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Reprogramming of Rat Hepatoma Cells with Fibroblast-specific Protein Twist1

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2019

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COMMITTEE IN CHARGE OF CANDIDACY

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Abstract

In mammalian development, a complex system comprised of regulatory signals causes tissue distinction with unique structures as well as functions. Gene expression controls these functions through specific combinations of transcription factors and cofactors that influence cell differentiation by both activation and repression of genes.

Whole genome microarray studies of fibroblasts have identified candidate genes that can serve as master regulators of fibroblast identity. A previous study showed that Prrx1 and Snai2 play important roles in activating expression of fibroblast identity, and Snai2 overexpression in hepatoma cells (Fg14) activated expression of fibroblast specific genes. Moreover, Snai2 overexpression resulted in repression of liver specific genes. This thesis addresses whether Twist1 has a similar effect as Snai2. More specifically, the objective of this work is to determine how Twist1 transfection of hepatoma cells (Fg14) affects fibroblast specific and hepatoma specific genes.

qPCR analysis revealed that Twist1 was successfully over-expressed in pooled Fg14 transfectants and individual clones compared to the non-transfected cells. Following these experiments, expression of several important genes in hepatic and fibroblast function were monitored. Results show that, of the 7 fibroblast specific genes tested, Twist1 overexpression activated two genes, Prrx1 and Sema3a, in Fg14 hepatoma cells. However, the remaining 5 fibroblast genes were not affected. Twist1 overexpression was found to not affect expression of the 10 hepatoma-specific genes tested.

Based on these results, unlike results obtained with Snai2 and Prrx1 overexpression studies, Twist1appears to have limited potential to reprogram hepatoma cells toward the fibroblast phenotype.

Dedicated to my family

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Table of Contents

Abstract……...…...…**iv**

Acknowledgement……**vii**

Table of Contents……...….……**viii**

List of Figures…….**x**

List of Tables……..…..**xii**

Chapter 1: Introduction……...….**1**

1.1. Gene Regulation in Multicellular Organisms……...…**1**

1.2. The Elements of Transcriptional Regulation……**3**

1.3. Transcription Factors and Gene Regulation…….**4**

1.4. Liver Development and Hepatocellular Carcinoma…….…**6**

1.5. Fibroblast as a Cell Model System…….…..**9**

1.6. Twist-related Protein 1 (Twist1)……...……..**10**

1.7. Epithelial-Mesenchymal Transition (EMT) and Regulation of EMT…….…**18**

1.8. Project Overview and Goals of the Study……...……**22**

Chapter 2: Materials and Methods……**23**

2.1. Cell Culture…….……**23**

2.2. RNA Isolation……...……..**24**

2.3. cDNA Synthesis……..**25**

2.4. Primer Design…….**25**

2.5. Quantitative-Polymerase Chain Reaction (qPCR)……..……**27**

2.6. Statistical Analysis……..……**28**

Chapter 3: Results……...…….**29**

3.1. Expression Comparison of Fibroblast and Hepatoma-specific Genes……...…….**30**

3.2. Twist1 Overexpression in Fg14-transfected Cells……..**30**

3.3. Twist1 Overexpression – Activation of downstream hepatoma-specific genes.....**31**

3.4. Effect of Twist1 overexpression on fibroblast-specific gene expression…...……**32**

Chapter 4: Discussion and Future Directions……...…**54**

4.1. Reprogramming the gene expression profile of hepatoma cells by Twist1 overexpression……...…**55**

4.2. Twist1 in Diseases……..**57**

4.3. Possible Factors Contributing to Variation in Expression Levels……..**58**

4.4. Future Directions……**59**

Chapter 5: References…….……**61**

List of Figures

1. Schematic of gene regulatory elements……..…**2**

2. Distal transcriptional regulatory elements and their functions of each……..……**4**

3. Schematic of transcriptional regulation……...**6**

4. Location of Twist1 gene in human……...**11**

5. Molecular structure of human Twist1 protein……..**12**

6. Amino acid sequences among human (H), mouse (M), xenopus (X), Drosophilia (D), leech (L) and C. Elegans (C)……...……**16**

7. Functional transition from epithelial to mesenchymal phenotype……...…….**19**

8. Three different types of EMT……..….**20**

9. Comparative expression of key liver-specific genes in hepatoma cells vs fibroblast cells…...**34**

10. Comparative expression of key fibroblast genes in hepatoma cells vs fibroblast cells……...**35**

11. Twist1 overexpression in transfected Fg14 rat hepatoma cells…….……..**36**

12. mTwist1 does not affect Hnf1a expression in Fg14 cells……..…….**37**

13. mTwist1 activates expression of Hnf4 in Fg14 cells…….…….**38**

14. mTwist1 activates expression of Hnf6 in Fg14 cells……..……**39**

15. mTwist1 effects on Alb expression in Fg14 cells……..…….**40**

16. mTwist1 effects on Pck1 expression in Fg14 cells…….……**41**

17. mTwist1 effects on Creg1 expression in Fg14 cells……..……**42**

18. mTwist1 effects on Hnf3 expression in Fg14 cells…….....**43**

19. mTwist1 effects on Serpina1 expression in Fg14 cells……..……**44**

20. mTwist1 effects on Kng1 expression in Fg14 cells……..……..**45**

21. mTwist1 effects on Fgb1 expression in Fg14 cells……**46**

22. mTwist1 effects on Prrx1 expression in Fg14 cells……..……..**47**

23. mTwist1 effects on Snai2 expression in Fg14 cells……...……**48**

24. mTwist1 effects on C-fos expression in Fg14 cells……**49**

25. mTwist1 effects on Shox2 expression in Fg14 cells……..……**50**

26. mTwist1 effects on Spp1 expression in Fg14 cells……..…**51**

27. mTwist1 effects on Cola1a expression in Fg14 cells……..**52**

28. mTwist1 effects on Sema3a expression in Fg14 cells……...…….**53**

List of Tables

1. Primers used in the quantitative Polymerase Chain Reaction (qPCR) for hepatoma cells……**26**

2. Primers used in the quantitative Polymerase Chain Reaction (qPCR) for fibroblast cells…....**27**

Chapter 1

Introduction

1.1. Gene Regulation in Multicellular Organisms

It is vital to have an in-depth comprehension of gene regulation to better understand many processes in life organisms. The discovery of the molecular machines that transcribe human genes, namely RNA polymerases I, II and III (Pol I, II and III), was a milestone in the research area of eukaryotic transcription [1][2][3] and paved the way to understanding the evolution of transcriptional regulation in eukaryotes. One of the main challenges in the area is to identify all the functional elements in this very complex genome, a subset of which is functional elements[4].

The expression of eukaryotic protein-coding genes occur mostly at the transcription initiation level. Most eukaryotic genes are transcribed by RNA polymerase II and regulated by two separate families of DNA sequences which are both cis-acting elements that have recognition sites to amplify or suppress transcription. The first family belongs to a promoter that contains a core promoter and nearby regulatory elements, whereas the second family belongs to distal regulatory elements such as enhancers, silencers, insulators and locus control regions (LCR). An illustration of a typical gene regulatory elements is given in Figure 1[4] and more details are presented in the next section.

There is much variation in eukaryotes in the quantity of the genes that are transcribed into both coding and noncoding RNA species[5]. The human genome has roughly 20,000 protein-coding genes and even more non protein-coding genes (ncRNA)[6]. Several ncRNAs have been shown to take part in gene expression control via modulation of transcriptional or

posttranscriptional processes such as fine tuning the levels of target messenger RNAs (mRNAs) or selecting chromatin regulators to modify gene expression[7].

Figure 1. Schematic of gene regulatory elements. Taken from[4]

Three groups play a role in accurate transcription in eukaryotic cells. These are: general transcription factors (GTF) activators which are promoter-specific proteins and coactivators. GTFs gather around the core promoter in order to form a transcription preinitiation complex (PIC) which leads RNA polymerase II to the transcription initiation site. A complete RNA polymerase II elongation complex occurs after several steps including promoter melting, clearance, and escape[8]. PIC sitting on the core promoter is only enough for low levels of basic transcription. In order to trigger more transcription activity, the second group (activators) come into play.. Activators are sequence-specific proteins with separable activation domain[9]. Activators are believed to work by increasing PIC formation through the interactions with transcriptional targets[10]. The last group that plays a role in correct transcription is coactivators. Coactivators do not have sequence-specific DNA binding, they are used by DNA-bound activators[11]. Although different in structure, coactivators' functions are very similar to activators which stimulate PIC formation and modify chromatin.

1.2. The Elements of Transcriptional Regulation

There are six different elements that have a significant role in the transcription process as can be seen in Figure 1. The first one is the core promoter which is described as the region at the starting point of a gene that acts as the docking site for both the PIC assembly and basic transcriptional machinery. It dictates position and direction of the transcription[12]. The second element is the proximal promoter which is the region that neighbors upstream from the core promoter. One important characteristic is to house activators via their binding sites[4]. The core promoter and the proximal promoter together make up the promoter part.

