The Role of Prrx1 and Snai2 as Master Regulators of Fibroblast Identity

Huda A. Alzahrani

Eastern Illinois University

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The Role of Prrx1 and Snai2 As Master Regulators of Fibroblast Identity

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Abstract

Mammalian development involves a complex system of regulatory signals and reactions, leading to the creation of distinct tissues with specific structures and functions controlled by various gene expression profiles. Tissue-specific gene expression is by controlled specific combinations of transcription factors and cofactors that dictate cell differentiation via activation and repression of genes in mammalian cells.

Whole genome microarray analyses of fibroblasts have revealed candidate genes that may serve as master regulators of fibroblast identity, such as Prrxl and Snai2, that activate downstream genes such as Bmp3, Twist, Shox2, Cfos, Slug, Sema3A, Sppl, and Collal. A previous study showed that Prrx1 and Snai2 play important roles in activating expression of fibroblast identity using somatic cell hybrids as model systems. Based on these results, we sought to examine the role of Prrx1 and Snai2 in affecting the phenotype of liver-derived cells using hepatoma cells as a model system.

Expression plasmids containing Prrx1 and Snai2 expression cassettes were transfected separately into Fg14 rat hepatoma cells using a standard transfection technique, then G418-resistant clones were selected (pooled and individually) and monitored for over-expression of Prrx1 and Snai2. RNA was extracted and used to synthesize cDNA. Quantitative real-time polymerase chain reaction (qPCR) was carried out using specific primer pairs and signals normalized to GAPDH mRNA levels. In addition, we observed alteration morphology and cell migration using Scratch Assays.

qPCR analysis revealed that both Prrx1 and Snai2 were successfully over-expressed in pooled Fg14 transfectants and individual clones compared to non-transfected cells. Next we monitored
expression of several genes known to be important in liver and fibroblast function. Results show that Snai2 activated several genes (Twist, Cfos, Shox2, and Bmp3) in Fgl4 hepatoma cells. Although several other fibroblast genes (SEMA3A and Spp1) were relatively unaffected. Prrxl overexpression resulted in activation of (Cfos, Twist, Shox2, Bmp3, and Col1a1) expression in Fgl4 hepatoma, but (SEMA3A and Spp1) were unaffected.

Based on these findings we hypothesized that overexpression of fibroblast-specific transcription factor Prrxl and Snai2 would result in loss of hepatoma phenotype. We therefore monitored expression of a panel of liver-specific genes using qPCR analysis. Results show that several liver related genes are repressed by these transcription factors, leading to partial loss of the hepatic phenotype. We observed that the cells re-acquired a spindle-shaped morphology and more significantly an enhanced migration capability, which is reminiscent of parental fibroblast cells. In conclusion, these results suggest that candidate core Prrxl and Snai2 serve as a useful starting point for studying transcriptional control of cell identity and reprogramming of mammalian cell types.
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Chapter 1

Introduction

1.1. Gene regulation in eukaryotes

Understanding of cellular development requires a strong knowledge of gene regulation. Multiple molecular mechanisms regulate cellular differentiation and functional specialization (Neph et al., 2012). Inappropriate activation or repression of key genes can result in birth defects, cancer, and even death. Even though individual cells of multicellular organisms generally have the same genome, each cell type has dissimilar sets of active genes, producing distinct sets of proteins thereby ensuring each cell type is specialized for its role. Consequently, gene regulation defines cell structure and function, which contribute to overall functions in the organism. Gene regulatory factors that control the expression of genomic information are diverse and complex.

Gene expression is defined as the process by which the genetic code - the nucleotide sequence - of a gene is used to direct protein synthesis (Tomizawa et al., 1997). Cell identity is controlled by the activity of transcription factors (TFs) that recognize and bind particular sequences in the genome and regulate gene expression (D’Alessio et al., 2015). Mechanisms responsible for establishment and maintenance of tissue-specific gene expression have been well studied over several decades using different methods (Chen et al., 2014, Christoforou et al., 2017, L’ecuyer et al., 1999). The primary regulation of gene expression is at the level of transcription (Neph et al., 2012, Kaplan et al., 2009). Chromatin packaging has a direct effect on transcriptional activity by limiting access to TFs, and eukaryotic gene regulation is dependent on chromatin structure in all stages of development (Bannister and Kouzarides, 2011, Karlic et al., 2010). Selective transcription is the basis for tissue-specific gene expression (Reviewed in Lodish et al., 1997).
Factors that play a role in gene regulation include tissue/sequence-specific TFs, general TFs (RNA polymerases with pre-transcriptional initiation complex factors such as TFIIA, TFIIIB, TFIID, TFIIIE), chromatin regulators, co-activators, co-repressors and other regulatory proteins (Chi and Carey 1996, Ha et al. 1993).

Transcription initiation requires cis-regulatory DNA sequences that include the promoter and enhancer regions which are recognized by specific DNA binding domains of TFs for gene activation (De Simone et al., 1987, Li et al., 1988). These TFs often contain domains which are responsible for functional interactions. Examples of commonly known domains are homeodomains (encoded by homeobox sequences), zinc finger motifs, and winged helices (Fork head box). TFs also commonly interact with additional proteins such as co-factors and other regulators that do not bind DNA sequences directly (Studitsky et al. 1995).

TFs bind directly to specific target sequences in a highly specific manner (Gourdeau and Fournier 1990). Many studies have shown that mutations in TF binding sites lead to a loss or decrease of gene activation (for example-Costa et al. 2003). Epigenetic phenomena including chromatin remodeling, histone modification and environmental conditions also play a major role in gene regulation. DNA methylation, histone acetylation and heterochromatin modeling all affect gene activity (Annunziato et al. 2000, Freeman- et al. 2000). The repression of transcriptional machinery is due to the inactive chromatin state resulting from methylation of tissue-specific promoters and histone de-acetylation (Ben-Shushan et al. 1992, Yoshida et al. 1995). During gene expression, the TFs are known to de-methylate tightly packed DNA to induce DNase I-hypersensitive sites (Cam et al. 2009, Nahon et al. 1988).
Gene activation/repression of most, if not all, genes in the genome are controlled not simply by a single factor, but through cooperative action of the numerous factors (Figure 1). In addition, gene expression is affected by transcription (initiation, elongation, and termination), mRNA processing, transport, translation, and protein stability (Hewish and Burgoyne 1973). Two well-known families of cis acting transcriptional regulatory DNA elements include (a) a promoter, composed of a core promoter and close (proximal) regulatory elements and (b) distal regulatory elements, that contain enhancers, silencers, insulators, or locus control regions (LCR) (see Fig. 2). These cis-acting transcriptional regulatory elements contain recognition sites for trans-acting DNA-binding TFs, which function either to enhance or repress transcription (Maston et al. 2006).

Eukaryotic Genomes (detailed) https://www.quia.com/jg/1277396list.html

**Figure 1:** Types of gene regulation including chromatin structure, translation of mRNA, RNA processing, RNA stability, and post-translation modification.
Figure 2. Schematic of a typical gene regulatory region. The promoter, which is composed of a core promoter and proximal promoter elements, typically spans less than 1 kb pairs. Distal (upstream) regulatory elements, which can include enhancers, silencers, insulators, and locus control regions, can be located up to 1 Mb pairs from the promoter. These distal elements may contact the core promoter or proximal promoter through a mechanism that involves looping out the intervening DNA.

1.2. Gene Regulatory Elements:

1- Chromatin Remodeling and Histone Modifications.

Chromatin structure is regulated by modification of either the DNA, or the histone proteins associated with the DNA. Chemical modification of histones includes phosphate, methyl, or acetyl groups (Lodish et al, 2000, Dahlin et al. 2015). DNA methylation tends to occur in area called CpG islands, often located in promoter regions of the genes, containing stretches with a high
frequency of CG bases. Deacetylated histones and highly methylated (hypermethylated) DNA regions are transcriptionally inactive and tightly coiled (Marchesi and Bagella 2013).

2- **Enhancers and Transcription.**

Enhancers are regulatory DNA sequences that stimulate the transcription of a gene physically placed at a distant location from the promoter. Enhancers come into nearby proximity with target promoters with the looping away of intervening sequences (see Fig. 2). Enhancers are usually some hundred base pairs long and cover clusters of different 4- to 8-bp DNA sequences that are recognized by several TFs. If TFs bind to enhancers, there is subsequent activation of transcription by recruitment of coactivators and RNA polymerase II (Spitz and Furlong 2012).

3- **The Promoter and the Transcription Machinery.**

The principal function of the promoter region is to bind the TFs that drive the initiation of transcription. The promoter region is immediately upstream of the coding sequence. The TATA box resides within the promoter region located just upstream of the transcriptional start site. Transcription occurs when RNA polymerase binds to the transcription initiation complex. To initiate transcription, a TFIID is the first to bind to the TATA box (Nikolov and Burley 1997). Binding of TFIID recruits other TFs, including TFIIB, TFIIE, TFIIF, and TFIH to the TATA box. RNA polymerase can then bind to its upstream sequence when this complex is assembled. Next, when bound along with the TFs, RNA polymerase is phosphorylated. This releases part of the protein from the DNA to activate the transcription initiation complex and places RNA polymerase in the correct orientation to begin transcription. DNA-bending protein brings the enhancer sequences, which can be quite a distance from the gene, in contact with TFs and mediator proteins (Figure 3) (Lodish et al, 2000).
Figure 3. An enhancer is a DNA sequence that promotes transcription. Each enhancer is made up of short DNA sequences called distal control elements. Activators bound to the distal control elements interact with mediator proteins and TFs. Two different genes may have the same promoter but different distal control elements, enabling differential gene expression.

1.3. TFs and Gene Regulation.

TFs ultimately direct recruitment of RNA polymerases to initiate transcription gene expression. Tissue-specific TFs play an essential role in defining cell type (Wilson et al. 2010). These factors are components of Transcriptional Regulatory Networks (TRNs) in somatic cells and are understood to maintain tissue identity. Recent discoveries have established that genes are not completely activated or deactivated through TFs, only increased or decreased in expression (Neph et al. 2012). Therefore, there is a high degree of interaction with other gene products to control expression of a single gene, so maintaining cell specific subnetworks is required to
coordinate biological functions and maintain cellular phenotypes. Most TFs are not related structurally, but they have important and diverse functions and may cross-talk during cell differentiation and lineage specification processes (Marchesi and Bagella 2013). Recent studies have suggested that expression of these lineage specific transcription factors can convert adult somatic cells from one type to another (Chen et al., 2014). Determination of cell fate is determined by lineage specific TFs regulating the expression of tens or hundreds of target genes. Thus, role of TFs in regulation of tissue specific gene expression is critical.

1.4. Fibroblasts.

Fibroblasts are the most common type of cells found in connective tissues. These cells synthesize a collection of extracellular molecules and collagen fibers (extracellular matrix) that are used to maintain a structural framework for many tissues and play an important role of tissue repair in animals (Darby et al. 2014). The extracellular matrix is the basis for connective tissues in animals (Sixt, 2012). Connective tissues are structural frameworks which support organs. A fibroblast can therefore also be defined in simple terms as a cell that manufactures and maintains connective tissues.

Connective tissue is one of four main types of tissues in the body grouped into fibroblasts, cartilage cells, bone cells, fat cells and smooth muscle cells, all of which are specialized for the secretion of collagenous extracellular matrix and are together responsible for the architectural framework of the body. Connective-tissue cells play an essential part in the support and repair of almost every tissue and organ, and the adaptability of their differentiated character is a significant feature of the responses to many types of damage (Alberts et al., 2002). Fibroblast morphology is based on a branched cytoplasm which surrounds a speckled nucleus having an elliptical shape containing two or more nucleoli. Although fibroblasts produce multiple collagens in all tissues of
the body, synthesis and deposition of collagen are not often addressed in the identification of fibroblasts since fibroblasts globally remodel the collagen matrix (Tamariz and Grinnell 2002).

