein translation can resume(275). For the mildly affected morphants who survive beyond 5dpf it would be interesting to question whether H2afv is deposited at similar to sites to that of control fish once the morpholino has been diluted out and translation resumes. Disrupted methylation patterns may lead to altered deposition with potential consequences for transcriptional regulation later in life. Another study in *Arabidopsis* revealed enrichment of H2A.Z at genes regulating response to environmental stress and H2A.Z mutants responded poorly to stress stimuli such as heat or cold(276). Another question to ask could be if a similar effect is observed in morphants. Mildly affected morphants could be subjected to standard stressors in zebrafish models such as heat shock, oxidative stress or ethanol treatment. Mortality and compensatory response could then be observed and compared to controls.

Finally, H2A.Z also has well established roles in DNA damage response and genome stability(277, 278). Another limitation of this study was that due to time constraints these roles were overlooked in favour of studying methylation. Levels of DNA damage in morphants could be initially assessed through whole mount staining for the DNA damage marker γ H2AX(279). Genome stability has previously been examined in zebrafish through use of phosphor Histone 3 (PH3) staining as a marker for mitosis or metaphase spreads to analyse chromosome behaviour(280). The developing nervous system is highly susceptible to defects in DNA repair pathway as developing neurons undergo rapid phases of proliferation. If mutation occurs which is not correctly repaired this can be propagated through the developing nervous system, incurring serious consequences(281). Defects in the DNA repair pathway could therefore be contributing to the neural phenotype, especially given its slightly later onset (approximately 2dpf) at which point defects will have had time to accumulate.

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