The remaining four elements are called distal regulatory elements. An interesting question to ask can be how these distal regulatory elements function from a distance. According to studies, DNA-looping, in which the DNA loops out so that core promoter and distal elements can be brought together is responsible[13]. Of them, enhancers increase the transcription capacity and perform similar to proximal promoter[14][15]. They regulate the transcription in a spatial or temporal-specific way and work regardless of the distance or orientation of the promoter[15]. The second distal regulatory element is silencers which have similar structure but act in the opposite way of enhancers. They house binding sites for repressors which are negative transcription factors[16]. Another element is insulators that block the genes from being affected by the neighboring genes. This effectively limits the affecting boundary of the other regulatory elements[4]. Two main characteristic of insulators are blocking enhancer-promoter communication and averting the spread of suppressive chromatin. Insulators are also called boundary elements. The last element in this category is locus control regions (LCRs). As the name suggests, they regulate an entire locus or gene cluster[17] with enhancing specific activity being the most important characteristic. They function in a similar way to enhancers and silencers.

Figure 2. Distal transcriptional regulatory elements and their functions of each. Taken from[4]

1.3. Transcription Factors and Gene Regulation

Transcription factors bind to DNA by occupying DNA sequences at control elements (cis-elements) where they make use of and regulate the transcription apparatus[5]. Transcriptional regulation occurs at two different but interconnected levels. The first level includes transcription factors and transcription apparatus whereas the second level includes chromatin and its regulators[5] (Figure3).

Transcription factors regulate gene expression through binding to enhancer elements and selecting RNA polymerase II and cofactors to target genes[18]. Transcription factors regulate transcription by synergistically binding to individual enhancers from core promoters via physical contact which involves looping of the DNA that contains enhancers and the core promoters[19].

As mentioned before, transcription factors bind to cofactors which are protein complexes that leads to activation (termed coactivators) and repression (termed corepressors). By making use of coactivators, transcription factors are believed to assist transcription initiation. After the transcription is initiated by RNA polymerase II, only a short distance of 20 to 50 bp is transcribed due to pause control factors. At the next step, these paused polymerases transition to elongation with the help of positive transcription elongation factor b (P-TEFb) which can be a component of super elongation complex (SEC).

The second level of transcriptional regulation involves chromatin (Figure 3, right). The main unit of chromatin is the nucleosome that is governed by protein complexes capable of mobilizing and altering its histone components. Mobilizing nucleosomes with the help of ATP-dependent chromatin complexes help them access to the transcription apparatus A[20]. There are also a variety of histone-modifying enzymes that modify nucleosomes of each active gene[21]. The alteration helps create a dynamic environment for chromatin modification as RNA polymerase is used in the process of initiation and elongation of the RNA species. There are several types of repressed chromatin[22] which are embedded: one type contains nucleosome alterations by the Polycomb complex which are silent but ready for activation at later stages of development[23] and the other type is completely silenced[24].

Figure 3. Schematic of transcriptional regulation. Taken from[5]

1.4. Liver Development and Hepatocellular Carcinoma

Being the largest gland in mammals, the liver has endocrine functions such as insulin-like growth factors, angiotensinogen, and thrombopoietin secretion and exocrine properties like bile secretion[25]. It is the main player in a variety of bodily functions such as glycogen storage, drug detoxification, metabolism control, overseeing cholesterol synthesis and transport and plasma protein secretion. Liver diseases such as hepatic fibrosis, cirrhosis and hepatitis tend to have a high morbidity rate and are reported to be the fourth leading cause of death in the United States among middle-aged people[25]. Comprising 2-5% of the whole body mass and being mostly made from

hepatocytes, this organ is responsible for many functions, including purification of proteins and production of growth factors, signaling molecules and hormones.

Being mostly comprised of hepatocytes, the liver has a very complicated tissue architecture. The liver lobule can be thought of the main building block of the liver. Plates of hepatocytes are separated by sinusoidal capillaries are arranged around central veins. Hepatocytes make up 78% of the liver and are responsible for protein and bile secretion, detoxification, cholesterol, urea and glucose regulation, acute phase response and blood clotting[26]. There are other types of cells in the liver. Cholangiocytes (bile duct cells) make up 3% of the liver and form bile ducts, control bile flow and pH and secrete water and bicarbonate as necessary. Endothelial cells line the lumen of blood vessels. Sinusoidal cells which are responsible for transferring of molecules between serum and hepatocytes, scavenging of waste, secretion of cytokine and antigen presentation. Pit cells are in charge of cytotoxic activity, while Kupffer cells scavenge foreign material and secrete cytokines. Finally, hepatic stellate cells are in charge of maintaining extracellular matrix, vitamin A synthesis, and retinoid deposition.

Developing cardiac mesoderm has an indispensable role during the hepatic cell fate phase based on the studies done on mouse and chick embryos[27][28]. Fibroblast growth factor (FGF) family is responsible for these inductive signals as FGF1 and FGF2 are capable of substituting cardiac tissue at the Albumin kickoff which is known to be characteristic to hepatic cell fate[29]. FGF-induced (including Fgf1, Fgf2, Fg8, and Fg10 which are believed to have overlapping functionality[30]) hepatic gene expression is regulated via mitogen-activated protein kinase (MAPK) pathway in the cardiac mesoderm during the hepatogenesis knockoff phase[31]. FGF signaling necessity is conserved throughout t evolution, as *Xenopus*, chick and Zebrafish embryos exhibit the same requirements[32].

Cells going through a hepatocyte cell fate acquire a certain gene expression and physiological profile during the embryonic and postnatal phases[33]. There is a complex network of transcription factors in the hepatocyte gene expression regulation. The main players in this network are HNF1a, HNF1β, FoxA2, HNF4a, HNF6, and LRH-1 [Nr5a2] working by occupying each other's promoters and thus form the regulatory circuity[34]. HNF1b, FoxA2, and HNF6 are involved in controlling the hepatic gene expression kickoff during the specification and liver bud formation[25]. HNF1a does not seem to have a big impact during embryogenesis which is thought to be the HNF1β occupying the HNF1 binding sites initially but in the adulthood phase HNF1β occupied promoter sequences were observed to be bound by HNF1a[35]. HNF4a does not seem to affect hepatic speciation although subsequent differentiation is blocked[36]. In fetal hepatic progenitors, the lack of HNF4a has a serious drawback[34] and preserving character in mature ones are reported in the literature[37].

Even though there are many diseases associated with liver malignancy hepatocellular carcinoma (HCC) is the most common one and is the ninth leading cause of cancer deaths in the United States[38]. HCC is more prevalent in males than females by a ratio of 2.4 to 1 with a higher strike rate for people with Eastern and Southern Asia, Middle and Western Africa, Melanesia and Micronesia backgrounds[39]. Chronic liver disease, cirrhosis, viral hepatitis, and excessive alcohol consumption are the leading risk factors of HCC[38]. Chronic medical conditions, namely obesity (which is believed to increase the risk by 1.5 to 4 times) and diabetes mellitus (due to the liver's important role on glucose metabolism), also increase the risk of HCC. Treatment of HCC patients is rather limited since the diagnosis are often done on patients with advanced HCC which have gone through some liver damage already[38]. Among the treatments surgical techniques such as resection[40], liver transplantation[41] as well as nonsurgical techniques such as

transarterial chemoembolization[42], transartetial radiation[43], percutaneous local ablation[44] and microwave ablation[45] exist.

Many of the known processes driving liver function have been identified through the study of hepatoma cells which makes them favorable as a model for liver function. For example, several key liver-specific transcriptional regulators have been identified using cultured liver-derived cells[46]. More recently, hepatoma x fibroblast cell hybrids were used to identify candidate genes possibly involved in driving both hepatic and fibroblast cell function using whole genome approach. Of the genes identified as highly fibroblast-specific, two genes, Snai2 and Prrx1, were shown to be able to reprogram cells hybrids to a fibroblast phenotype. Furthermore repression of either of these genes in a fibroblast cell line resulted in dramatic reprograming of cells into a variety of cell lineages, including chondrocyte and adipocyte cell phenotypes. Overexpression of these genes in a hepatoma cell line resulted in repression of hepatoma genes and activation of fibroblast genes.

1.5. Fibroblast as a Cell Model System

Within the body's connective tissues, fibroblasts are the most common cell type. Their main role is to secrete the components of the extracellular matrix (ECM)[47]. Fibroblasts are a family of very heterogeneous multifunctional cells which have an important role in development processes[48]. They are also instrumental in repairing tissue damage from inflammation and injury since they can produce different types of paracrine immune modulators[49]. Fibroblasts are also thought be involved in tumor development and progression as cancer and chronic inflammation exhibit some analogies[50].

Fibroblasts are found in most of the tissues and organs in the body and is associated with extracellular matrix (ECM) molecules[51]. Fibroblasts derive embryonically from mesenchymal

origin with a variety of phenotypic characteristics[52]. More recent findings suggest that the source of fibroblasts after injury or disease can come from epithelial-mesenchymal transition (EMT) and mesenchymal stem cells which are derived from bone marrow and tissue[53].