Rather, fibroblasts can be defined based on morphological characteristics that can differ by location within the organism. Therefore, the most important feature of fibroblasts is their morphologic heterogeneity with diverse appearances depending on their location and activity. Fibroblast morphology includes flat, spindle shaped cells with various processes deriving from the main cell body. Fibroblasts have a unique characteristic: lack a basement membrane, whereas most permanent cell types such as those of the heart contain a basement membrane (Souders et al. 2009).

The terms "fibroblast" and "fibrocyte" are used to describe different forms of the same cell; fibroblasts are the active state of the cell while fibrocytes are the less active state of the cell. An active fibroblast can be differentiated from inactive ones by examining their endoplasmic reticulum, which is abundantly rough compared to inactive fibrocytes with a condensed rough endoplasmic reticulum (Pilling et al., 2009). Although there is a tendency to refer to these different forms as fibroblasts, the suffix "-blast" in cellular biology is used in reference to a cell in an activated state of metabolism. The appearance of fibroblasts depends on their location within the body and their activity making them morphologically heterogeneous. Within this framework, fibroblasts which are ectopically transplanted can retain positional memory of the location where they previously resided (Hannan et al., 2018).
**Fibroblasts in Development.**

The development of a fibroblasts is a complex subject due to their wide variety of locations and origins. During organogenesis, understanding where the cells come from is an important endeavor and essentially aids in knowing how the cells migrate to their location within the tissue. Depending on the stage of development, fibroblasts can arise from various sources. Fibroblasts are mesenchymal cells which occur in all tissue types that derive from the mesoderm during embryonic development (Kendall and Feghali 2014). Fibroblasts are believed to originate from mesoangioblasts which have the ability to differentiate into mesodermal tissues (fibroblasts) or endothelial cells (Souders et al. 2009). The origin of this progenitor (mesoangioblasts) in the bone marrow is the hematopoietic stem cell. Hematopoietic stem cells express many lineage markers of vascular cells such as VEGF-R2 and CD34. Additionally, studies have indicated that in adult animal cells, the fibroblast population is contributed by pericytes and mesenchymal stem cells of the bone marrow (Cossu and Bianco 2003). Albert et al. (2002) stated that, in certain conditions and situations, the process of epithelial-mesenchymal transition (EMT), whereby epithelial cells give rise to fibroblasts, is a crucial source of fibroblasts development. EMT is the evolution through which epithelial cells lose their polarity.

**Fibroblast Functions.**

The primary function of fibroblasts is the maintenance of the structural integrity of the connective tissues, through the secretion of extracellular matrix proteins such as glycoproteins, proteoglycans and collagen. Apart from their function as structural components in connective tissues, fibroblasts have a significant role in immune response to tissue injury. Following tissue injury, fibroblasts respond to wound healing through proliferating and by migration due to chemotaxis to the sites of tissue injury to restructure the extracellular matrix (ECM) as a scaffold...
for tissue regeneration. Furthermore, fibroblasts serve functions in inflammation and immune cell recruitment to sites of tissue injury (Kendall and Feghali-Bostwick 2014)

According to Darby et al. (2014), fibroblasts are early players in initiating irritation in response to invading microorganisms. In the process of wound healing, fibroblasts in an acute wound exhibit an immediate response to factors delivered by activated platelets. Some of the released factors include platelet-derived growth factor-BB, proteases, and chemokines (Chiquet et al. 2015). Fibroblasts, by presenting receptors on their surfaces, are able to induce chemokine synthesis, which triggers immune cells to respond by activating cascading events that clear the intrusive microorganisms.

Another important function of fibroblasts is monitoring any deviation from tissue homeostasis which is commonly referred to as changes in mechanical stress. The process by which fibroblasts detect any variations in the mechanical pressure within their extracellular matrix is through integrin connectors which substantially link the extracellular matrix with the cell’s cytoskeleton enabling transmission of force in both directions (Chiquet et al., 2015; Darby et al., 2014). Externally applied force results in an extracellular strain which in turn is transmitted to the cytoskeleton of the cell. Fibroblasts then counter the applied pressure through cytoskeleton traction due to the increase in cytoskeletal stress.

Fibroblasts are also significant in tumor mediation, since immune regulation of tumors is largely determined by the remodeling of the extracellular matrix which has a responsibility of regulating a variety of functions of the vital organs. Sewell-Loftin et al. (2017) state that extracellular matrix remodeling is very common in many tumor types derived from epithelial cells.
Types of Fibroblasts and Their Functions.

As mentioned above, fibroblasts are extensively distributed connective tissue cells that are found in all vertebrate organisms, so there are a variety of fibroblasts types having distinct functions in animals. Fibroblasts produce connective tissues which are important in supporting the organs in which they are produced. Fibroblasts can also show phenotypes that depend on their tissue origin and functional state (Slany et al., 2014). In this thesis, different phenotypes of fibroblasts and their functions are described.

The most abundant cell types of periodontal connective tissues are the gingival fibroblasts (GF). The GF has a primary function of repairing periodontal tissues and can also be used in stem cells for periodontal tissue engineering (Lee et al. 2013).

GFs have a critical role in the maintenance of gingival tissues and subsequently are their major constituents. Human gingival fibroblasts express a variety of molecules on their surfaces including protease-activated receptor-1. Other molecules expressed by GF include CD55, CD99, and CD63. Apart from the GF, another type of fibroblast in humans is the cardiac fibroblasts; the cardiac fibroblasts are one of the permanent cellular constituents of the heart in addition to myocytes and endothelial cells. The function of the cardiac fibroblast is to maintain the normal operation of the cardiac tissue and similarly in cardiac remodeling during conditions such as hypertension (Souders et al. 2009). In addition, cardiac fibroblasts are involved in the secretion of cytokines and growth and factors (Souders et al., 2009).

Another type of fibroblasts are dermal fibroblasts. These are located in the dermis of the skin and facilitate skin recovery post-injury. Slominski et al. (2016) established that human dermal fibroblasts express CYP11A1 10, which can interact with vitamin D3 to produce unique
CYP11A1-dependent hydroxyderivatives. Additionally, the dermal fibroblasts produce protein molecules fibronectin and laminin which are constituents of the extracellular matrix.

Yet another type of human fibroblasts is the human uterine fibroblasts (HUF), which are isolated from the uterine wall. In conjunction with additional cells, such as immune cells and epithelial cells, fibroblast cells in the uterus support an immune system which is essential for the optimization of conditions for fertilization and pregnancy (Braundmeier et al. 2012).

Finally, Human Vas Deferens Fibroblast (HVDF) and Human intestinal fibroblast (HIF) are other types of fibroblasts isolated from the vas deferens and human intestinal tissues, respectively. Table 1 describes various types of fibroblast, their functions, and from where they are isolated.

Table 1. Fibroblasts types, their location, and function.

<table>
<thead>
<tr>
<th>Type</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gingival Fibroblasts</td>
<td>Repairing periodontal tissues</td>
</tr>
<tr>
<td>2</td>
<td>Cardiac Fibroblast</td>
<td>Maintaining normal function of the cardiac and its subsequent remodeling.</td>
</tr>
<tr>
<td>3</td>
<td>Dermal Fibroblast</td>
<td>Guaranteeing skin recovery from injury</td>
</tr>
<tr>
<td>4</td>
<td>The Human Uterine Fibroblast (HUF)</td>
<td>Optimizing conditions for fertilization and pregnancy in conjunction with other cells. Remodeling the uterine tissues</td>
</tr>
</tbody>
</table>
Moreover, fibroblasts have the ability to secrete several different types of fibrous proteins which are described in table 2, depending on where they are and what type of connective tissue they are form.

**Table 2. Fibroblasts secrete several different types of fibrous proteins, their location, and function.**

<table>
<thead>
<tr>
<th>Fibrous proteins</th>
<th>Function</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>Strong fiber. Collagen is the most abundant protein in the human body. Provides tensile and compressive strength for organs and tissues.</td>
<td>Tendons, Ligaments</td>
</tr>
<tr>
<td>Elastin</td>
<td>Resilient protein. Helps tissues return to their previous shape after they have been stretched</td>
<td>Skin, Smooth muscle</td>
</tr>
<tr>
<td>Reticular fibers</td>
<td>The scaffold form for cells Make collagen type 111</td>
<td>liver, spleen, and lymph nodes are loaded with reticular fibers</td>
</tr>
</tbody>
</table>
Marker genes and specific TFs driving fibroblast identity.

There are a variety of genes that are essential in determining fibroblast identity. One such gene is Tbx20, which is a gene that encodes the TF TBX20 in cardiac fibroblasts. Studies have established that Tbx20 is instrumental in the early development of the heart and the remodeling of the yolk sac vasculature (Furtado et al. 2016). Furtado et al. (2014) further argue that Tbx20 and other genes such as Gata4, Mef2c, Tbx5, Mmp3, Hand2 and Nkx2-5 are highly enriched in the cardiac fibroblast. Another gene driving fibroblast development is a smooth muscle actin (αSMA) whose expression is the hallmark of mature myofibroblasts (Hsiao et al. 2016).

Fibroblast genes have been studied in this laboratory using a cell hybrid model system combined with whole genome analysis. Evidence suggests that specific genes such as Prrx1 and Snai2 have the ability to act as master regulators that can activate fibroblasts function. Other candidate genes identified which appear to be markers of fibroblast identity include Bmp3, Twist, Shox2, Cfos, Sema3a, and Spp1.

Recent results by Ray et al. (2016), suggest that SNAI2 (Snail Family Transcriptional Repressor 2) is an influential activator or repressor of expression of genes in mammalian cells. SNAI2 expression is ubiquitous and may affect tumor invasion as it alters the expression of E-cadherin and vimentin genes. According to Mistry et al. (2014), SNAI2 is an inducer of the epithelial to mesenchymal transition (EMT) which mediates cell migration during development and tumor invasion. Moreover, SNAI2 gene is zinc finger protein (Mistry et al, 2014). This protein is involved in the process through which epithelial cells lose their cell polarity and subsequently acquire invasive and migratory properties. SNAI2 is therefore responsible for mediating cell migration during development or the invasion of tumor (Pioli et al. 2015 and Mistry et al. 2014).
Cell migration plays a very vital role in many biological processes such as cancer growth, tissues repair, and embryogenesis. For example, fibroblasts have a significant influence in remodeling of the extracellular matrix, especially during tumor invasion related to epithelial cells, and SNAI2 acts as a master regulator of fibroblast function during such a process. As a mediator of cell migration, SNAI2 regulates epidermal progenitor cells which together with the fibroblast maintain adult skin homeostasis.

Another protein, PRRX1 (Paired-Related Homeobox 1) (Zhu et al. 2017) is a master regulator of IPF (idiopathic pulmonary fibrosis) fibroblasts and an instrumental gene driving fibroblasts identity. Froidure et al. (2016) argue that PRRX1 isoforms are upregulated in IPF fibroblasts and their overexpression inhibits the expression of platelet-derived growth factor (PDGF) migration thereby promoting IPF survival after serum deprivation (Froidure et al. 2016). The regulation of mesenchymal cell fate is carried out by PRRX1 TFs and simultaneously stand at the center of a network coordinating fibroblasts differentiation. Subsequently, this makes PRRX1 a master regulator of fibroblast identity.

According to Zhu et al. (2017) and Froidure et al. (2016), the modulation of fibroblast phenotype is directed by PRRX1 and consequently involved in IPF (idiopathic pulmonary fibrosis). Fibroblast to myofibroblast differentiation is a very significant aspect of IPF and is controlled by PRRX1, since the overexpression of PRRX1 promotes the control, survival and migration of lung fibroblasts (Froidure et al., 2016).

The family of paired-related homeobox TFs, which includes Prrx1a, Prrx1b, and Prrx2, is implicated in regulation of mesenchymal cell fate, including myogenesis and skeletogenesis (Baowen et al. 2012). Collagen, type I, alpha 1 (COL1A1) and fibroblast specific protein 1 (FSP-1) are highly expressed in fibroblasts, with FSP-1 being the standard marker for fibroblast identity,
since the promoter/enhancer region driving this gene is active in fibroblasts (Strutz et al. 1995). Österreicher et al. (2011) suggested that levels of FSP-1 expression in different organs is a marker of fibroblasts from different organs when they undergo remodeling of their tissues. Additionally, FSP-1 is used to identify fibroblast derived from the extracellular matrices of several organs including the heart, lungs, or liver.

Another marker of fibroblasts identity is a type III intermediate filament protein (vimentin) which subsequently has been used as a marker for mesenchymal-derived cells such as fibroblasts. Furthermore, a better understanding of complex pathologies associated with fibroblasts would be helped by methods to positively identify fibroblasts under a wide range of cellular states using their shape with a combination of positive staining with vimentin (Goodpaster et al. 2008).

Similarly, Fibroblasts Surface Antigen (FSA) is one of the main glycoprotein antigens identified in connective tissues. Prolyl-4-hydroxylase is another marker used for fibroblast identity as a result of its use as a putative marker for collagen-producing cells.

Finally, recent research shows that discoidin domain receptor 2 (DDR2) is expressed specifically by cardiac fibroblasts and is important in a variety of cellular functions; such as activating expression of the collagen receptor (a cell surface receptor) as well as growth, migration, and differentiation that can represent markers of fibroblast cells (Souders et al., 2009).

**Fibroblast specificity.**

During tissue remodeling and cell migration, differentiated cell types that have lost tissue-specific cues are considered to be drivers of fibroblast identity (Strutz et al., 1995). Subsequently, fibroblast are fully differentiated cells having their own identity and functions even though fibroblasts of different origin show heterogeneity in abundance, behavior, and identity.
Additionally, signaling intermediate proteins and specific transcriptions factors are reported to stimulate the conversion of fibroblasts to different cell types such as myoblasts (Tapscott et al., 1998), osteoblasts (Rutherford et al., 2002), and adipocytes (Wu et al., 1996). According to Strutz (1995), fibrosis (which is a tissue degenerative disease) is a result of the excessive synthesis of fibroblast cells and deposition of extracellular matrix substances such as proteoglycans, collagen types I and III, and fibronectin. Instances of fibrosis in tissues such as liver, skin, and lung show excessive fibroblast differentiation which affects the surrounding tissues. In fact, for development and maintenance of several tissue specific phenotypes require a strong relationship with fully differentiated fibroblasts. Existing research indicate that blood, bone and cartilage are highly dependent on fibroblast cells with fibroblasts playing a crucial role in bone, cartilage, and blood tissue specialization (Baksh et al., 2004).

**Fibroblasts in Disease.**

One of the primary function of fibroblast is the secretion of the extracellular matrix proteins to maintain the structural integrity of the tissues. However, when the accumulation of extracellular matrix in the connective tissues is undesirable, the resultant condition is termed fibrosis, a common connective tissue disease. Fibrosis is the thickening and scaring of connective tissues leading to notably scleroderma (systemic sclerosis). The process of connective tissue scarring and deposition in healing and the pathological state of surplus deposition of fibrous tissue is well be described through fibrosis. The function of fibroblasts in the development of fibrosis includes their activation and stimulation, initiated when immune cells release soluble factors which activate fibroblasts. The occurrence of fibrosis can be witnessed in different parts of the body due to inflammation or damage. Some common examples include lung (pulmonary fibrosis) and heart. Katsumata et al.
(2017) suggested that liver fibrosis that is the most common outcome of chronic liver injuries of different etiologies by accumulation of extracellular matrix proteins.

The role of fibroblasts in diseases can also be examined through cancer-associated fibroblasts (CAFs) which is a cell type that promotes tumorigenic aspects through remodeling of the extracellular matrix (Sewell-Loftin et al., 2017). Most cancers originate from epithelial cells that can be the result of fibrosis (Hinck and Næthke 2014). For example, non-cancerous cells cooperate with cancer cells, with fibroblasts affecting the biology of a tumor.

Also, certain biological processes of fibrous cells promote the spread of cancers by regulating the microenvironment of a tumor, affecting tumor size and tumor invasion of adjacent tissues (metastasis). The most important of these vital processes are wound healing, inflammation, fibrosis, angiogenesis, and their associated biochemical machinery (Mueller and Fusenig, 2004). Liu et al., (2011) reported that metastasis occurs when fibroblasts secrete the ECM proteins that modulate tumor progression and remodeling of the connective tissue surrounding a tumor. This result plays a critical role in cancer progression by allowing cancerous cells to be released from a contained tumor into the vascular system.

**Fibroblasts in Medicine.**

Fibroblasts play an important role in mammals. Not only are they responsible in the synthesis of the extracellular matrix, but also collagen, providing structure of tissues in animals and helping to repair damaged tissues. They can also migrate and accumulate in different areas to facilitate wound healing especially following injury. There is much interest to understand how to repair body tissues damaged by illnesses and injuries using fibroblasts.
Studies by Kendall and Feghali-Bostwick (2014) have shown that fibroblasts are not only effector cells in fibrosis, but also are potential targets for several therapies. Various disorders have been described as being targets of potential strategies which could inhibit or completely end fibrotic responses. Several fibroblast-specific molecules that could be of use in developing therapies include Endostatin, vitamin d3, and components of the Wnt-like signal transduction pathway. Endostatin is a super inhibitor of angiogenesis, which has gone through several clinical tests and emerged as an agent which can prevent the growth of a tumor mass. It not only inhibits vessel formation, but also endothelial cell proliferation. Endostatin is a purple peptide, which is a wide spectrum inhibitor that could interfere with the action of growth factors like endothelial growth factor and fibroblast growth factor.

Vitamin D, is another area of therapeutic interest, as it serves several functions. The main function is to regulate the absorption of minerals in the intestines, calcium and concentrations of phosphate in the blood. It also helps in maintaining the integrity of bones in the body. Studies however have shown a linear correlation between the absence of Vitamin D3 and many extra-osseous disorders. The improvement of vitamin D3 presence in the body is therefore a medicinal strategy to treat fibrotic disorders. In additionally, Wnt is a framework signaling protein which regulates the production, migration and determination of the cell-fate. The ligands of this protein enable frizzled receptors and encourage the activation of glycogen synthase kinase. Eventually, the end result is the contribution of collagen production and wound healing.

With fibroblasts being the most abundant cells in the skin, making up about 70% of the dermal cells, they constantly maintain the skin structure and contribute to healing post-injury. Chronic wounds such as diabetic foot ulcers are not easily managed using conventional treatment since fibroblasts sequestered from a diabetic foot ulcer display low cellular activity. While
fibroblasts derived from normal human tissues, exhibit high proliferative possibility and slow growth factor production, fibroblasts from diabetic foot ulcer exhibit low levels of intercellular communication in which growth factors orchestrate the wound healing process (Han et al. 2009).

According to the study carried out by Han et al. (2009), fresh fibroblasts allografts showed positive therapeutic outcome for diabetic foot ulcers. The study by Han et al. gives a feasible medical practice through which cultured fibroblast allografts could be used to successfully improve problematic wounds by secreting numerous growth factors and cytokines that regulate cell proliferation, modify the inflammatory process, and induce angiogenesis. Additionally, the transplanted fibroblasts also produce three-dimensional extracellular matrices which are comprised of collagen and other proteins. Even with a viable approach of treating diabetic foot ulcer using transplanted fibroblasts, Han et al. still suggests further investigation to determine the optimal number of transplanted fibroblasts and the fate of the fresh fibroblasts. Other curative properties of fibroblasts are still under investigation with researchers proposing numerous medicinal uses for them, such as in non-surgical lift procedures.

1.5. Models to Understand Differential Gene Expression:

1- Cell Culture as a Model.

Cell culture is a feasible model by which the mechanisms of differential gene expression can be understood. Cell culture is the removal of plant or animal cells from their natural residence and placed in an artificial environment that is favorable for their growth. The removal of the cells can be from tissues that are then disaggregated by mechanical means or enzymatic means before cultivation. Additionally, the cells can be derived from a cell line that has already been established. The application of cell culture is mainstream in molecular and cellular biology through the
provision of outstanding models for studying cell physiology and biochemistry. Cell culture is also
applied in biomedical manufacture through development and screening of drugs.

Cell culture provides a means through which information from a gene used to manufacture
protein or other molecules is used to understand regulation of gene expression. Cell culture
provides a basis by which cell physiology and biochemistry can be studied. Moreover, the ability
to grow a large number of cells or viruses in a cultured environment is significant in improving
knowledge of the genetic material of a cell, thus enabling the use of information provided by
genomic and transcriptomic project to describe gene product functions and interactions.

2- Fibroblasts as a Model.

The most common type of connective tissue in animals is fibroblasts cells that make up the
structural framework composed of the extracellular matrix and collagen. Subsequently, fibroblasts
are a favorite subject for cell biological studies because they are the easiest cells to grow in culture..
Fibroblast transfection is a commonly used method, and fibroblast transfection reagents are
commercially available. Gene expression is frequently studied using fibroblast cells and tissues.
Skin fibroblast cells are easily acquired and can be cultured in a conducive environment to grow
large amounts of the cell and simultaneously maintain their stability for several generations
(Logotheti et al., 2015). Nowinski et al. (2004) provided a model through which gene expression
can be understood and studied using fibroblast cells. The study is based on gene expression of
fibroblast cells throughout the wound restorative progression in which keratinocytes communicate
with fibroblasts, thereby determining different gene expression to reestablish a functional
epidermis. This study suggests that keratinocytes disturb the activity of fibroblasts gene expression
throughout the process of epithelialization. Therefore, fibroblasts serve as a model which can be
used to understand differential gene expression.
3- Somatic Cell Hybridization.

One model used to understand fibroblast-specific gene function is through the use of somatic cell hybrids. Somatic hybrids have been used as a significant tool to examine the extinction of tissue-specific traits for over 40 years (Barski et al. 1960). This technique was among the first used to study the molecular mechanisms underlying tissue specific gene expression. Somatic cells hybrids are culture lines which are formed through the fusion of somatic cells derived from different or the same species. One viable process by which cell hybrids are achieved is by fusing cells in the presence of Sendai virus which facilitates the fusion to form a hybrid cell (See Figure 4). Sendai virus is used as a fusogen, since spontaneous fusion of cells occur at a low frequency (Marro et al. 2014). To guarantee that the fused cells are real hybrids, karyotype analysis is performed on parental and hybrid cells hence the hybrid cells have twice the chromosome average of the parental cells. Hence, a hybrid cell contains a full complement of the chromosomes from the original cell parents.

![Figure 4. Flow chart showing fusion of somatic cells hybrids.](image-url)
Immediately after a successful fusion of mouse X human cells, human chromosomes are more commonly eliminated. However, studies have indicated that intra-species hybrid (rat hepatoma and rat fibroblast) have a stable genotype that maintain tetraploid karyotype a well as expression profiles and are suitable as a model to understand gene regulation. For example, using somatic cell hybridization, extinction of lineage-specific genes has been studied in the pituitary cells and liver. Somatic cell hybridization has been used as a technique for studying repression of lineage specific genes in muscles, liver, pituitary cells, and myeloid cells (Ringertz and Savage.1976).

In somatic cell hybrids, some tissue specific genes are silenced, a phenomenon which is known as extinction (Bulla et al., 2012). Extinction includes regulatory mechanisms that inhibit the expression of specific genes for specific cell types during development. Gene silencing occurs due to the loss of tissue-enriched TFs.

Somatic cell hybridization has been studied intensively in our lab to study extinction of fibroblast-specific functions to provide an understanding of the regulation of fibroblast-specific gene and the upstream regulators. Recently, Ray et al. (2016) studied genome-wide gene silencing of fibroblast cells using the somatic cell hybridization approach. Here, rat hepatoma cells and rat fibroblasts were fused to generate FR (2) hybrids. The advantage of using intra-species cells to generate hybrids is that the resultant hybrid cell demonstrates a stable genotype with minimal chromosomal loss.
1.6. DNA Microarray Analysis.