One of the most important functions of fibroblasts is the generation and control of ECM of the tissue or organ. Fibroblasts do this function by synthesizing and secreting collagens, proteoglycans, fibronectin, tenascin, laminin and fibronectin. They are very active cells and each cell is thought to synthesize 3.5 million procollagen molecules per day[54]. Fibroblasts also generate matrix metalloproteinases, their inhibitors, and tissue inhibitors of metalloproteinase that regulate extracellular degradation. Another important function of fibroblasts is regulation of tissue interstitial fluid volume as well as pressure through interaction of β1 integrin receptors[55]. The last important function of fibroblasts is the involvement in wound healing and repair which includes clot formation and platelet degranulation followed by releasing mediators to attract relevant cells to the wound area[56]. It replaces the matrix with a more mature ECM and subsequently heals the wounded site.

1.6. Twist-related Protein 1 (Twist1)

Twist1 was first identified in *Drosophila* as an indispensable gene for mesoderm formation and differentiation into different distinct tissue types as well as dorsal-ventral patterning in the early embryo development phase[57]–[60]. The name was given since the *Drosophila* embryos lacking Twist1 gene died at the end of embryogenesis looking “twisted”[60]. The Twist1 gene encodes a transcription factor that is comprised of a basic helix-loop-helix (bHLH) domain as well as an amino-acid motif[61]–[63]. The important role of Twist1 in the development of mesoderm has been established. For example, Twist1 gene mutations in human cause *Saethre-Chotzen* syndrome which is an autosomal dominant inheritance disease[64]. Similar effects were observed

in gene-ablation studies involving Twist1 null mice[65]. More recently, a crucial role Twist1 plays in cancer metastasis has emerged in the literature[66]–[68].

Belonging to the bHLH family which is characterized by the formation of a domain consisting basic amino acids sitting next to two amphipathic α-helices that are separated by an inter-helical loop[61]. The α-helices moderate the interaction with another bHLH factor and cause the production of a dimer that binds to Nde1 E-box which has an indispensable role for a variety types of organogenesis[63]. There are three subfamilies within bHLH family. Class A proteins are omnipresent among the mammalians. Class B proteins are specific to tissue expression and form dimers with class A molecules in order to bind to E-boxes. The final class C subfamily do not form dimers with other classes. Twist1 belongs to the family of class B[69].

Figure 4. Location of Twist1 gene in human. Taken from[70]

Human Twist1 gene has two exons and one intron and is mapped to 7p21.2[71] as shown in Figure 4. The first exon contains an ATG site which is followed by 202 amino-acid residues that are in turn followed by an untranslated portion (45-bp) in exon 1, 536-bp intron and another untranslated portion in exon 2[57]. Human Twist1 molecular weight is approximately 21 kDa, and has a theoretical isoelectric point of 9.6. This protein has more polar amino-acid residues near the NH_2 ends whereas more nonpolar residues at the COOH ends. Hence, NH_2 terminus is more hydrophilic.

Human Twist1 protein and mouse Twist1 protein are 96% identical with the bHLH domain between residue^{109} to residue^{163} has exactly the same homology[72]. Molecular structure of human Twist1 protein is shown Figure 5. Moreover, bHLH domains of Twist1 is mostly conserved among many species such as human, mouse, frog, *Drosophila*, leech and *Caenorhabditis elegans*. ^{109}Q – T^{121} region of human Twist1 protein is the main region in charge of binding to DNA. Furthermore, Loop-Helix II region also plays a role in the DNA binding process. Not only does Twist1 form dimer in order to bind to DNA, it also interacts with MyoD which is responsible for muscle differentiation. Hence, this interaction leads to suppression of muscle differentiation[73].

Figure 5. Molecular structure of human Twist1 protein. Taken from[57]

Twist box (also called WR motif) which is found between 20 and 55 amino acids from COOH-terminal to bHLH area is protected among vertebrates. This region has 100% homology among human (corresponding to between residue^{180} and residue^{202}), mouse and *Xenopus*[57]. On the contrary, a more historic species such as *C. elegans* does not contain a WR dipeptide. This WR

domain is necessary for the transactivation of Twist1 and any genetic mutation in this area results in *Saethre-Chotzen* syndrome for humans[74].

Aside from Twist1, there is also Twist2 protein. The Twist2 protein in humans has 160 amino-acid residues and it has only 68% identical homology-wise. However, the amino-acid sequences in bHLH and Twist box domains are essentially identical and it is the reason for their mutually redundant functions[57].

Being a major player in organogenesis, Twist1 is mostly expressed in tissues derived from mesoderm. In *Drosophilia*, it has been shown that cellular blastoderm stage is the first phase Twist1 is detected[75]. In the early gastrulation era, Twist1 expression is observed to be high in the mesodermal layer of the embryos. Late gastrulation phase comes and Twist1 can be observed in the cells of germ band mesodermal layer as well as anterior midgut primordium. In the later stages, Twist1 expression weakens but can still be seen within the mesodermal layer of somatopleura and splanchnopleura. After birth happens, it can be observed in adult mesenchymal cells such as muscle stem cells[76][77]. It is worth mentioning that at all developmental stages, Twist1 is localized in the nuclei since it is a nuclear protein.

As another important model, Twist1 is first observed at embryonic day 7.5 during mouse embryogenesis in the anterior-lateral mesoderm underneath the head folds, inside the primitive streak epiblast as well as in scattered cells in the amniotic cavity[77][78]. Afterwards, Twist1 is observed sequentially in the metameric segments, the neural crest derived head mesenchyme, the first aortic arches, the lateral mesoderm, the second, third and fourth branchial arches, the anterior limb buds, and finally, the posterior limb buds[79]. Twist1 is likely to be expressed initially along a dorsoventral gradient pattern until the headfold phase, followed by expression along the rostro-caudal axis of the embryos[57].

Unfortunately, dominant Twist1 expression areas during embryo development stages for humans are not as developed as *Drosophilia* and mouse, although there have been reports in the literature. Twist1 expression was strongly observed in the placenta. More specifically, in the fetal region of the placenta which is unsurprisingly comes from the mesoderm[71]. Less strong expression levels were observed in the adult heart and skeletal muscle. Even weaker signals were seen in the kidney and pancreas whereas brain cells which are derived from ectoderm and lung and liver cells which are derived from endoderm did not express any Twist1 signal. Considering cell lines derived from human, WI-38 cells (fetal fibroblasts from lung), human peritoneal mesothelial cells and endometrial fibroblasts as well as mesenchymal stem cells derived from bone marrow showed Twist1 signals[80]. To summarize, Twist1, an indispensable gene for organogenesis, is mostly expressed in the mesoderm-derived cells. After birth, it is mostly expressed in adult stem cells.

Twist1 is a vital regulator of many biological processes. Required for the proper development of mesenchyme derivatives, Twist1 executes a set of downstream target genes[81]. However, the exact molecular mechanism of Twist1's role on mesenchymal tissue formation is not developed[69].

One signaling Twist1 is involved is Fibroblast Growth Factor (FGF) signaling pathway[82]. In *Drosophilia* Twist1 activates the expression of DFR1 and in *C. elegans* eg1-15[69]. As mentioned before, mutations in Twist1 causes *Saethre-Chotzen* syndrome which is believed to be due to the necessity of Twist1 for FGF signaling during morphogenesis[83].

Apart from FGF signaling, Twist1 is also involved in Bone morphogenetic protein (BMP) and probably Transforming Growth Factor-β (TGF-β) signaling[84]. As opposed to other bHLH proteins, Twist1 has the ability to build functional homodimers (T/T) as well as heterodimers with

E12 (T/E). Homodimers and heterodimers have different actions and influence different gene sets' expression. It is possible to pinpoint the ratio between these dimers by checking relative levels of Twist1 and helix-loop-helix Id proteins as Twist1 tends to form heterodimers when Id proteins are absent and homodimers when present[84]. Id proteins are a different class of HLH and compete with Twist1 by forming dimers with E12 and inhibiting heterodimer formation by Twist1s[85]. In light of this knowledge, in the areas where Twist1 and Id1 are expressed together such as fronts of cranial structures, genes regulated by T/T dimers are expressed. On the flip side, T/E regulated genes are expressed in mid-sutures and it has been shown to suppress BMP signaling[82][86]. As BMP signaling activates Id expression, it leads to more homodimer (T/T) formation. This makes up a positive feedback loop that is believed to cause the early closure of the sutures[57]. Similar effect takes place in the limb bud for the case of FGF signaling[87]. Twist1 is also found to be indispensable for Bmp4 expression in the apical ectoderm[87].

An opposite feedback loop, namely negative feedback, happens in Tumor necrosis factor (TNFα) which activates both proapoptotic and antiapoptotic pathways. In order to prevent apoptosis, TNFα activated NF-κB upregulates Twist1 as well as Twist2. In response to this, Twist1 and Twist2 reacts with a subunit of NF-κB to inhibit cytokine genes expression[57]. This negative feedback loop is thought to have a role in impeding overactivation of cytokine expression[88].