DNA microarray (DNA chip or biochip) analysis is a tool used to study the expression of all genes of a tissue in a single experiment (Hihara et al., 2001). The practical application of DNA microarrays is in its ability to diagnose diseases or test genetic predisposition. Microarray analysis utilizes microchips that contain anchored arrays of short DNA probes; labelled nucleic acids are applied to the arrays and subsequent imaging and data processing carried out to monitor hybridization to specific probes. Each data point produced by a DNA microarray hybridization experiment represents the ratio of expression levels of a particular gene under two different experimental conditions (Brown et al., 2000). Through a DNA microarray analysis, differential patterns of gene expression can be studied thereby giving clues to their biological roles.

Through microarray experiments, genes of comparable functions have been studied to take on similar expression patterns thus offering biological significance and assigning functions to genes. A DNA microarray analysis can also be used to examine different gene expression in human fibroblast cells. Furtado et al. (2014) showed that DNA microarray analysis is essential in understanding cardiac fibroblast function to establish operational therapeutic managements for heart failure. Microarray analysis datasets have important issues when it comes to data noise; however, Furtado et al. (2014) state that using a robust lmpFit method is instrumental as it is noise-tolerant as regarded within the bioinformatics community.

One viable case study in which DNA microarray has been utilized is by Ray (2016) using whole genome microarray experiments conducted using RNA isolated from parental cell hybrid line. This allowed for analysis if 31,042 target genes. Ray (2016) confirm the repression of genes in somatic cell hybrids by q-RRT-PCR. The results of the study indicated fibroblast TFs, Prrx1 and Snai2 are repressed in hybrid cells (Ray, 2016).
1.7. Cell Migration.

The development and maintenance of multicellular organisms require cell migration (Ray, 2016). During embryonic development, immune response, and wound healing processes, coordinated cell movements take place in distinct directions to specific locations. Cells are activated in response to specific external signals (mechanical and chemical). Errors during cell migration have detrimental outcomes leading to conditions such as intellectual disability, tumor formation, and metastasis. Additionally, abnormal cell migration during embryogenesis leads to birth defects while lack of cell migration within the body of an adult negatively affects wound healing process and inflammation response (Sixt, 2012). An understanding of the cell migration process is important to development of novel therapeutic strategies which when initiated can help mitigate invasive tumor cells.

Staying within a highly viscous environment, cells need to produce force to help them move and can achieve such movement through different mechanisms. Prokaryotic organisms, as well as less complex eukaryotic cells such as the sperm cell, make use of cilia or flagella for locomotion. Complex eukaryotic cell migration consists of different migration mechanisms with the two most common migration scenarios entailing blebbing motion and crawling motility. As fibroblasts synthesize extracellular matrix, they can undergo migration when responding to injury or inflammation. Fibroblasts move in a type of ameboidal-type cell migration; this is a type of cell migration which is accomplished by the extension and retraction of a pseudopodium. Sixt (2012) argue that fibroblasts migrate in 2-D (two dimensional) surfaces by developing lamellipodia-actin abundant extensions.

Hulkower and Herber (2011) describe various methods through which cell migration is studied including Scratch Assays, Transmembrane Assays, Microfluidic Chamber Assay, and Cell
Exclusion Zone Assays. According to Hulkower and Herber (2011), the Scratch Assay is a simple low-cost method which is used to measure cell migration in a controlled environment outside an organism (in vitro). The Scratch Assay method entails capturing images at several time points after creating a scratch in a cell monolayer. Performing this procedure allows quantification of the rate of cell migration. Another method for studying cell migration is Transmembrane Assay which uses a chamber separated into two compartments by a porous filter membrane to allow the study of leukocyte migration in response to antibody-antigen complexes as a chemotactic agent (Hulkower & Herber, 2011). Through this assay, a solution which is to be tested for chemotactic activity is seeded on one side while the cells are seeded on the other side of the membrane. Finally, the number of cells which have migrated to the other side of the membrane in response to the chemotactic agent are counted microscopically. The Microfluidic Chamber Assay uses microfluidic systems whereby cells are introduced in a small port and stick to the chamber bottom while the test agents are added to the larger port hence providing a basis by which cells are imaged to measure migration in response to the test agent (Hulkower & Herber, 2011). Cell Exclusion Zone Assays are similar to Scratch Assays and Microfluidic Assays. However, the Cell Exclusion Zone Assays is characterized using a barrier to prevent cultured cells from growing and making healthy blood cells (engraftment).

1.8. Objective and the Goals of this study.

A previous study using whole genome microarray analysis and somatic cell hybridization showed that Prrx1 and Snai2 play important roles in activating expression of fibroblast identity in somatic cell hybrids as model systems (Ray, 2016). To extend these studies, we addressed the questions of whether fibroblast specific transcription factors Snai2 and Prrx1 could reprogram
hepatoma cells to a fibroblast phenotype. To this end, the objective is to overexpress these genes and monitor both liver- and fibroblast- specific gene expression.
Chapter 2

Materials and Methods

2.1. Cell Culture

The cells used in this study were rat hepatoma cell line (FG14) originally obtained from a rat liver tumor by Mary Weiss and colleagues. These cells have been studied widely and genetically modified to allow for increasing our understanding of liver function. Cells were maintained in a medium containing 1:1 Ham’s F12/Dulbecco’s modified Eagle’s medium (FDV) with 10% fetal bovine serum (FBS) plus 5 µg/ml of penicillin and streptomycin (GIBCO). All cells were incubated in water-jacketed incubator at 37°C in a humid 5% CO2. After two days, cells were split, counted cells by using a hemocytometer, and prepared for transfection.

2.2. Cell Transfection

Cells were transfected with candidate genes through the process of lipofection using commercially available reagents (Invitrogen). In this procedure, DNA is introduced into the cells using liposomes. The liposomes trap the DNA and fuse with the cell membrane of the target cells, releasing the DNA into the cells. Expression vectors containing candidate genes (PRRX1 and SNAI2) purchased from Origene, Inc. were introduced separately into the rat hepatoma cell line (FG14) by lipofection. 6-Well cell culture plates were used for the transfection of candidate genes. 0.5 ml of FDV media without penicillin and streptomycin (Pen/Strep) was added to a microcentrifuge tube. DNA (1 µg/µl) along with 5 µl Lipofectamine Plus reagent (Invitrogen, Inc) was added and mixed gently for five minutes at room temperature. Then, 5 µl of Lipofectamine LTX reagent was added and mixed gently by pipetting and incubated for 30 min at room temperature.
Cells to be transfected were plated at a density of $10^6$/well in a 6-well format the day before transfection. Media on cell plates was removed by pipetting. The transfection mixture prepared in the microfuge tubes were added in the wells of the plates and incubated at 37° C in 5% CO$_2$ incubator for 6 - 8 hrs, rocking periodically. After 6 - 8 hrs, medium was replaced with FDV containing 10% fetal bovine serum plus penicillin and streptomycin and incubated for 2 days at 37° C in 5% CO$_2$ incubator. For stable transfection, cells were split into various dilutions (typically several replicate T-25 flasks at 1:20, 1:10 and 1:5 dilutions) in complete medium plus 500 µg/ml G418 and incubated for 2-3 weeks. After that, G418 resistant clones were pooled (10—50 clones per pool) or picked individually then expanded into larger plates until the cells could be lysed and RNA extracted.

To monitor transfection efficiency, cells were also transfected with a Green Fluorescent Protein (GFP) expression plasmid. Typically, 4-6% transfection efficiency was achieved as determined by counting the number of GFP positive cells 48 hours post-transfection. In addition, a no-DNA control plate was used a negative control to ensure that no G418 –resistant cells were present in the cell line being transfected.

2.3. RNA Extraction

RNA was extracted from 70-80% confluent monolayers (approximately $10^7$ cells) using a Qiagen RNeasy Mini Kit (Cat #74104) following the manufacturers protocol with the addition of a DNAse I (Cat #79254) digestion step, as per protocol. Briefly, nutrient medium was removed from the culture dish, and cells were lysed by treating with 350 ul highly denaturing guanidine isothiocyanate (GITC) containing RLT buffer and β-mercaptoethanol and mixed by rocking the plate, then scraped and transferred into a Qiashredder column in a 2 ml collection tube. Samples were centrifuged 2 minutes at 15,000 rpm to homogenize. The flow-through was mixed with 70%
ethanol by pipetting, the mixtures were transferred to RNeasy columns in collection tubes and centrifuged at 10,000 rpm for 15 seconds and flow through discarded.

Next, RWl buffer was used to wash the resin and centrifuged 10,000 rpm for 15 secs followed by digestion with DNase I in RDD buffer (Qiagen Cat #79254) for 15 minutes at room temperature. To prepare DNasel, 10 ul of DNAsel was added to 70ul RDD (supplied with DNasel kit). Next, RWI buffer was applied to onto the RNeasy column and centrifuged 10.000rpm for 15 seconds. The RNeasy column was transferred to a new 2 ml collection tube, and the RNeasy column was washed twice with 500ul RPE and centrifuged after each wash. Finally, the RNeasy column was transferred to a 1.5 ml tube, and the RNA was eluted by adding 40 µl RNase free water into the column and centrifuging at 14,000 rpm for one minute. The RNA samples were collected in microfuge tubes and stored at 70° C. A Bio line spectrophotometer with a nanodrop plate reader was used to determine the concentration and purity of the RNA.

2.4. cDNA Synthesis

Complementary DNA was obtained using the reverse transcriptase enzyme from a mature, fully spliced, messenger RNA. The mature RNA was purified from the cells using affinity chromatography (Ying, 2004). The complimentary DNA strand was then created by the addition of the poly-T oligonucleotides that anneal to the poly-A tails of the RNA. The second strand was generated by first digesting the RNA strand using RNase H followed by synthesis of the complementary strand to yield double stranded DNA.

The MasterAmp High Fidelity RT-PCR kit (Applied Biosystems, part# 4368814) was used to generate cDNA from purified RNA. Reaction mixtures contained (10X RT Buffer, 25uM dNTP Mix, 10uM RT Random Primers, MultiScribe and 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 Bio­
rad Thermal Cycler was used to synthesize cDNA using 25 °C for 10 min, followed by 37° C for 2 hours, 85°C for 5 min, then 4° C until reactions could be transferred to a microfuge tube and stored at -20° C. The stored cDNA tubes were diluted to the appropriate concentrations used for quantitative PCR (qPCR).

2.5. Primer Design

Primer design was done using software provided by NCBI. This program has been optimized to identify primer pairs that hybridize to single gene targets at temperatures between 55 and 65 °C and produce short amplicons when using qPCR. Typically, the melting temperature of the primers can be calculated using nearest neighbor thermodynamic calculation. Specificity of the primers can be improved by ensuring the 3' ends are not very sticky. The intermolecular homology within the primers should be avoided to reduce the chances of the formation of the snap backs that interfere with the annealing process (Lin and Brown, 1992).