Another very important role Twist1 plays besides the role in organogenesis is the expression of in aggressive tumors in a variety of cancer types such as breast cancer[66], hepatocellular carcinoma[89], prostate cancer[90], gastric cancer[91], esophageal squamous cell carcinoma[92], bladder cancer[93] and pancreatic cancer[94]. Twist1 has a presence in cancer initiation, progression as well as metastasis[57]. Amino acid sequences of well characterized species are shown in Figure 6.

```
H   ------------------------------------------------------------
M   ------------------------------------------------------------
X   ------------------------------------------------------------
D   MMSARSVSPKVLLDISYKPTLPNIMELQNNVIKLIQVEQQAYMQSGYQLQHQQQHLHSHQ 60
L   ------------------------------------------------------------
C   ------------------------------------------------------------

H   ---------------------------------------MMQDVSSSPVSPADDSLSNSE 21
M   ---------------------------------------MMQDVSSSPVSPADDSLSNSE 21
X   ---------------------------------------MMQEESSSPVSPVD-SLSNSE 20
D   HHQQHHQQQHAQYAPLPSEYAAYGITELEDTDYNIPSNEVLSTSSNQSAQSTSLELNNNN 120
L   ---------------------------------------MMSPSFPTTSSSHHLRRLTQQ 21
C   ------------------------------------------------------------

H   EEPDRQQPPSG-------------------------------------------------K 33
M   EEPDRQQPASG------------------------------------------------K 33
X   EELDKQQS------------------------------------------------K 29
D   TSSNTNSSGNNPSGFDGQASSGSSWNEHGKRARSSGDYDCQTGGSLVMQPEHKKLIHQQQ 180
L   QQRQRRQPASTDSVIKNCLFNNKNDTSTHLTTHLQGVNKIYFHNNNNCSNDSNNNSVDLK 81
C   ------------------------------------------------------------

H   RGGRKR------------RSSRRSAGGGAGPGGA--------------------------- 55
M   RGARKR------------RSSRRSAGGSAGPGGA--------------------------- 55
X   RGCRKR------------RSARKSP---------------------------------- 42
D   QQQQQHQQQIYVDYLPTTVDEVASAQSCPGVQSTCTSPQSHFDFPDEELPEHKAQVFLPL 240
L   PATKKRKKNIKKLTKSDSRPIRTSESTKNDLQQNNV-------------------VKVEL 122
C   ------------------------------------------------------------

H   AGGGVGGGDEPGSPAQGKRGKKSAGCGGGGGAGGGGG----SSSGGGSPQSYEEL----- 106
M   TGGGIGGGDEPGSPAQGKRGKKSAGGGGGGGAGGGGGGGGGSSSGGGSPQSYEEL----- 110
X   --------EDPDSPISVKRNKK-------------------ASSTGSSPQSFEEL----- 70
D   YNNQQQQSQQLQQQQPHQQSHAQMHFQNAYRQSFEGYEPANSLNGSAYSSSDRDDMEYAR 300
L   DQSDNNTAENEDSKSSVKDSKNNNHCNNTAPTTTITTTPSINFNTQTTTSTYRKLPNATT 182
C   -----MVARRKGERVVRKNEVEN------------------------------------- 18
                $    #

H   ------------------------------------------------------------
M   ------------------------------------------------------------
X   ------------------------------------------------------------
D   HNALSSVSDLNGGVMSPACLADDGSAGSLLDGSDAGGKAFRKPRRRLKRKPSKTEETDEF 360
L   NNIAYNHNGSGKNYYSNYNSHNNSPP------------------------------LSL 211
C   ------------------------------------------------------------

H   QTQRVMANVRERQRTQSLNEAFAALRKIIPTLPSDKLSKIQTLKLAARYIDFLYQVLQSD 166
M   QTQRVMANVRERQRTQSLNEAFAALRKIIPTLPSDKLSKIQTLKLAARYIDFLYQVLQSD 170
X   QSQRVMANVRERQRTQSLNEAFSSLRKIIPTLPSDKLSKIQTLKLASRYIDFLCQVLQSD 130
D   SNQRVMANVRERQRTQSLNDAFKSLQQIIPTLPSDKLSKIQTLKLATRYIDFLCRMLSSS 420
L   QTQRVLANVRERQRTQSLNDAFSQLRKIVPTLPSDKLSKIQTLKLATRYIDFLYDQLENN 271
C   VQQRACANRRERQRTKELNDAFTLLRKLIPSMPSDKMSKIHTLRIATDYISFLDEMQKNG 78
      **$ ** ******#$**#**  *####*##****#***#**##*# **$**    $$$

H   ELDS--------------------------KMASCSYVA---------HERLSYAFSVWR 191
M   ELDS--------------------------KMASCSYVA---------HERLSYAFSVWR 195
X   ELDS--------------------------KMASCSYVA---------HERLSYAFSVWR 155
D   DISLLKALEAQGSPSAYGSASSLLSAAANGAEADLKCLRKANGAPIIPPEKLSYLFGVWR 480
L   KQQQQQHQQQQQQQHQS-----------MDEFANGGYVSGLNGSPVISSDKLGYAFSVWR 320
C   CKLYGHSIFDEKRGYNLQSAFNMWRGNNGYTPIAGPSQLPPLQSAHIPPPAPSSIPPHCL 138
                                                        $
H   MEGAWSMSASH----------------------------- 202
M   MEGAWSMSASH----------------------------- 206
X   MEGAWSMSASH----------------------------- 166
D   MEGDAQHQKA------------------------------ 490
L   MEGAWQMNKSELNNNGCYLRNGSSAGGSSNNVRDGSVIG- 359
C   MPQPWYQTCPPPKQEFHELCPISTPNPNSNPNQLTPIHWQ 178
    *       $
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Figure 6. Amino acid sequences among human (H), mouse (M), xenopus (X), Drosophilia (D), leech (L) and C. Elegans (C). Taken from[57]

Most well-known effects of Twist1 in cancer is the promotion of cancer cell (epithelial-mesenchymal transition) EMT and metastasis. Metastasis has many steps including EMT, local invasion, intravasation, transportation, extravasation, proliferation at another site and production of overt metastatic lesions[95]. Just as it does for development, Twist1 exhibits a similar behavior by supporting the cells to undergo EMT and move to form secondary tumor sites[96].

Although Twist1 plays an important role in many types of cancers, the way it expresses, functions and the molecular mechanisms affected in this process vary for each type of cancer. Due to the topic of the thesis, I will cover only the hepatocellular carcinoma (HCC). HCC is a metastatic tumor characterized by rapid growth[57]. It has been shown that Twist1 overexpression has a positive correlation with HCC metastasis and a negative correlation with E-cadherin expression[89]. The demonstration of higher levels of Twist1 and lower levels of E-cadherin corresponding to higher metastatic capability implies that Twist1 inhibits E-cadherin expression and activates EMT alterations[89]. Upregulation of vascular endothelial growth factor (VEGF) and N-cadherin by Twist1 in HCC points to Twist1's role in HCC angiogenesis[97]. Another study demonstrated the increased mobility effect of overexpression of Twist1 on HCC cells[98]. In a study[99] involving Twist1 with two other major EMT regulators (Snail and Slug) the synergistic effect of Twist1 and Snail in enhancing HCC metastasis was demonstrated. Co-expression of Snail and Twist1 decreased E-cadherin and deteriorated prognosis significantly compared to Twist1 expression alone whereas Slug expression was not significant. Further verification comes from a study demonstrating Twist1's effect on increased motility, invasiveness via inhibiting E-cadherin expression and upregulation of N-cadherin[100].

1.7. Epithelial-Mesenchymal Transition (EMT) and Regulation of EMT

Epithelial tissue is comprised of tightly-packed epithelial cells and is useful to protect the body from outside hazards as this tight packing of cells provide the required structural robustness due to strong cell-cell and cell-matrix interactions[101]. As opposed to epithelial tissue mesenchymal tissue is a loose connective tissue that exists in early embryo. Composed of fibroblast-like shaped cells, mesenchymal cells are highly mobile thanks to their loose interactions[102]. Being undifferentiated, mesenchymal cells have stem cell properties.

In the beginning phase of an organism's development, there is only a single cell which is the fertilized egg. As time passes, the multiplied cells from the egg have to differentiate into three separate tissue types which are endoderm, ectoderm and mesoderm. The differentiation process was once believed to end after a certain time passes[101] but more recent evidence showed differentiation continues in response to physical damage and stress[103][104]. Epithelial-mesenchymal transition (EMT) is a biological process of epithelial cells gaining mesenchymal properties via physical and biochemical changes, and is the mechanism for cellular diversity. As a result of this process, epithelial cells lose their rigid architecture and become mobile, apoptosis resistant cells just like mesenchymal cells. Figure 7 shows the process of EMT[105]. After the transition, these cells move around the body to perform tasks such as tissue reconstruction and repair[106]. After migrating to the necessary part of the body, these cells undergo a reverse transition called mesenchymal-epithelial transition (MET) to become rigid, immobile cells once again[107].

Figure 7. Functional transition from epithelial to mesenchymal phenotype. Taken from[105]

Besides being a key player in for embryonic development, EMT also plays an important role in tumorigenesis since it promotes invasive and metastatic properties of epithelial cancer cells according to animal studies conducted[108]. Some signaling pathways involved in EMT during embryonic development are present in EMT during carcinogenesis[101]. It was suggested that these epithelial cells from the primary tumors become motile through the process of EMT and move away from the primary tumor area and cause secondary tumors at distant places where they have metastasized. They again become rigid and immotile once again through the process of MET[106]. This behavior has been observed for breast, ovarian, colon, and esophageal cancer[109].

It is important to state there is not only one type of EMT and each one occurs for a specific type of requirement. Type I EMT is linked to implantation, embryo formation, and organ development and thus pivotal in cell type differentiation. It is possible to go back to form epithelial cells through the process of MET. Type II EMT, on the other hand, occurs in reaction to an inflammation and stops once the inflammation is reduced. Hence, type II EMT occurs during tissue repair and damage healing. Type III EMT is found in solid tumors and makes the epithelial cells

invasive, leading to metastasis. A schematic showing all three types of EMT is illustrated in Figure 8[105].

Figure 8. Three different types of EMT. Taken from[105]