In this study, the primers were ordered from Integrated DNA Technologies Inc. Three primers sets were designed for each gene to be tested, and the primer set that produced optimal amplification with lowest background signal for both the parental hepatoma cells and fibroblasts cells were used for further experiments. We monitored expression of several genes known to be important in hepatic and fibroblast function. Primer pairs used for this study as well as predicted melting and annealing temperatures are given in the Tables 3 and 4.
Table 3. Primers used in the quantitative Polymerase Chain Reaction (qPCR), primer sequences, melting temperature and annealing temperature of primers for the parental hepatoma cells.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Tm (°C)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNF1</td>
<td>F- 5’CCAATCTGAAAGAGCTGGAGAAC 3’</td>
<td>57.1</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5’AGGTACGACTTTGACCATTTCATCTTG 3’</td>
<td>55.6</td>
<td></td>
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<tr>
<td>rHNF3 (4)</td>
<td>F- 5’ TGGCAGAACTCCATCGTCATCT 3’</td>
<td>60.2</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>R- 5’ GCCATCAAGCCAGTTGTCATCAGTCT 3’</td>
<td>60.5</td>
<td></td>
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<tr>
<td>HNF4</td>
<td>F- 5’ TGAGCTGAGGATTGACATCAAC 3’</td>
<td>56.6</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5’ AACTGGATCTGCTGATGTCATCAGTCT 3’</td>
<td>56.5</td>
<td></td>
</tr>
<tr>
<td>HNF6</td>
<td>F- 5’ CAGTGCTCTAAAGCAAGATAAA 3’</td>
<td>54.5</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5’ CAGTGCTGTGGAAGACAGATAAGA 3’</td>
<td>54.3</td>
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<td>SERPINA1</td>
<td>F- 5’ CCTATAACCGGAGCTGGTCCAT 3’</td>
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<td>64</td>
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<td>R- 5’ TTGCGAGGATGTCACCTCTGT 3’</td>
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<td>ALB</td>
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<td>R- 5’ TTCCACCAAAGACCCACTA 3’</td>
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<td>R- 5’ GTGCAATGGAATGACAGACACGTGCT 3’</td>
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<td></td>
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</tbody>
</table>
Table 4. Primers used in the quantitative Polymerase Chain Reaction (qPCR), primer sequences, melting temperature and annealing temperature of primers for the parental fibroblasts cells.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Tm (°C)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Prrx1</td>
<td>F- 5'TGT CTI TGA GCG GAC ACATTA 3'</td>
<td>$54.2$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5'GAA CTT GGC TCT TCG GTTCT 3'</td>
<td>$54.9$</td>
<td>60</td>
</tr>
<tr>
<td>Rat Prrx1</td>
<td>F- 5'GAA CCG AAG CTG GGA GAAA 3'</td>
<td>$54.9$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5'AGG AGA GAGAGAGAGAGAGAGAGA 3'</td>
<td>$54.9$</td>
<td>60</td>
</tr>
<tr>
<td>Snai2</td>
<td>F- 5'CTG GAT ACT CCT CAT CTITGGG 3'</td>
<td>$54.5$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5'CTCTTCGTCACTAATGGGACTT 3'</td>
<td>$54.1$</td>
<td>60</td>
</tr>
<tr>
<td>C-Fos</td>
<td>F- 5'GCT GGT GCA TTACAGAGAGAA 3'</td>
<td>$54.8$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5'GTGTGGTTTTACGCACAGATAAG 3'</td>
<td>$54.3$</td>
<td>60</td>
</tr>
<tr>
<td>Twist</td>
<td>F- 5'TCG CTG AAC GAG GCA TTT 3'</td>
<td>$54.8$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5'GCC AGT TIG AGG GGT TCT TGA AT 3'</td>
<td>$55.1$</td>
<td>60</td>
</tr>
<tr>
<td>Shox2</td>
<td>F- 5'CTG AAG GAT CGC AAA GAG GATG 3'</td>
<td>$55.6$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5'CGT TGA GTT GTC TCA GGG TAAA 3'</td>
<td>$55.2$</td>
<td>60</td>
</tr>
<tr>
<td>Spp1</td>
<td>F- 5'CAG CCA AGG ACC AAC TAC AA 3'</td>
<td>$55.0$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5'TGCCAA ACT CAG CCA CTT 3'</td>
<td>$54.8$</td>
<td>60</td>
</tr>
<tr>
<td>Bmp3</td>
<td>F- 5'CTA GAG GCT AGA GGG AGA ACTT 3'</td>
<td>$54.9$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5'GAC AGA GAG ACA GAG ACA GAGA 3'</td>
<td>$54.8$</td>
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<tr>
<td>Colla1</td>
<td>F- 5'ACT GGT ACA TCA GCC CAA AC 3'</td>
<td>$55.0$</td>
<td>60</td>
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<tr>
<td></td>
<td>R- 5'GGA ACC TTC GCT TCC ATA CTC 3'</td>
<td>$55.3$</td>
<td>60</td>
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<tr>
<td>Sema3a</td>
<td>F- 5'GGG AGA AGA CTG TAT CTT C 3'</td>
<td>$55.1$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R- 5'GATGGGGCACTGATGAATCTAGG 3'</td>
<td>$55.1$</td>
<td>60</td>
</tr>
</tbody>
</table>
2.6. Quantitative-Polymerase Chain Reaction (qPCR)

q-RT-PCR assays were performed in triplicate for each gene tested. All the cDNA samples were diluted and concentration (5 ng/µl) was standardized among the samples. A final volume of 20 µl of reaction mixture contained 2 µl of specific cell line cDNA template (5 ng/µl), 2 µl of both forward and reverse gene-specific primers (0.5 µM) (IDTDNA), 6 µl of sterile nuclease free water, and 10 µl of Fast SYPR® Green Master Mix (Applied Biosystems), with the total volume of the reaction mixture at 20 µl. The list of primers used for genes and their Tm are described in Tables 3 and 4. An Applied Biosystems StepOne qPCR machine was used to amplify cDNA. Typical reactions included 40 cycles, with the first step at 95° C for 3 min, followed by second step at 5° C above melting temperature (Tm) for the primers 30 min for extension/ annealing, and an extension step at 60° C for 60 min. Also, the control used in the RTqPCR experiments contained 2 µl of gene specific primer (0.5 µM) (IDTDNA), 8 µl of sterile nuclease free water, and 10 µl of Fast SYPR® Green Master Mix (Applied Biosystems), but were without target cDNA.

The cDNA quality was tested against the reference gene GAPDH. Duplicate assay was performed for each cell line and the reaction was repeated three times. Raw threshold (Ct) value was averaged after the amplification for each cell line. The amplified target genes in each cell line were normalized to the respective GAPDH Ct-value, generating a delta-Ct value (Act). Fold differences in gene expression were determined with a delta delta-Ct (ΔΔCt) calculation. The ΔCt of the control cell line was subtracted from the test 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). Standard deviation (SD) and P-value of ΔΔCT in three biological replicates was calculated using a threshold to select significant transcriptional regulation edges.
2.7. Cell Migration Assay

The scratch assay is an easy way to monitor cell migration in vitro. In this study we monitored cell migration of Prxl and Sna2 transfected Fg14 cells (Fg14-Prxl & Fg14-Sna2) compared to the non-transfected cells (Fg14 hepatoma & RAT1 fibroblasts) (Hulkower and Herber, 2011). In this technique, using a sterile 12 well plate, a marker was used to draw horizontal lines at the base of the plate. One ml of culture cells containing ~10^6 cells were added to each of the wells and incubated for 24 hours at 37°C that allowed to attach, spread, and form a confluent monolayer. The next day, a 200μl micropipette tip was used to scratch a trough in each well by dragging the pipette tip along the entire length of the well such that the scratch was perpendicular to horizontal line of the marker.

The 12 well plate was then visualized using light microscopy connected to a computer installed with imaging software. Images were acquired at 0, 4, 8, 12 hours and so on until the scratch was completely or nearly filled in.

![Image of Scratch Assay](image)

**Figure 5. Images of Scratch Assay.** A “wound” is introduced into a confluent monolayer of cells (A) by drawing a tip across the cell layer (B). The denuded area is imaged to measure the boundary of the wound at pre-migration (C) and after cells have migrated inward to fill the void (D).
2.8. Photomicrography of cells

To monitor differences in cell morphology of the transfected and non-transfected Fg14 cells and RAT 1 fibroblasts, cells were plated onto coverslips placed in a 6 well plate and incubated overnight at 37°C in a 5% CO2 incubator. The following day, forceps were used to remove coverslips containing the cells, and the coverslips were placed on a slide with cells facing the slide. PBS was added as needed to prevent drying of the cells the images were collected on 400x magnification using phase 2 rings.
Chapter 3

Results

As a model to understand the role of candidate genes Prrx1 and Snai2 in regulation of fibroblasts-specific gene expression, the hepatoma cell line Fg14 was used. Previous results from our laboratory showed that these two genes, originally identified as candidate master regulatory genes in a genome-wide screening of gene expression in hepatoma x fibroblast cell hybrids (Bulla, 2010), could remodel cell hybrids to become more fibroblastic in nature. The goal of this study was to determine whether these genes could drive expression of fibroblasts-lineage specific transcription factors (TFs) in a fully differentiated hepatoma cell line.

To this end, expression plasmids containing Prrx1 (paired related homeobox) and Snai2 (snail family transcriptional repressor 2) genes in expression cassettes in a neo plasmid were transfected individually into the Fg14 cells and transfected cells obtained using G418 selection. G418 resistant clones were picked individually or pooled and expanded into 100mm cell culture dishes. RNA was then extracted and used to generate complimentary DNA (cDNA) that was then used in the qPCR reactions to monitor expression of target genes.

3.1. Prrx1 overexpression in Fg14-transfected cells

First, Prrx1 gene expression was compared between the RAT1 fibroblasts cells and the Fg14 cells using rat-specific primer. As shown in Figure 6, Prrx1 gene expression is robust in the RAT1 fibroblasts, but nearly undetectable in the Fg14 cells. We next asked whether the transfected mouse Prrx1 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...
qPCR analysis revealed that Prrxl was successfully over-expressed in pooled Fg14 transfectants as well as individual clones compared to the non-transfected Fg14 cells (Figure 7).

**Figure 6. Prrxl is highly fibroblast specific.** Rat specific primers were used to monitor Prrxl expression in the Fg14 rat hepatoma and Rat1 fibroblast cells by qPCR analysis. The fold activation was normalized to GAPDH levels using the $\Delta\Delta$CT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.
Both the pooled clones well as the 6 individual clones tested expressed the introduced Prrx1 gene at levels >1000 fold higher than the signal in the Fg14 cells (Figure 7). Note that, because these are mouse-specific primers, background signals in the rat-derived Fg14 cells could either be due to cross-hybridization to rat sequences, or spurious hybridizations to other sequences resulting in background amplification. For this reason, cycle number differences compared to the parental Fg14 cells are shown.

3.2. Prrx1 Overexpression – Activation of downstream fibroblast-specific genes

Next, we asked whether Prrx1 overexpression in hepatoma cells (Fg14) could affect expression of downstream fibroblasts genes. Pooled Fg14-Prrx1 cells were tested for fibroblast-specific gene expression using qPCR. Results showed that Prrx1 activates several fibroblast-specific genes. Of the 9 fibroblast-specific genes tested, several of these (Cfos, Colla1, Shox2, Twist, and Bmp3) were activated in Fg14-Prrx1 pooled cells (Figure 8). Levels of activation ranged from 4-7-fold for Twist, Shox2 and Bmp3 to 15-25-fold for Colla1 and C-Fos. Other genes (Spp1, Slug, SemA3 and Sna2) were unaffected.