During EMT, gene expression levels are altered as down-regulation of epithelial markers such as E-cadherin and up-regulation of mesenchymal markers such as vimentin and N-cadherin occurs[104]. The most important marker for EMT is the E-cadherin which plays a significant role in cell-cell adhesion[107]. Since E-cadherin is important for epithelial cells to be immotile, reduction of E-cadherin levels leads to increase in invasiveness and change of adenoma to carcinoma. Hence, expression of E-cadherin is inversely correlated with tumor grade and stage[110][111]. E-cadherin levels are regulated both at the genomic and transcriptional level. It is possible to reduce activation of E-cadherin gene CDH1 by mutations as well as loss of CDH1 heterozygosity and hyper-methylation of CDH1 promoter[107]. Activation of EMT-inducing

pathways is possible through the use of growth factors such as fibroblast growth factor, epidermal growth factor, hepatocyte growth factor and transforming growth factor (TGF-β). The last factor is instrumental in many processes in the body including regulating growth, as well as differentiation and migration of cells during cancer progression, and metastasis[112].

The crucial players of EMT pathways are also E-cadherin repressors. One of them is Snail which is a zinc finger DNA binding protein present in normal growth as well as tumorigenesis and has been shown to be an important mediator of EMT in human and mouse invasive carcinoma cells[112]. Another important EMT inducer is Twist, a helix-loop-helix transcription factor. Twist inhibits E-cadherin via binding to E box motifs which are also directed by Snail and overexpression of both inducers has been shown to be linked to loss of E-cadherin as well as increase in N-cadherin in human gastric cancer[113]. Many EMT-inducing transcription factors act synergistically. For example, in human cells Twist1 activates Snail2 by directly binding to its promoter area and these two transcription factors are believed to be conserved through evolution and play an important role during embryogenesis and tumor metastasis[114].

Other studies on EMT focused on the effect of epigenetic alterations on up-regulation of epithelial genes and down-regulation of mesenchymal genes during EMT[112]. TGF-β supports the epigenetic control of EMT by inducing a DNA methyltransferase, namely DNMT1. It also reduces the heterochromatic marker, di-methylation of Lys9, and increases euchromatin marker[108]. Snail expression, in various types of carcinoma, is related to the E-cadherin promoter hyper-methylation by binding to the promoter region. Afterwards, Snail uses a complex including histone deacetyltransferases 1 and 2. Using this complex results in histone H3/H4 acetylation and an increase in histone H3 methylation that in turn induces heterochromatic structure and restrains E-cadherin expression[112].

As an environmental factor hypoxia, defined as pressure of O_2 levels being less than 10 mm Hg, is correlated with normal development and tumor progression[101]. Low oxygen environment up-regulates hypoxia inducible factor (HIF-1) which interferes with the hypoxic response by producing a transcription factor that activates transcription of target genes[104]. Hypoxia conditions up-regulate Snail and Twist, possibly through TGF-β stimulation[108] by inducing Snail and Twist expression which reduces the expression of E-cadherin.

1.8. Project Overview and Goals of the Study

The most important roles Twist1 plays are involvement in organogenesis and the expression of aggressive tumors in a variety of cancer types including hepatocellular carcinoma. Besides Twist1, Snai2 is another EMT regulator according to previous studies[99]. Previous studies showed that Snai2 overexpression in hepatoma cells (Fg14) activated expression of fibroblast specific genes[115]. Moreover, Snai2 overexpression resulted in repression of liver specific genes. This thesis addresses whether Twist1 has a similar effect as Snai2. More specifically, the objective of this work is to determine how Twist1 transfection of hepatoma cells (Fg14) affects fibroblast specific and hepatoma specific genes.

Chapter 2

Materials and Methods

2.1. Cell Culture

All cells were stored in a medium that contains 1:1 Ham's F12/Dulbecco's modified Eagle's medium (FDV) which consists of 10% fetal bovine serum (FBS) (GIBCO BRL) and 5 µg/100 ml penicillin-streptomycin (GIBCO) at a temperature of 37° C with 5% humidity CO_2 water-jacketed incubator. FG14 cells were derived from the parental cell line H4IIEC3 cells, isolated from a rat tumor by Weiss and colleagues. RAT2 cells are a thymidine kinase negative variant of the rat fibroblast cell line RAT1 and were obtained from American Tissue Type Collection (ATCC).

Commercially available Lipofectame kit from Invitrogen was used to transfect the cells with candidate genes. Briefly, DNA was mixed with a liposome reagent, trapping the DNA inside the liposomes and allowing liposomes to fuse with cell membranes to release the DNA into the cells. Expression vectors containing candidate gene TWIST1 was purchased from Origene, Inc. Six-well cell culture plates were used for the transfection process of candidate genes. Specifically, 0.5 ml of FDV media without penicillin and streptomycin (Pen/Strep) was added to a micro-centrifuge tube., DNA (1 µg/µl) was added, mixed then and 5 µl Lipofectamine Plus reagent was added to the mixtures and mixed gently for five minutes at room temperature. Finally, 5 µl of Lipofectamine LTX reagent was added to the mixture and was mixed gently by pipetting and incubated for half an hour at room temperature.

Cells to be transfected were introduced in a 6-well format the day before transfection. The transfection mixture that was prepared in the microfuge tubes before was added in the wells of the

plates and incubated at 37°C with 5% CO_2 for 6 to 8 hours while rocked. Afterwards, medium was substituted with FDV containing 10% fetal bovine serum, penicillin and streptomycin and incubated for 2 days at 37°C in 5% CO_2 environment. Cells were split into various dilutions (1:20, 1:10 and 1:5 dilutions) in complete medium plus 500 µg /ml G418 and was incubated for 2 to 3 weeks. After this process, G418 resistant clones were either pooled (10 to 50 clones per pool) or picked individually then expanded into larger plates until the cells could be lysed and ready for RNA extraction.

To determine the transfection efficiency, cells were also transfected with a Green Fluorescent Protein (GFP) expression plasmid. Based on GFP positive cells after 48 hours, we estimated between 4 and 6% transfection was achieved Furthermore, a DNA free control plate was used a negative control to make sure no G418–resistant cells were present in the cell line being transfected.

2.2. RNA Isolation

RNA extraction from confluent cell lines was performed using an RNeasy Mini Kit from Qiagen, with a DNase-I step modification. Briefly, the medium was removed, and the denaturing reagent guanidine isothiocyanate (GITC) and β- mercaptoethanol (RLT buffer) were used to lyse the cells. Following this procedure, lysed cells were placed in a collection tube for homogenization by centrifugation through a QiaShreddr (Qiagen) mini-column. One volume of ethanol was added followed by application of an RNeasy column in a collection tube. RW1 buffer was used to wash the resin filter and digested with DNase I for 15 min at room temperature. The filter was washed with RW1 again first and RPE buffer second followed by quick centrifugation after each treatment. RNA was then eluted from resin filter by addition of 40 µl to the column followed by centrifugation at 10,000 x g for 1 minute. Extracted RNA samples were transferred to microfuge tubes and stored

at -70° C. Nano-drop spectroscopy at 260 and 280 nm was used to determine concentration and purity of the purified RNA.

2.3. cDNA Synthesis

A mature, fully spliced, messenger RNA was used to acquire complementary DNA with the help of reverse transcriptase enzyme. In this procedure, Poly-T oligonucleotides anneal to poly-A tails of the RNA and addition of it creates the required complimentary DNA strand which was generated by first digesting the RNA strand using Ribonuclease H. Afterwards, complementary strand synthesis yields the double stranded DNA.

To produce cDNA from previously purified RNA, MasterAmp High Fidelity RT-PCR kit from Applied Biosystems was used. Reaction mixtures contained 10X RT Buffer, 25uM dNTP Mix, 10uM RT Random Primers, MultiScribe, sterile nuclease free water and 1 µg RNA in a final 20 µl volume. The RNA mixture was incubated at 37° C for 30 minutes. The settings in the Bio-rad Thermal Cycler used to synthesize cDNA are: 25°C for 10 min, followed by 37°C for 2 hours, 85°C for 5 min, then 4°C until final products are transferred to a microfuge tube and stored at -20°C. Afterwards, the stored cDNA tubes were diluted to the previously determined concentrations to be used for quantitative PCR.

2.4. Primer Design

Primer design was done using software provided by NCBI which is programmed to optimize and identify primer pairs that hybridize to single gene targets at appropriate temperatures (between 55 and 65°C in our case) and produce short amplicons when using qPCR. The suitable primers were purchased from Integrated DNA Technologies Inc. Three primers sets were designed for each gene to be tested. The best performing primer set (producing optimal amplification with