To verify the effect of Prrx1 overexpression on activation of fibroblast-specific genes, we next tested expression profiles for individual Fg14-Prrx1 clones, focusing on genes that showed activation in the pooled transfectants. While C-Fos expression was found to be nearly 100-fold higher expressed in the RAT1 cells compared to Fg14 cells, the Fg14-Prrx1 pool and clones showed variable activation, ranging from 4 to 52-fold (Figure 9). Similarly, while Colla1 expression was found to be >1000-fold higher expressed in the RAT1 cells compared to Fg14 cells (data not shown), the Fg14-Prrx1 pool and clones showed variable activation, ranging from 3 to 15-fold (Figure 10).
Figure 7. mPrrxl overexpression in transfected Fg14 rat hepatoma cells. Mouse-specific primers were used to monitor over-expression of the introduced mouse Prrxl gene in the Fg14 rat hepatoma cell line. Fgl4 cells were stably transfected with a mouse Prrxl expression plasmid, and G418-resistant clones pooled or selected individually. Prrxl expression of 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 set for each trial.
Prrxl over-expression activates some fibroblast-specific genes in Fg14 hepatoma cells

![Bar chart showing fold activation of various genes](image)

Figure 8. Prrxl activates several fibroblast-specific gene expressions (Cfos, Collal, Shox2, Twist, and Bmp3) in Fg14 rat hepatoma cells. Pooled Fg14-Prrxl were tested for fibroblasts specific genes expression using qPCR of cDNA derived from Fg14 cells. The fold activation was 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 9. Prrxl strongly activates C-Fos expression in Fg14 rat hepatoma cells. Prrxl-overexpressing Fg14 cells (pooled and individual clones) were tested for C-Fos expression using qPCR of cDNA derived from Fg14 cells. C-Fos expression was found to be nearly 100-fold higher expressed in the RAT1 cells compared to Fg14 cells. The fold activation was 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 10. **Prrxl modestly activates Coll1a1 expression in Fg14 rat hepatoma cells.** Prrxl-overexpressing Fg14 cells (pooled and individual clones) were tested for Coll1a1 expression using qPCR of cDNA derived from Fg14 cells. Coll1a1 expression was found to be >1000-fold higher expressed in the RAT1 cells compared to Fg14 cells. The fold activation was 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.
3.3. Snai2 overexpression in Fg14-transfected cells

We next tested whether overexpression of a second fibroblast TF, Snai2 (aka Slug), could activate fibroblast-specific genes in the Fg14 hepatoma cells. A mouse Snai2 expression plasmid was transfected into the Fg14 cells and G418-resistant clones expanded and tested. Using rat-specific primers which cross-hybridized to mouse Snai2 cDNA sequences, qPCR analysis revealed that Snai2 was successfully over-expressed in pooled Fg14 transfectants as well as individual clones compared to the non-transfected Fg14 cells (Figure 1). Both the pooled clones as well as the 6 individual clones tested expressed the introduced Snai2 gene at levels >1000 fold higher than the signal in the Fg14 cells, which is nearly undetectable.

3.4. Snai2 Overexpression – Activation of downstream fibroblast-specific genes

We next asked whether Snai2 overexpression in hepatoma cells (Fg14) could affect expression of downstream fibroblast genes. Pooled Fg14-Snai2 cells were tested for fibroblast-specific gene expression using qPCR. Results showed that Snai2 activated several fibroblast-specific genes. Of the 8 fibroblast-specific genes tested, several of these (C-fos, Shox2, Twist, and Bmp3) were activated in Fg14-Snai2 pooled cells (Figure 12). Levels of activation ranged from 3-16-fold for (Twist, Shox2 and Bmp3) to 25-fold for C-fos. Other genes (Spp1, Prx1, SemA3 and Col1a1) were unaffected.
Figure 11. Snai2 over-expression in the Fg14 rat hepatoma cell line. Fg14 cells were stably transfected with a mouse Snai2 expression plasmid, and G418-resistant clones pooled or selected individually. Snai2 expression of cDNA derived from isolated mRNA was monitored by qPCR analysis. The cycle # was 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. Snai2 activates additional fibroblasts specific genes (Cfos, Twist, Shox2, and Bmp3) expression in Fg14 rat hepatoma cells. Pooled Snai2-expressing Fg14 and clones (Fg14-Snai2-Pooled) were tested for fibroblasts specific gene expression using qPCR of cDNA derived from extracted RNA. The fold activation was normalized to GAPDH levels using the \( \Delta \Delta CT \) method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.
To verify the effect of Snai2 overexpression on activation of fibroblast-specific genes, we next tested expression profiles for individual Fg14-Snai2 clones, focusing on genes that showed activation in the pooled transfectants. While C-Fos expression was found to be nearly 100-fold higher expressed in the RAT1 cells compared to Fg14 cells, the Fg14-Snai2 pool and clones showed variable activation, ranging from 9 to 70-fold (Figure 13). Likewise, Twist expression was found to be >1000-fold higher expressed in the RAT1 cells compared to Fg14 cells (data not shown), but the Fg14-Snai2 pool and clones showed variable activation, ranging from 16 to 90-fold activation (Figure 14).

While Shox2 expression was found to be >1000-fold higher in the RAT1 cells compared to Fg14 cells (data not shown), the Fg14-Snai2 pool and clones showed variable activation, ranging from 3 to 7-fold (Figure 15). Bmp3 expression was found to be >1000-fold higher in the RAT1 cells compared to Fg14 cells (data not shown), the Fg14-Snai2 pool and clones showed variable activation, ranging from 1 to 5.5-fold (Figure 16).

3.5. Effect of Snai2 and Prx1 overexpression on hepatoma gene expression

Based on the above findings, we hypothesized that overexpression of fibroblast-specific transcription factor Prx1 and Snai2 would result in partial loss of the hepatoma phenotype. We therefore monitored expression of a panel of liver-specific genes using qPCR analysis. First, we asked whether the transfected mouse Prx1 in hepatoma cells (Fg14) could affect liver-specific genes. Pooled Fg14-Prx1 cells were tested for liver-specific gene expression using qPCR. To ensure that our primer sets could detect differences in expression levels, we tested cDNAs derived from Fg14 hepatoma cells and the RAT1 fibroblasts. All genes tested showed >1000-fold higher expression in the Fg14 cells compared to the RAT1 fibroblasts (results not shown).
Figure 13. Snai2 strongly activates Cfos expression in Fg41-Snai2 clones. Fg14-Snai2 pooled and clones were tested for Cfos expression using qPCR of cDNA derived from extracted RNA. C-Fos expression was found to be nearly 100-fold higher expressed in the RAT1 cells compared to Fg14 cells. The fold activation was normalized to GAPDH levels using the ΔΔCT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set each trial.
Figure 14. Snai2 strongly activates Twist expression in Fg14-Snai2 clones. Fg14-Snai2 pooled and clones were tested for Twist expression using qPCR of cDNA derived from extracted RNA. Twist expression was found to be >1000-fold higher expressed in the RAT1 cells compared to Fg14 cells. The fold activation was 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.
Snail2 over-expression activates Shox2

Figure 15. Snail2 modest activates Shox2 expression in Fg14-Snai2 clones. Fg14-Snai2 pooled and clones were tested for Shox2 expression using qPCR of cDNA derived from extracted RNA. Shox2 expression was found to be 1000-fold higher expressed in the R AT1 cells compared to Fg14 cells. The fold activation was 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. Snai2 modest activates Bmp3 expression in Fg14-Snai2 clones. Fg14-Snai2 pooled and clones were tested for Bmp3 expression using qPCR of cDNA derived from extracted RNA. Bmp3 expression was found to be >1000-fold higher expressed in the RAT1 cells compared to Fg14 cells. The fold activation was normalized to GAPDH levels using the $\Delta\Delta$CT method for each cell line. The experiments were repeated at least 3 times, with triplicate reactions set for each trial.
Of the 11 liver-specific genes tested, several of these (Cregl, Pckl, Hnf4, Serpina6, Hnf6, Serpina3n, Fgb4, Serpinal, and Kngl) were repressed in Fgl4-Prrxl pooled cells compared to the parental Fgl4 cells (Figure 17). Levels of repression ranged from 40 to 200-fold for Hnf4, Pckl, Serpina6, Hnf6, and Serpina3n, to 7 to 23-fold for Cregl, Fgb4, SerpinaAl, and Kngl. Other genes (Alb and Hnfl) were unaffected.

To verify the effect of Prrxl overexpression on repression of liver-specific genes, we next tested repression profiles for individual Fgl4-Prrxl clones, focusing on genes that showed repression in the pooled transfectants. The Fgl4-Prrxl pool and clones showed strong repression of Serpinal, ranging from 40 to 134-fold (Figure 18). Similarly, other hepatoma genes showed strong repression, including Hnf6 (20 to 314-fold), Serpina3n (34 to 142-fold), Serpina6 (64 to 90-fold), and Hnf4 (75 to 246-fold) (Figure 18). Expression of other hepatoma genes were also found to be repressed in the Fgl4-Prrxl clones. Repressed genes included Fgb (20 to 27-fold), Kng (34 to 71-fold), Pckl (26 to 34-fold), and Cregl (3.5 to 4.5-fold) (Figure 19).

We next asked whether the transfected mouse Snai2 in hepatoma cells (Fgl4) could affect liver-specific genes. Pooled Fgl4-Snai2 cells were tested for liver-specific gene expression using qPCR. Results showed that Snai2 repressed several liver-specific genes. Of the 11 liver-specific genes tested, several of these (Alb, Pckl, Hnf4, Serpina6, Hnf6, Serpina3n, Fgb4, SerpinaAl, and Kngl) were repressed in Fgl4-Snai2 pooled cells. (Figure 20). Levels of repression ranged from 29.5-163.5-fold for (Hnf4, Serpina6, Kngl and Serpina3n) to 5.5-14-fold for (Pckl, Hnf6, Alb, Fgb4, SerpinaAl). Other genes (Cregl and Hnfl) were unaffected.

To verify the effect of Snai2 overexpression on repression of liver-specific genes, we next tested repression profiles for individual Fgl4-Snai2 clones, focusing on genes that showed repression in the pooled transfectants. The Fgl4-Snai2 pool and clones in Serpina6 showed
variable repression, ranging from 85 to 89-fold (Figure 21). Likewise, Kngl (4 to 22-fold), Hnf6 (8 to 14-fold, Serpina3n (9 to 14-fold) and Hnf4 (9 to 12-fold) showed repression, although at more modest levels. Finally, three liver-specific genes tested (Pck1, Alb, and Fgb4) showed repression ranging from 2 to 5-fold (Figure 21).

Figure 17. Repression of liver-specific genes in Prrx1 transfected pooled clones (Fg14-prrx1 pool) in the Fg14 hepatoma cell line. RNA levels of indicated genes were determined by RT-qPCR and compared to levels in non-transfected Fg14 hepatoma cells. The fold repression was 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.
Effect of Prrxl on repression of liver-specific genes in Fg14 hepatoma cells

Figure 18. Fold repression of liver-specific genes (Hnf4, Hnf6, Serpina3n, SerpinaA1, and Serpina6) were affected strongly by Prrxl (pooled and individual clones) in the Fg14 hepatoma cell line compared to non-transfected Fg14 hepatoma cells line as determined by RT-qPCR. The fold repression was 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.
Effect of Prrx1 on repression of liver-specific genes in Fg14 hepatoma cells

Figure 19. Fold repression of liver-specific genes (Fgb4, Kng1, and Pck1) were affected strongly by Prrx1 (pooled and individual clones) in the Fg14 hepatoma cell line as well as (Creg1) was affected lower by Prrx1. Both were compared to non-transfected Fg14 hepatoma cells line as determined by RT-qPCR. The fold repression was 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.
Effect of Snai2 on repression of liver-specific genes in Fg14 Heptoma cell line

Figure 20. Repression of liver-specific genes in Snai2 transfected pooled clones (Fg14-snai2 pool) in the Fg14 hepatoma cell line. RNA levels of indicated genes were determined by RT-qPCR and compared to levels in non-transfected Fg14 hepatoma cells. The fold repression was 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.
Effect of Snai2 on repression of liver-specific genes in Fg14 hepatoma cells

Figure 21. Fold repression of liver-specific genes (Hnf6, Serpina3n, Serpina6, and Hnf4) were affected strongly by Snai2 (pooled and individual clones) in the Fg14 hepatoma cell line as well as (Kng1, Pck1, Alb, and Fgb4) were affected moderate by Snai2 (pooled and individual clones) in the Fg14 hepatoma cell line. Both were compared to non-transfected Fg14 hepatoma cells as determined by RT-qPCR. The fold repression was 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.
3.6. Cell Migration Assay (scratch method) and Morphology

Migration is a key property of live cells and is critical for normal development, immune response, and disease processes. The process of migration assay occurs in most cells, but it is considered a hallmark assay for the fibroblasts. Based on these results, we sought to examine the migration of both Prrx1 and Snai2 in affecting the phenotype of hepatocytes. We asked whether Snai2 and Prrx1 overexpression in hepatoma cells (Fg14) could affect migration of transfected Fg14 cells compared to the parental of Fg14 and RAT1 cells.