lowest background signal for both cells of interest) namely the parental hepatoma cells and fibroblasts cells were used for subsequent experiments. Genes whose expression was known to be significant in hepatic and fibroblast function based on the literature was monitored using qPCR. Primer pairs in this study and their predicted melting and annealing temperatures are given in the Tables 1 and 2.

Table 1. Primers used in the quantitative Polymerase Chain Reaction (qPCR), primer sequences, melting temperature and annealing temperature of primers for the parental hepatoma cells.

Primer	Primer Sequence	Melting Temperature	Annealing Temperature
Hnf1	F-5' CCATTCTGAAAGAGCTGGAGAAC3' R-5' AGGTACGACTTGACCATCTTTGC3'	57.1 55.6	60
Hnf4	F-5' TGGCAGAACTCCATCCGTCATTC3' R-5' AACTGGATCTGCTCGATCATCTG3'	56.6 56.5	60
Hnf6	F-5’ CAGTGGCTCTAAGCACAGTAA 3’ R-5’ CAGTGTGGTGGAACAGATAAGA 3’	54.5 54.3	60
Serpina1	F-5’ CCTATACCGGGAGCTGGTCCAT 3’ R-5’ TTGCGAGTGTCACCCTTGCT 3’	60.3 59.5	64
Alb	F- 5’ CATCCTGAACCGTCTGTGTG 3’ R-5’ TTTCCACCAAAGACCCACTA 3	55.7 55.8	60
rKng1(1)	F- 5’ AACACAATTGCCGCCTTCTCACAG 3’ R-5’ GTGCAATGGAATGACCAAGTGCCT 3’	60.2 60.2	64
rFgb1 (4)	F-5’ AAGGAGACAAGGTGAAGGCACACT 3’ R-5’ AAGAACATGCCGTTGTGGATGCTC 3’	60.3 59.9	64
rPck1 (2)	F- 5’ CGCTATGCGGCCCTTCTTT 3’ R-5’ CGTGAAAGATCTTGGGCAACT 3’	58.3 57.5	60
Gapdh	F-5’ TGATTCTACCCACGGCAAGTT 3’ R-5’ TGATGGGTTTCCCATTGATGA 3’	56.5 54.2	60
rCreg1 (3)	F-5’ CATCAGACACCCTGAGATGAAA 3’ R-5’ GGTCCACCAAAGTAGTCCAAA 3’	54.4 54.8	60

Table 2. Primers used in the quantitative Polymerase Chain Reaction (qPCR), primer sequences, melting temperature and annealing temperature of primers for the parental fibroblast cells.

Primer	Primer Sequence	Melting Temperature	Annealing Temperature
Prrx1	F-5' GAACCGAAGCTGGGAGAAA 3' R-5' AGGAGAGAGAGAGAGAGAGAGA 3'	54.9 54.9	60
Snai2	F-5' CTGGATACTCCTCATCTTTGGG 3' R-5' CTCTTCGTCACTAATGGGACTT 3'	54.5 54.1	60
C-Fos	F-5' GCTGGTGCATTACAGAGAGAA 3' R-5' GTGTGTTTCACGCACAGATAAG 3'	54.8 54.3	60
Twist	F-5' TCGCTGAACGAGGCATTT 3' R-5' GCCAGTTTGAGGGTCTGAAT 3'	54.8 55.1	60
Shox2	F-5' CTGAAGGATCGCAAAGAGGATG 3' R-5' CGTTGAGTTGTTCCAGGGTAAA 3'	55.6 55.2	60
Sppl	F-5' CAGCCAAGGACCAACTACAA 3' R-5' TGCCAACTCAGCCACTT 3'	55.0 54.8	60
Bmp3	F-5' CTAGAGGCTAGAGGGAGAACTT 3' R-5' GACAGAGAGACAGAGACAGAGA 3'	54.9 54.8	60
Col1a1	F-5' ACTGGTACATCAGCCCAAAC 3' R-5' GGAACCTTCGCTTCCATACTC 3'	55.0 55.3	60
Sema3a	F-5' GGGACGAGACTTTGCTATCTTC 3' R-5' GATGGGCACTGATGAATCTAGG 3'	55.1 55.1	60

2.5. Quantitative-Polymerase Chain Reaction (qPCR)

All the cDNA samples were diluted to a concentration of 5 ng/µl for all the samples. A final volume of 20 µl of reaction mixture contained 2 µl of cDNA template at a concentration of 5 ng/µl, 6.75 µl of sterile nuclease free water, 10 µl of Fast SYBR® Green Master Mix purchased from Applied Biosystem and 1.25 µl of gene specific primer at a 0.5 µM concentration (IDTDNA). To quantify the expression of mRNA level, Applied Biosystem thermocycler was used. The first step was done at 95° C for 3 sec which is followed by an annealing step happening at 5°C above the primer melting temperature for 30 sec for 40 cycles. A final melt curve step was performed at 95°C for 15 sec, followed by going down to 60°C for 1 minute and a final step at 95°C for 15 sec. The control used for qPCR is comprised of 8.75 µl of sterile nuclease free water, 10 µl of Fast

SYBR® Green Master Mix and 1.25 µl of gene specific primer at a concentration of 0.5 µM (IDTDNA). The reference gene used to test the cDNA quality was Gapdh. Duplicate assay was performed for each cell line and reactions were repeated three times for reproducibility purposes.

Raw threshold (Ct) values were calculated by taking the average after the amplification for each of the cell lines. The amplified target genes in each hepatoma and fibroblast cells were normalized to Gapdh for the respective Ct-value, generating a delta-Ct value (ΔCt). Fold differences in gene expression were determined with a delta delta-Ct (ΔΔCt) calculation. The ΔCt of the control cell line (Fg14) was subtracted from the hepatoma variant ΔCt value, generating a ΔΔCt value. Using a log base 2 scale, the calculated difference was placed into the ΔΔCt equation: 2(ΔCt (sample – experimental) − (ΔCt Control).

2.6. Statistical Analysis

Statistical significance was determined using student t-test assuming unequal variance. Significance value was accepted at $p < 0.05$ level.

Chapter 3

Results

We used hepatoma cell line Fg14 as a model to study the role of candidate gene Twist1 in the regulation of fibroblasts-specific gene expression. The results can be regarded as an extension of past studies from our laboratory which originally identified candidate master regulatory genes in a genome-wide screening of gene expression in hepatoma x fibroblast cell hybrids that could remodel cell hybrids to become more fibroblastic in nature[46]. Previous studies showed that Snai2 overexpression in hepatoma cells (Fg14) activated expression of fibroblast specific genes[115]. Moreover, Snai2 overexpression resulted in repression of liver specific genes. This thesis addresses whether Twist1 has a similar effect as Snai2. More specifically, the objective of this work is to determine whether Twist1 overexpression in hepatoma cells (Fg14) can reprogram these hepatoma cells to become more fibroblast-like. To that end, we monitored fibroblast specific and hepatoma specific gene expression in response to Twist1 overexpression.

Expression cassettes containing the mouse Twist1gene in a neo plasmid vector was transfected into the Fg14 cells and G418 selection applied. There was only one pool and three total clones. A parallel non-transfected negative control was included which, as expected, resulted in no G418 resistant clones. G418 resistant clones were picked individually or pooled and expanded into 100mm cell culture dishes. Following this procedure, RNA was extracted and used to generate complimentary DNA (cDNA) that was later used in the qPCR reactions to monitor expression of target genes.

3.1. Expression Comparison of Fibroblast and Hepatoma-specific Genes

We first compared gene expression profiles of key liver-specific and fibroblast -specific genes between the RAT1 fibroblasts cells and the Fg14 cells using rat-specific primers. For hepatoma cells, we measured expression of genes encoding transcription factors known to be important in driving the liver phenotype. These included transactivator genes Hnf4, Hnf1, Hnf6 as well as Creg1, a gene recently identified in our laboratory as being important for liver function. Results show that of these genes are highly liver-specific, with levels 100-1000 fold higher in the hepatoma cells compared to those in the RAT2 fibroblast cell line (Fig. 9). We next measured expression of a panel of fibroblast specific genes known to be important in driving the fibroblast phenotype. These included Prrx1, Snai2, C-fos, Shox2, Sppl, Col1a, and Sema3a and the gene of interest, Twist1. As expected, result show that Fg14 cell expression of these genes is 2 to 3 orders of magnitude lower than in the RAT1 fibroblasts (Fig 10).

3.2. Twist1 Overexpression in Fg14-transfected Cells

As shown above, Twist1 is expressed at levels >100 fold lower than those in the RAT2 fibroblasts. We next asked whether the transfected mouse Twist1 gene was expressed in transfected Fg14 cells. To do this, mouse-specific primers were used, as the rat primers failed to cross-hybridize to the mouse cDNA sequences (results not shown). Results from RT-qPCR analysis show that mouse Twist1 was successfully over-expressed in pooled Fg14 transfectants as well as two (clones 4 and 8) of the three individual clones compared to the non-transfected Fg14 cells (Fig. 11). A third clone (clone 6) showed no detectable expression of the Twists1 transgene, and therefore serves as a negative control for non-specific effects.

As mentioned above, both the pooled clones well as the two out of the three individual clones tested expressed the introduced Twist1 gene at levels >2500 fold higher than the signal in

the Fg14 cells (Fig. 11) . It is important to mention that since the used primers are mouse-specific, background signals in the rat-derived Fg14 cells could either be due to cross-hybridization to rat sequences or spurious hybridizations to other sequences.

3.3. Twist1 Overexpression – Activation of downstream hepatoma-specific genes

The next set of experiments were done to address the question of whether Twist1 overexpression in hepatoma cells (Fg14) could affect expression of downstream hepatoma-specific genes. Regulation of downstream genes in distinct cell types depends largely upon transcriptional control. It is known that regulation and/or activation of liver specific downstream genes necessitates binding of hepatocyte nuclear factors and co-activators to promoter regions. Due to the established features of Twist1 and its fibroblast specificity, we asked if it could alter liver-specific downstream genes (which would suggest reprogramming of these cells). Expression of 10 different key liver markers was monitored. In each case, expression profiles were compared between the non-transfected Fg14 cells and the Twist1-transfected cells (pools and three clones).

All values obtained from the thermal cycle were controlled for Gapdh expression for each cell line tested. All cells lines showed Gapdh levels with 3 cycles and expression results obtained for each gene was normalized to these differences in Gapdh levels. This assumes that Gapdh levels are unaffected by introduction of the transgene and instead reflects technical variation in RNA isolation, cDNA production, or assay preparation.

For some of the hepatoma-specific genes including Hnf1, Creg1, Hnf3, and Serpina1 (see Figs 12, 17, 18 and 19, respectively), levels were within 3-fold of the non-transfected Fg14 cells. For other genes including Hnf4, Alb, Pck1, Kng1 and Fgb (see Figs. 13, 15, 16, 20 and 21, respectively) an unexpected increase in expression was observed in clones compared to the Fg14 parental line. For example, both Alb and HNF4 showed 5-15 fold activation in the transfected

clones compared to the pooled transfectants. Clone 6 produced the highest levels, despite the fact that we were unable to detect expression of the introduced transgene in this clone (see Fig. 11), leading us to conclude that the levels detected for these genes were independent of Twist1 expression.

Of the 10 genes tested, only Hnf6 showed a possible effect on gene expression due to the in introduction of the mouse Twists1. The pooled transfectants showed a 6-fold increase, with clones 4 and 8 having a more modest 2-fold increase compared to either non-transfected on non-expressing Fg14 cells, Therefore, little if any effect on liver-specific genes expression was observed by overexpression of the mouse Twist1 gene.

3.4. Effect of Twist1 overexpression on fibroblast-specific gene expression

In the next section, we tried to answer the question of whether Twist1 overexpression in hepatoma cells (Fg14) could affect expression of downstream fibroblast genes. As described above fibroblast-specific genes expressed at levels more than 100-fold below those in the Fg14 hepatoma cell line (Fig10). Therefore, Pooled Fg14-Twist1 cells were tested for fibroblast-specific gene expression using qPCR. Expression of 7 different key fibroblast markers was monitored, in each case, expression profiles were compared between the non-transfected Fg14 cells and the Twist1-transfected cells (pools and three clones).

All values obtained from the thermal cycle were controlled for Gapdh expression for each cell line tested. All cells lines showed Gapdh levels with 3 cycles of each other, results obtained for expression of each gene was normalized to these differences in Gapdh levels. This assumes that Gapdh levels are unaffected by introduction of the transgene and instead reflects technical variation in RNA isolation, cDNA production, or assay preparation.

For some of the fibroblast-specific genes including Snai2, C-fos, Col1a1, and Sema3a (see Figs. 23, 24, 27, and 28) expression levels were 20 to 90% lower than that of the non-transfected Fg14 cells with an unexpected increase in expression for Sema3a clone 4. Additionally, Snai2 expression levels in Fg pool is comparable to non-transfected Fg14 cells expression levels. For the genes Prrx1, Shox2, and Sppl (see Figs. 22, 25, and 26) expression levels were 20 to 95% lower than non-transfected Fg14 cells with no expression for some cases, specifically, Prrx pool, Shox2 clone 8, Sppl pool and clone 6.

Of the 7 genes tested, only Prrx1 showed significant expression levels for clone which is 20 to 90 fold compared to non-transfected Fg14 cells while the pool has expressed levels of half the intensity.

Figure 9. Comparative expression of key liver-specific genes in hepatoma cells vs fibroblast cells. Rat-specific primers were used to monitor expression of known liver-specific genes in the Fg14 rat hepatoma cell lines and RAT1 fibroblasts cells. cDNA derived from isolated mRNA was monitored by qPCR analysis. The cycle numbers shown are compared to those obtained from the RAT1 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions sets for each.

Figure 10. Comparative expression of key fibroblast genes in hepatoma cells vs fibroblast cells. Rat-specific primers were used to monitor expression of known liver-specific genes in the Fg14 rat hepatoma cell lines and RAT2 fibroblasts cells. cDNA derived from isolated mRNA was monitored by qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions sets for each.

Figure 11. Twist1 overexpression in transfected Fg14 rat hepatoma cells. Mouse-specific primers were used to monitor over-expression of the introduced mouse Twist1 gene in the Fg14 rat hepatoma cell line. Fg14 cells were stably transfected with a mouse Twist1 expression plasmid, and G418-resistant clones pooled or selected individually. Cellular mRNA was isolated, converted to cDNA, and monitored by qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 12. mTwist1 does not affect Hnf1a expression in Fg14 cells. Rat-specific Hnf1a primers were used to monitor Hnf1a expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 13. mTwist1 activates expression of Hnf4 in Fg14 cells. Rat-specific Hnf4 primers were used to monitor Hnf4 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were performed in triplicate.

Figure 14. mTwist1 activates expression of Hnf6 in Fg14 cells. Rat-specific Hnf6 primers were used to monitor Hnfn6 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were performed in triplicate.

Figure 15. mTwist1 effects on Alb expression in Fg14 cells. Rat-specific Alb primers were used to monitor Alb expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 16. mTwist1 effects on Pck1 expression in Fg14 cells. Rat-specific Pck1 primers were used to monitor Pck1 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 17. mTwist1 effects on Creg1 expression in Fg14 cells. Rat-specific Creg1 primers were used to monitor Creg1 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 18. mTwist1 effects on Hnf3 expression in Fg14 cells. Rat-specific Hnf3 primers were used to monitor Hnf3 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 19. mTwist1 effects on Serpina1 expression in Fg14 cells. Rat-specific Serpina1 primers were used to monitor Serpina1 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 20. mTwist1 effects on Kng1 expression in Fg14 cells. Rat-specific Kng1 primers were used to monitor Kng1 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 21. mTwist1 effects on Fgb1 expression in Fg14 cells. Rat-specific Fgb1 primers were used to monitor Fgb1 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 22. mTwist1 effects on Prrx1 expression in Fg14 cells. Rat-specific Prrx1 primers were used to monitor Prrx11 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 23. mTwist1 effects on Snai2 expression in Fg14 cells. Rat-specific Snai2 primers were used to monitor Snai2 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 24. mTwist1 effects on C-fos expression in Fg14 cells. Rat-specific C-fos primers were used to monitor C-fos expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 25. mTwist1 effects on Shox2 expression in Fg14 cells. Rat-specific Shox2 primers were used to monitor Shox2 expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 26. mTwist1 effects on Spp1 expression in Fg14 cells. Rat-specific Spp1 primers were used to monitor Spp1 expression in transfected cell using qPCR analysis**.** The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 27. mTwist1 effects on Cola1a expression in Fg14 cells. Rat-specific Cola1a primers were used to monitor Cola1a expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Figure 28. mTwist1 effects on Sema3a expression in Fg14 cells. Rat-specific Sema3a primers were used to monitor Sema3a expression in transfected cell using qPCR analysis. The cycle numbers shown are compared to those obtained from the Fg14 cells and normalized to Gapdh levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.

Chapter 4

Discussion and Future Directions

Transcription factors (TFs) have a very important role in activation of genes within a genome to regulate development in mammalian cells. As described in the literature many control mechanisms, namely histone modification, chromatin regulation, transcriptional control, post-transcriptional regulation and cell-cell contact, regulate gene expression in fibroblast cells[116]. Promoter-proximal elements and enhancers initiate transcriptional activity where transcription factors bind[117]. Fibroblast-lineage TFs bind to regulatory regions of certain genes in a synergistic fashion that leads to activation of these genes to form fibroblast identity in all distinct tissues in complex organisms.

Fibroblast-specific gene expression was studied in our laboratory using cell hybrids as model system combined with whole genome microarrays. The genes were identified by comparing whole genome transcriptome microarray data of hepatoma cells and hepatoma x fibroblast hybrid cells along with parental fibroblast cells by observing the fold differences in expression. The experiments identified potential master regulators of fibroblast cell fate (fibroblast-specific TFs), and their putative regulatory role in fibroblast differentiation. Among the identified genes are transcription factors (e.g. Prrx1/Pmx, Slug, Snai2, Shox2, CFOS, Twistl, Hox-d10, and Msxl) as well as fibroblast-specific downstream such as Sema3a, (cell signaling) Sppl, Bmp3 (cell differentiation), and Collal (structural organization).

Among the fibroblast-specific candidate genes identified that have putative roles in the maintenance of the fibroblast phenotype were transcription factors Snai2 and Twist1. A previous study from our laboratory found out Snai2 was highly expressed in fibroblasts, silent in hepatoma

cells, and repressed in hepatoma x fibroblasts cell hybrids. Moreover, forced over-expression of these genes in the hybrid cells leads to activation of fibroblast gene expression and resulting fibroblast-like morphology. We asked the question of whether a similar effect could be observed for Twist1. The goal of this study was to determine the ability of a fibroblast-lineage specific TF to remodel hepatoma cells.

4.1. Reprogramming the gene expression profile of hepatoma cells by Twist1 overexpression