In this technique, using a sterile 12 well plate, a marker was used and draw horizontal lines at the base of the plate. Culture cells were added to each of the wells incubated for 24 hours at 37°C to allow cells to attach, spread, and form a confluent monolayer. Next, a 200-microliter pipette tip was used to dislodge cells along the entire length of the well such that the scratch was perpendicular to horizontal line of the marker. Plates were then visualized by microscopy and images captured using imaging software. Images were acquired at several time points (4,8,12, and 16 hrs.) or until the scratch was completely or nearly completely filled in. We used this cell migration to differentiate migration behavior between hepatoma (Fg14), fibroblasts (RAT1), Fg14-Snai2 clone 6 and Fg14-Prrx1 clone 6. Results show that the Prrx1 or Snai2 transfected cells had enhanced migratory capabilities of transfected cells compared to the parental Fg14 cells (Figure 22 and 23).

Next, we asked whether the cell morphology of the transfected hepatoma cells was altered. We compared the morphology of two of the clones, (Fg14-Prrx1 clone 6 and Fg14-Snai2 clone 6)
to the parental Fg14 cells as well as to the RAT2 fibroblasts cells. Results show that the transfected hepatoma cells have a morphology more consistent with that of the fibroblasts than the hepatoma parental cells (Figure 24).

Taken together, our results show that overexpression of fibroblast-specific TFs Snai2 and Prrxl have the ability to reprogram the hepatoma cells to a fibroblasts phenotype that includes activation of expression of fibroblast genes and repression hepatoma gene expression (see summary in Figure 20), resulting in a change to a fibroblast cell morphology.
Fg14  RAT1  Fg14-Prrx1Cl6  Fg14-Snai2 Cl6

Time = 12 hours

Fg14  Fg14-Prrx1Cl6  Fg14-Snai2 Cl6

Time = 16 hours
Figure 22. Cell Migration Assay (The scratch method). Difference in cell migration capabilities in hepatoma (Fg14), fibroblasts (RAT1), Fg14-Snai2 clone 6 and Fg14-Prrxl clone 6 overexpressed in hepatoma Fg14. Resulting have enhanced migratory capabilities of transfected cells (Prrxl or Snai2) compared to the parental cells (Fg14 and RAT1).

Migration in different cell lines

![Graph showing cell migration distance over time for different cell lines](image)

Figure 23. Difference in cell migration capabilities in hepatoma (Fg14), fibroblast (RAT1), Fg14-Snai2Cl6 and Fg14-PrrxlCl6 overexpressed clones in hepatoma cells. The graph determines, the distance of the cells migration that were calculated by the ruler using Centimeter (cm)
Figure 24. Morphology changes due to Snai2 or Prrx1 overexpression in hepatoma cells line Fg14. Comparison of cell morphology between parental cells (Fg14 hepatoma cells and rat fibroblast cells RAT-1) with (Fg14- Prrx1-clone 6 and Fg14-Snai2-clone 6). Prrx1 and Snai2 overexpressed clones produced a spindle-shaped morphology which more closely resembled the fibroblast cells.
Figure 25. Summary of fibroblast gene activation and liver gene repression observed in the Fg14 hepatoma cells transfected with Prrxl and Snai2 TFs.
Chapter 4

Discussion

In mammalian cells, transcription factors (TFs) play a major role in activation and repression of genes within a genome to regulate development. Gene expression in fibroblast cells is regulated by multiple control mechanisms including histone modification, chromatin regulation, transcriptional control, post-transcriptional regulation and cell-cell contact (Panduro et al, 1987). Initiation of transcriptional activity requires promoter-proximal elements and enhancers (Lodish et al, 2000) to which transcription factors bind. As with all distinct tissues in complex organisms, fibroblast-lineage TFs bind to regulatory regions of certain genes in a synergistic fashion resulting in activation of genes that dictate fibroblast identity.

Fibroblast-specific gene expression has been recently studied in this laboratory using cell hybrids as a model system combined with whole genome microarrays. The strategy to identify these genes was to compare whole genome transcriptome microarray data of hepatoma cells and hepatoma x fibroblast hybrid cells with that of parental fibroblast cells, based on their fold differences in expression. These experiments identified potential master regulators of fibroblast cell fate (fibroblast-specific TFs), and their putative regulatory role in fibroblast differentiation. Genes identified included transcription factors (e.g. Prrx1/Pmx, Slug, Snai2, Shox2, CFOS, Twist1, Hox-d10, and Msx1) 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 Prrx1 and Snai2, which were used for further analysis of tissue-specific gene expression in fibroblasts. These genes were highly
expressed in fibroblasts, silent in hepatoma cells, and repressed in hepatoma x fibroblasts cell hybrids. Forced over-expression of these genes in the hybrid cells resulted in activation of fibroblast gene expression and resulting fibroblast-like morphology. Based on these results (described in the results section), we examined the role of Prrxl and Snai2 in affecting the phenotype of liver-derived cells using hepatoma cells (Fgl4) as a model system. The rat hepatoma cell model in the current study has been used for over 50 years. The goal of this study was to determine the ability of fibroblast-lineage specific TFs to remodel hepatoma cells.

A master regulatory gene is one which is at or near the top of a regulatory "hierarchy" and as such can have a strong influence in driving the cellular networks responsible for cellular identity. A majority of the genes considered master regulatory genes encode transcription factors which then activate many downstream tissue-specific genes. However, the concept of a master regulatory gene has been criticized for being an oversimplified paradigm which does not take into account multifactorial influence on a number of cell fates. Our study focused on the role of Prrxl and Snai2 as master regulators of fibroblast identity.

Paired related homeobox 1 (PRRX1), also termed PMX1, PRX1, and PRX-1, is a protein which is a member of a family of paired homeobox proteins confined to the nucleus. This protein has been shown to act by enhancing the binding of serum response factor to DNA, thereby indicating PRRX1 functioning as a transcriptional coactivator. PRRX1 also plays a role in the formation of various mesodermal muscle types through its role in the activating expression of the muscle creatine kinase (MCK) gene by binding to a A/T-rich element in the MCK gene enhancer. The expression of PRRX1 is broadly expressed in fat (RPKM 27.9), endometrium (RPKM 34.6), gallbladder, heart, and 15 other tissues (National Center for Biotechnology Information, 2018).
PRRX1 has also been known to play a role in the epithelial to mesenchymal transition during development (Saika et al., 2007), as well as in regulation of mesenchymal cell fate, including myogenesis, osteoblastogenesis, and chondrogenesis (Lu et al. 2011, Martin et al. 1995). Moreover, the PRRX1 family of TFs are connected with other transcription factors and signaling proteins in cascades leading to gene regulation during fibroblast differentiation. Chen et al. (1993) reported that the target of many of these transcription factors appears to be the osteopontin gene, which encodes a matrix structural protein that serves as a component of bone matrix as well as serving as a marker gene for fibroblast identity.

SNAI2 (Snail Family Transcriptional Repressor 2, also termed SLUG) is a member of the Snail family of zinc finger transcriptional regulators in mammals (Mistry et al, 2014). Studies suggests that SNAI2 is a TF that regulates gene expression during the epithelial-mesenchymal transition (EMT) and plays a very significant role in cell migration during tumor invasion or development (Mistry et al. 2014). SNAI2 has a vital role during embryogenesis and in adult tissues to maintain the normal functioning of the cells after birth.

The significance of SNAI2 in cell migration is in its ability to downregulate the expression of E-cadherin resulting in highly structured epithelial cells to transitioning to a mesenchymal phenotype. This process significantly leads to gastrulation of mesoderm in which the single layered blastula is reorganized into a multilayered gastrula (Moly et al.,2016). SNAI2 also regulates epidermal progenitor cells which, together with fibroblasts, support adult skin homeostasis. Mutations within the SNAI2 gene are related to sporadic cases of neural tube defects which are associated with an anti-apoptotic activity. SNAI2 expression is ubiquitous in the endometrium, ovary and other 22 tissues in humans (National Center for Biotechnology Information, 2018). The Snail superfamily of zinc-finger transcription factors have also been implicated in the signaling
cascade that dictates left-right identity, as well as in the formation of appendages, neural
differentiation, cell division and cell survival (Nieto, 2002).

4.1. Reprogramming the gene expression profile of hepatoma cells by Prrxl and Snai2
overexpression

Results of the current study suggest that overexpression of Prrxl and Snai2 genes via
introduction of an expression plasmids into the FgI4 hepatoma cells results in activation of
fibroblast-specific TFs as well as downstream fibroblasts-specific genes. Although not studied
here, overexpression of PRRX1 and SNAI2 in the hepatoma cell line likely also activated other
fibroblast-specific TFs that work in collaboration with other TFs to define the drive lineage-
specific transcriptional regulatory circuitry.

We first verified previous results showing that both PRRX1 and SNAI2 are expressed at
levels >1000-fold higher than those in the hepatoma cells (Figs 7 and 11). We were able achieve
high level expression of both mouse PRRX1 and mouse SNAI2 in the rat hepatoma cells using
expression vectors from Origene which used a cytomegalovirus promoter/enhancer to drive
expression of the target genes (Du et al., 2012). It should be noted that while the rat primer sets
used to monitor expression of the Snai2 gene detected both rat and mouse transcripts, the mouse
Prrxl primers failed to hybridize to the rat Prrxl sequences. However, the results did show strong
expression of mouse Prrxl gene in the cells compared to non-transfected cells, both in the pooled
transfectants and the individual clones analyzed (Figs 6 and 7).

Importantly, over-expression of the mouse Prrxl gene in the hepatoma cell line resulted in
activation of several fibroblast-specific TFs and downstream genes, including Shox2, Bmp3, c-Fos,
and Twist. Likewise, overexpression of Snai2 in hepatoma cell line activated many of the

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same genes (Shox2, Bmp3, c-Fos, and Twist). This data was supported by monitoring expression in several individual clones, which largely reflected data from the pooled transfectants (Figs. 8 and 12) and (Figs. 9, 10, 13, 14, 15, and 16).

These data suggest that PRRX1 and SNAI2 act in a similar fibroblast regulatory network. However, the overlap was not complete: The Collal gene, a common marker for fibroblast identity, was strongly activated (15-fold) by Prrx1 (Fig 10), but no detectable activation was observed in the Snai2-trransfected cells (Fig 12). It was surprising that while both these genes independently activated a similar set of genes, neither SNAI2 nor PRRX1 overexpression activated expression of the other gene. This suggests that they TFs feed into the same regulatory pathway, but at different points in the pathway.

Our results show that Twist is markedly upregulated by both overexpression of Prrxl and Snai2 in the hepatoma cell line. Twist is a member of a family of basic helix-loop-helix transcription factors and a known mediator of mesodermal tissue development (Isenmann et al., 2009). It also has been reported to play an essential role in various stages of embryonic development (Ning et al., 2018).

Interestingly, several TFs have been identified as master regulators of the Epithelial–mesenchymal transition (EMT) program, including TWIST1, Snail family zinc finger 1 (SNAIL1), Zinc finger E-box-binding homeobox (ZEB), PRRX1, KLF4, SOX4, and SOX9 (Zhou et al, 2017), suggesting correlation between Snai2, Prrx1, and Twist in the pathways of EMT. During EMT in humans, SNAIL2 replaces SNAIL1 to make drive TWIST1 expression, on the human SNAIL2 promoter activating its transcription by TWIST1 specifically binds to the E-box (Casas et al., 2011). Through early embryogenesis, TWIST1 and SNAIL2 are also functionally connected (Martin et al, 2005). Our data support a direct correlation between TWIST and SNAIL2, an
interaction which has been reported to be conserved functionally through embryogenesis and tumor metastasis.

C-Fos was also found to be significantly upregulated by individual overexpression of Prrxl and Snai2 genes in hepatoma cell line. C-FOS is a transcription factor with a leucine zipper motif that heterodimerizes with members of the Jun family to form the Activator Protein (AP-1) TF. Prrxl has previously been shown to take part in the activation of CFOS/c-JUN complex of AP-1 that has an important role to drive the transcriptional activation of osteopontin gene and other osteoblastic markers (Nakamachi et al., 2007, Ohta et al. 1991). PRRX1 has a regulatory role on the CFos gene, and the heterocomplex of cFOS and cJUN is linked with osteoblast proliferation (Okazaki et al., 1992). Bai et al, (2006) suggests that PRRX1 is associated with CFOS. Additionally, abnormal expression of SNAI2 and C-FOS has been linked to obesity that can increase the risk of female reproductive tract cancers (Yang et al, 2012).

Both Shox2 and Bmp3 genes were found to be upregulated by overexpression of Prrxl and Snai2 genes in the hepatoma cell line. SHOX2 (short stature homeobox 2) is a member of the homeobox family of transcription factors. The growth and development of the vertebrate limb depend on homeobox genes of the Hox and Shox families (Neufeld et al. 2014). SHOX2 also plays an important element in the osteoblast differentiation (Gu et al. 2008) and SHOX2 and PRRX1 are both connected with osteopontin gene regulation. BMP2A and BMP3 (known as Bone morphogenetic proteins) are TFs that play an important role in fibroblast differentiation and in mesenchyme stem cell differentiation into multipotent osteoblastic, myogenic, adipogenic, chondrogenic cells (Matsubara et al. 2008).

Interestingly, Colla1 was upregulated by overexpression of Prrxl, but not Snai2, in the hepatoma cell line. The Colla1 gene encodes type I collagen, which is the most abundant form of
collagen that strengthens and supports many tissues in the body such as cartilage, bone, tendon, ligaments, the dermal layer of skin, and the sclera of the eye. A study by Palumbo et al. (2017) suggests a correlation between Twist and Collal expression in skin fibrosis. Matsushita et al. (2009) reported that ERK1 and ERK2 regulate osteoblast differentiation and bone formation through multiple regulator genes including Prrxl, RunX2, Cfos, Shox2, Bmp3, Twist). Based on the information above, fibroblasts and osteoblasts may have common precursor cells, mesenchymal stem cells, which may indicate that, during bone development, fibroblasts might be the precursor of bone cells (Shrestha, 2011).

Previous studies from our lab showed that overexpression of Prrxl in hepatoma x fibroblast hybrids was able to strongly activate the Snai2 gene. Likewise, Snai2 overexpression activated the Prrxl gene in these cell hybrids (Ray, 2016). However, results presented in the current study showed that neither gene could activate the other. This suggests that the hybrid cells are more flexible regarding reprogramming than are the hepatoma cells.

Because the above results suggest that several genes involved in fibroblast identity and function could be activated by the introduction of a single transgene, we hypothesized that overexpression of fibroblast-specific transcription factors Prrxl or Snai2 would also result in a partial loss of the hepatoma phenotype. We therefore monitored expression of a panel of hepatoma-specific genes (both those encoding transcription factors and downstream genes) including (Cregl, Pck1, Hnf4, Serpina6, Hnf6, Serpina3n, Fgb4, SerpinaA1, Alb, Hnf1 and Kngl) using qRT-PCR analysis. Results show that, in the Prrxl-transfected pooled clones, 9 of the 11 liver-specific genes tested were repressed at levels ranging from 3 fold (Cregl) to 200-fold (SerpinaA6). This effect was verified by monitoring repression of several of these genes in individual transfected clones, with results generally similar to those found in the pooled clones (Fig. 17,18 and 19).
Likewise, the pooled Snai2 transfectants as well as individual Snai2-expressing clones were found to repress expression of mostly the same liver genes as those in the Prrx1 transfected cells, with levels of repression being similar (Figs 20 and 21).

4.2. Reprogramming of morphology and migratory properties of hepatoma cells by Prrx1 and Snai2

In addition to Prrx1 and Snai2 having a strong effect on gene expression profiles in the hepatoma cells, our results also suggest that both Prrx1 and Snai2 contributed to reprogramming of hepatoma cells to a more fibroblast-like phenotype in two ways. First, we observed restoration of the fibroblast phenotype as shown by their acquisition of a more spindle shaped morphology in contrast to that of the hepatoma cell (Fig 24). Second, we observed the transfected hepatoma cells have an increased migratory ability compared to the parental cells that became more pronounced at the 12 and 16-hour times points (Fig 22 and 23).

As discussed above, cell migration is a very important cellular process for the normal development of tissues and organs. Fibroblast are able to migrate within tissues through the formation of lamellipodia. The migration of fibroblast relies on the initiation of focal adhesion kinase (FAK) and a corresponding interaction with tenascin-C (McKean et al., 2003). Experiments using FAK-null embryos suggest that FAK affects embryogenesis by both controlling cell proliferation as well as cellular morphology and migration. An increase in cell spreading and migration on fibronectin is made possible by the stable stimulation of FAK (McKean et al. 2003) provide a different possibility that FAK stimulates the expression of extracellular matrix genes and proteins which are significant in creating migratory tissues environment even though the idea has not been fully established.
It has been recently shown in our lab by Ray (2016) using a chemotaxis-based cell migration assay to distinguish the migratory behavior between hepatoma, fibroblasts, and hepatoma fibroblast hybrid, that SNAI2 or PRRX1 overexpression in cell hybrids resulted in a partial reversion back their fibroblast phenotype. The results further showed that hybrids which exhibited forced overexpression of both SNAI2 and PRRX1 by $\geq 10$-fold show increased migration abilities when compared to the non-transfected hybrid.

According to Hulkower and Herber (2011), cell migration and invasion assays are critical for drug discovery to understand the correlation between compounds that inhibit migration and invasion in vitro. Our observations establish that Prrxl and Snai2 overexpressed in hepatoma cells (pools and individual clones) show enhanced the cell migration behavior (Fig. 22).

4.3. SNAI2 and PRRX1 in Diseases

Based on our results and other studies, Prrxl and Snai2 independently show the ability activate pathways that contribute to the fibroblast phenotype and possibly to direct disease progression. We observed cross talk between fibroblast lineage specific (Prrxl and Snai2) and downstream genes, including (e.g. C-fos, Twist, Shox2, Bmp3). Accordingly, Jiang and Pugh (2009) showed that both Prrxl and Snai2 functions are connected with super enhancers in several fibroblast cell types. Thus, we have identified Snai2 and Prrxl as strong fibroblast lineage specific candidate TFs that appear to play an essential role in determining and maintaining fibroblast cell fate.

Interestingly, about 10% of the transcription factors have a significant relationship in a very large number of human diseases (Bouhlel et al. 2015). The TFs that control pathways are important to decipher because they have the potential to define cell identity, enhance cellular reprogramming
for regenerative medicine, and improve our understanding of an abnormal transcription factors leading to disease (Saint et al. 2016).

Several studies have been reported that deregulation or upregulation of either Prrx1 and Snai2 lead to cancers in different tissues of fibroblasts (Hirata et al., 2014, Moreno-Bueno et al 2008) such as SNAI2 has been reported to regulate cell proliferation and invasiveness of metastatic prostate cancer cell lines (Baygi et al. 2010). Several other studies have likewise suggested the importance of SNAI2 in cancer development (Wang et al. 2011; Buehler et al. 2012). Likewise, downregulation of PRRX1 via the p53-dependent signaling pathway predicts poor prognosis in hepatocellular carcinoma (Fan et al. 2017). Furthermore, metastatic colonization has been reported to require the repression of PRRX1 (Ocaña et al. 2012).

4.4. SNAI2 and PRRX1 Knockout Mice

In Snai2 knockout mice revealed alterations in epidermal differentiation, adhesion, motility, and angiogenesis although genes that are direct targets of SNAI2 remain unknown and that wound healing is impaired in SNAI2 knockout mice (Mistry et al. 2014, Hudson et al. 2009). This finding is used to support the claim that SNAI2 expression is augmented in epidermal cells neighboring a wound consequently supporting epithelial-mesenchymal transition to permit relocation to the injury hence stimulating re-epithelization (Mistry et al., 2014).

In Prrx1 with knockout mice, it was reported that tumor weights and volume were greater Conversely, research by Du et al. (2011) suggested that Prrx1 knockdown decreases transforming growth factor-β (TGFβ) ligand expression and increases adipogenesis, whereas Prrx1 rises in adipose tissue through obesity. Therefore, Prrx1 knockdown enhanced adipogenesis by suppressing TGFβ signaling.
Thus, evidence suggests that Prrx1 and Snai2 are necessary to program and maintain fibroblast function. The results of this study suggest that the factors individually have the ability to reprogram a fully differentiated cell type into the fibroblast lineage and extend the results of Ray et al who reported the reprogramming of hepatoma x fibroblast hybrids by these TFs.

4.5. Conclusions.

We were able to show that candidate master regulator genes Prrx1 and Snai2 have a remarkable ability to reprogram a fully differentiated hepatoma cell line into a cell type with a partial fibroblast phenotype. We showed activation of several key genes encoding fibroblast-specific transfection factors (as well as and downstream genes) combined with strong repression of several liver-specific genes. Furthermore, these results suggest that both cellular morphology and migratory abilities can be altered by the liver-expression of either of these transcription factors.

This study also provides a proof-of-concept that master regulatory genes can be identified by comparing expression profiles of fibroblast with cell lines of other cell types. In our case, we compared profiles of fibroblasts with those of hepatoma and hepatoma x fibroblast hybrids. The inclusion of the cell hybrids in the analysis allowed identification of some genes that may not have been identified using comparisons of expression profiles of only fibroblasts and hepatoma cells. Importantly, the inclusion of cell hybrids allowed us to exclude a large number of candidate genes, as we required a >5-fold increase in expression of a gene in fibroblast compared to both hepatoma and hybrid cells.

This result of this study supports the idea that single TFs can direct tissue identity through activation of transcriptional regulatory networks that both drive some lineage specific genes as well as repress others. These results also demonstrate that fully differentiated cell types (e.g., hepatoma
cells) can be reprogrammed in a straight-forward way by overexpression of key transcription factors (e.g. Prrx1 or Snai2).

4.6. Future Directions.

Prrx1 and Snai2 candidate genes have shown a strong activation > 1000-fold of several fibroblast-specific genes and downstream gene that lead to strong repression of several liver-specific genes. We did not determine whether this occurred due to Prrx1 and Snai2 binding to the transcription factor promoters. This could be determined using software that predicts transcription factor binding sites (TFBS) paired with functional assays such as ChIP and microarray to detect the binding site sequences of Prrx1 and Snai2 on promoters. Also, we could use prediction software such as systematic Evolution of Ligands by Exponential Enrichment (SELEX), which has been shown to be a powerful tool to detect the binding sites and the interactions of transcription factors.

Gene knockdown experiments using RNAi could be used to reduce Prrx1 and Snai2 expression in the Fg14 cells and determine if fibroblast-specific genes and downstream gene have been affected or not. Additionally, comparisons between Prrx1 and Snai2 expression during embryogenesis in normal and Prrx1 and Snai2 with knockout mice model could help to clarify the role of the protein (Prrx1 and Snai2) in fibroblast function. It would also be useful to conduct whole-genome microarray analysis on the Fg14 -Prrx1 and Fg14-Snai2 cells compared to Fg14 cell and RAT1 cells to identify the extent of genomic remodeling that has occurred due to overexpression of these genes. Finally, these data would help us identify in vivo DNA-binding sites for downstream pathway proteins and provide a snapshot of the impact of these key TFs on the transcriptional activation/repression of interconnected TFS and other fibroblast-specific downstream targets.
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