In this study, we extended the results of a previous study from our lab which suggested that overexpression of Prrx1 and Snai2 genes via introduction of an expression plasmids into the Fg14 hepatoma cells results in activation of fibroblast-specific TFs as well as downstream fibroblasts-specific genes. We wanted to determine whether a similar effect can be observed for Twist1 gene. We were not able to observe significant activation of key fibroblast-specific TFs. Although not studied in here, we speculate that overexpression of Twist1 in the hepatoma cell line might activate other fibroblast-specific TFs that work in collaboration with other TFs to define the drive lineage-specific transcriptional regulatory circuitry.

We started with showing that the Twist1 gene is expressed at levels which are 1000-fold higher than those in the hepatoma cells (Figure 9). We were able achieve high level expression in mouse Twist1 in the rat hepatoma cells using expression vectors from Origene. The results showed strong expression of mouse Twist1 gene in the cells compared to non-transfected cells, both in the pooled transfectants and some of the individual clones analyzed (Figure 11).

Over-expression of the mouse Twistl gene in the hepatoma cell line resulted in activation of only one fibroblast-specific TFs and two fibroblast-specific downstream genes- Prrx1 and Sema3a, respectively (Figures 22 and 28) while the other genes, including C-Fos, Shox2, Snai2,

Sppl and Col1a1 (Figures 23, 24, 25, 26, 27) did not show significant activation in both the pool and clones.

Several TFs have been reported as master regulators of the epithelial–mesenchymal transition (EMT) program. These include Twist1, Snail family zinc finger 1 (SNAIL1), Zinc finger E-box-binding homeobox (ZEB), PRRX1, KLF4, SOX4, and SOX9[118]. Thus, it makes sense to assume correlation between Snai2, Prrx1, and Twist in the pathways of EMT. The Col1a1 gene, which is a common marker for fibroblast identity, was not activated by Twist1. A similar observation took place for Snai2-transfected cells per previous results from our lab, although Prrx1-transfected cells showed significant activation.

Prrx1 is expressed in mesenchymal tissues in adult mice and studies showed that Prrx1 has the capability of differentiating mesenchymal precursors. More specifically, Prrx1 was showed to inhibit adipogenesis by activating TGF-beta signaling[119]. On another note, Twist 1 was also showed to be a regulator of adipocyte gene expression although not likely to regulate differentiation[120]. This positive correlation might explain Prrx1 upregulation by overexpression of Twist1 gene in hepatoma cell line.

We observed a high activation level in some of the clones for Sema3a gene (Figure 28) which is a member of the semaphorin family and it is secreted by neurons and surrounding tissue which guides migrating cells and exons in developing systems. It also serves as an endogenous inhibitor of angiogenesis[121]. A similar condition occurs for Twist 1 gene. Studies showed that Twist1 is required for Tie2 expression and angiogenesis[122]. This might be a reason why Sema3a is upregulated by overexpression of Twist1 gene in hepatoma cell line.

4.2. Twist1 in Diseases

A very important role Twist1 plays besides the role in organogenesis is the expression of aggressive tumors in a variety of cancer types such as breast cancer[65], hepatocellular carcinoma[88], prostate cancer[89], gastric cancer[90], oesophageal squamous cell carcinoma[91], bladder cancer[92] and pancreatic cancer[93]. Twist1 has a presence in cancer initiation, progression as well as metastasis[57]. Most well-known effects of Twist1 in cancer is the promotion of cancer cell (epithelial-mesenchymal transition) EMT and metastasis. Metastasis has many steps including EMT, local invasion, intravasation, transportation, extravasation, proliferation at another site and production of overt metastatic lesions[94]. Just as it does for development, Twist1 exhibits a similar behavior by supporting the cells to undergo EMT and move away to form secondary tumor sites[95].

Although Twist1 plays an important role for many types of cancers, the way it expresses, functions and the molecular mechanisms affected in this process vary for each type of cancer. HCC is a metastatic tumor characterized by rapid growth[56]. It has been shown that Twist1 overexpression has a positive correlation with HCC metastasis and a negative correlation with E-cadherin expression[88]. The demonstration of higher levels of Twist1 and lower levels of E-cadherin corresponding to higher metastatic capability implies that Twist1 inhibits E-cadherin expression and activates EMT alterations[88]. Upregulation of vascular endothelial growth factor (VEGF) and N-cadherin by Twist1 in HCC points to Twist1's role in HCC angiogenesis[96]. Another study demonstrated the increased mobility effect of overexpression of Twist1 on HCC cells[97]. In a previous study[98] involving Twist1 and two other major EMT regulators (Snail and Slug) the synergistic effects of Twist1 and Snail in enhancing HCC metastasis were observed. Co-expression of Snail and Twist1 decreased E-cadherin and deteriorated prognosis significantly

compared to Twist1 expression alone, whereas Slug expression was not significant. Further verification comes from a study demonstrating Twist1's effect on increased motility, invasiveness via inhibiting E-cadherin expression and upregulation of N-cadherin[99].

On a further note, Twist1 gene mutations in human cause *Saethre-Chotzen* syndrome which is an autosomal dominant inheritance disease[63].

4.3. Possible Factors Contributing to Variation in Expression Levels

Most of the experimental findings exhibit large variation in expression levels and this section aims to address possible reasons of why they occur. Firstly, sample acquisition and handling before the RNA extraction can contribute to variation because of inappropriate sample collection and processing[123]. For example, incubating (37 °C) freshly-obtained mouse liver tissue for four hours significantly reduced the measured levels of some mRNAs[124]. Secondly, assessment of RNA concentration and purity is determined using a UV/Vis spectrometer. Since at 260 nm wavelength DNA can be detected in the case of DNA contamination overestimation of RNA concentration can occur. The most common way to determine RNA quality is to separate the RNA sample in an agarose gel and to visualize using a fluorescent dye[125]. This could have been checked before proceeding to qPCR while making sure reagents are still fresh. Another check that might have an impact on variability is the presence of more than one DNA fragment. This could significantly change the result and including melting curve analysis to make sure a single product is amplified[123]. Yet another factor to reduce the variability happens during data normalization. We have used GAPDH as reference gene but using multiple genes for normalization has been shown to reduce variability[126]. There are a couple of software programs to assess reference gene as both GAPDH and ACTB variation for a wide range of tissues has been shown in the literature[127]. Yet another reason of variation could be to due technical issues such as qPCR

instrument, the detection method, the reaction volume, and the liquid dispensing method. Instrument calibration and regular testing are important. Finally, levels of expression for most fibroblast genes were close to the detection limit for the RT-qPCR, (> cycle 35) making it likely that signals obtained could be suspect. .

On another note, fibroblast genes were expressed at very low levels in the hepatoma cells (often >100 fold lower than in the fibroblasts cells) which possibly explains why the RT-qPCR data was highly variable between transfected pools and clones.

4.4. Future Directions

In the experiments, for the Twist1 candidate genes where we observed strong activation we are not certain that the cause is Twist1 binding to promoters of the transcription factor promoters. This could be determined by a software that predicts transcription factor binding sites (TFBS) paired with functional assays such as ChIP-Chip and ChIP-Seq to detect the binding site sequences of Twist1 on promoters. There are several experimental methods to achieve this both in vitro and in vivo[128]. In vitro methods include Electro-Mobility Shift Assay (EMSA) which uses non-denaturing polyacrylamide gel as a molecular sieve to separate protein-bound DNA from unbound DNA, DNase I footprinting/protection assay which combines the cleavage reaction of DNase I with EMSA, Systematic Evolution of Ligands by EXponential enrichment (SELEX) that works by monitoring short, random oligonucleotide probes that are recognized by a TFBS of interest. More recently, in vivo approaches are getting more popular. Chromatin ImmunoPrecipitation (ChIP) assay is one such method.

Moreover, to reduce Twist1 expression in the Fg14 cells gene knockdown experiments can be utilized. This experiment would help us find out whether the fibroblast-specific as well as downstream genes get affected or not. Furthermore, cell culture models can be established to check

Twist1 expression levels during embryogenesis in normal and Twist1 knockout mice model to help explain the role of the protein in fibroblast function. Finally, conducting a whole-genome microarray analysis on the Fg14-Twist1 cells as opposed to Fg14 cells and Rat cells would pinpoint the degree of genomic remodeling as a result of over expression.

Chapter 5

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