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A novel mechanism for antipsychotic-induced metabolic dysfunction: modulation of the TGF-beta pathway

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A Novel Mechanism for Antipsychotic-Induced Metabolic Dysfunction: Modulation of the TGF-beta Pathway

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in

Biomedical Sciences

by

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2011
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SAR-Structure activity relationship
TGFβ-Transforming growth factor beta
RSMAD-Receptor regulated SMAD
CoSMAD- interacts with R-SMADs to participate in signaling
HTS-High throughput screen
CNS-Central nervous system
5-HT_{2C} –Serotonin 2C receptor
5-HT_{1A} –Serotonin 1A receptor
a2 –Alpha-2 adrenergic receptor
H1-Histamine 1 receptor
M3-Cholinergic receptor, muscarinic 3
SBE-Smad binding element
CMAP 2.0-Broad Connectivity Map 2.0
NJCC- NIH/JDRF Custom Collection-
MAPK-Mitogen-activated protein kinase
bHLH-basic-Helix-Loop-Helix
r-Pearson correlation coefficient
ADHD-Attention deficit hyperactivity Disorder
BMI-Body mass index
CDKI- Cyclin-dependent kinase inhibitor
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ABSTRACT OF THE DISSERTATION

A Novel Mechanism for Antipsychotic-Induced Metabolic Dysfunction: Modulation of the TGF-beta Pathway

by

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Antipsychotics are among the most prescribed drugs in the US. Although they are effective in treating an array of neurological disorders the drugs share a number of deleterious metabolic side effects. Through a high-throughput screening approach we identified Phenothiazine-based antipsychotics as modulators of insulin promoter activity in the human insulin expression cell line T6PNE. Both typical and atypical antipsychotics were found to modulate the insulin promoter at E-box sequences, with
chronic treatment being repressive. Mechanistic work identified the TGFβ pathway as being involved in the effect of antipsychotics on the insulin promoter, finding that antipsychotics activated SMAD3, a downstream effector of the TGFβ pathway, through a receptor distinct from the TGFβ receptor family, and known neurotransmitter receptor targets of antipsychotics responsible for their beneficial CNS effects. *In vivo* relevance of the findings in T6PNE was demonstrated by analysis of publically available gene expression data from the brains of humans treated with antipsychotics that showed altered expression of SMAD3 responsive genes. Finally, through a comparative cell line approach candidate genes for the direct target of the antipsychotics linking them to SMAD3 were identified. Since the TGFβ pathway and SMAD3 in particular are highly associated with obesity, insulin resistance, and type II diabetes this work raises the possibility that antipsychotics could be designed that retain the beneficial neurological effects while lacking the deleterious metabolic side effects that are a major clinical issue with their use.
CHAPTER 1:

INTRODUCTION-

METABOLIC SIDE EFFECTS OF ANTIPSYCHOTICS: POTENTIAL MECHANISMS AND MOLECULAR TARGETS

1.1 Background

An estimated 14.3 million Americans were taking antipsychotics in 2008, making them among the most prescribed drugs in the US [1]. Total sales increased almost 50% over a 4 year period ending in 2009 when they accounted for $14.6 billion in reported sales, more than any other class of drugs that year [2]. The recent increase in patients taking antipsychotics is mainly attributed to their rising use for diagnoses other than psychosis. They are being prescribed in greater numbers to children and adults alike for the treatment of bipolar disorder and autism, as well as substance abuse and gambling addiction. [3], [4], [5], [6].

Antipsychotic use began in 1952 soon after French pharmaceutical company Laboratoires Rhône-Poulenc first synthesized chlorpromazine to use as a sedative during surgery [7]. It was said to induce a state of indifference, or “chemical lobotomy,” and therefore tested on psychotic patients for whom lobotomy, along with electroconvulsive and insulin shock therapy, was then common practice [8].
Chlorpromazine proved to diminish the symptoms of psychosis in an efficient and precise manner, moving beyond basic sedation effects and improving emotional behavior and thinking patterns in patients [8]. Its use was quickly adapted and by 1964 around 50 million people had taken the drug [9].

1.2 Therapeutic targets of antipsychotics

The beneficial CNS effects of chlorpromazine first described by Laboratoires Rhône-Poulenc are thought to be primarily due to D$_2$ receptor blockade, although chlorpromazine has high binding affinities for serotonin and adrenergic receptor subtypes also thought to play a role in its therapeutic effects [10], [11].

Chlorpromazine is classified as a typical or first-generation antipsychotics, a group of compounds which all share the same high affinity for D$_2$ receptors [12], [13], [14]. A second class of antipsychotics, the atypical or second-generation antipsychotics, differ from typical antipsychotics by sharing a high 5HT$_2$/D$_2$ binding affinity ratio [15], [13], [14]. Both classes of antipsychotics are known to have promiscuous binding profiles, and although common targets exist each individual drug has a unique set of molecular targets. It has been proposed that antipsychotics need to be “dirty drugs” since schizophrenia is a multifactorial disease which can only be treated by a combined effect on the diverse panel of CNS targets of antipsychotics [13], [11], [16].
1.3 Side effects of antipsychotics

Although allowing for effective treatment of psychosis, the diverse molecular targets of antipsychotics lead to a number of deleterious side effects. The typical antipsychotics share movement disorders (e.g., dyskinesias) as a neurological side effect related to their potent D$_2$ antagonism. Atypical antipsychotics have higher serotonin affinities and faster disassociation from D$_2$ receptors allowing them to remain potent neuroleptics without inducing movement disorders. While the relative effectiveness of typical and atypical antipsychotics in treating schizophrenia is debated [13], [17], [11], the newer atypical antipsychotics are currently more prescribed due to the lack of these neurological side effects.

Other side effects have been reported in both classes of antipsychotics, including seizures, hyperprolactinemia, and an array of deleterious metabolic side effects. These include obesity, hyperlipidemia, insulin resistance, and diabetes and occur in alarmingly high frequencies. It has been shown that over 50% of individuals taking the antipsychotic clozapine become overweight [18], and antipsychotic treatment can more than double ones likelihood of developing diabetes [19], [20], [21], [12]. These metabolic disturbances are seen regardless of age, sex, or duration of antipsychotic exposure [22], [21]. They also seem to occur in equal frequencies among typical and atypical antipsychotics [23], [24], [25], [26], [27], [21], and although were first described with Chlorpromazine use in the late 1950s [28] the mechanisms responsible remain unclear [27], [13]. Given their widespread use, the metabolic side effects of antipsychotics are a major public health problem and there is a great need for a better understanding their origins.
1.4 The metabolic side effects of antipsychotics are due to both adipogenic and diabetogenic properties.

Since higher rates of diabetes among Schizophrenics were reported before the widespread use of antipsychotics [29], [30], [31] it has been suggested that pleiotropic effects of the disease causing mutations or factors relating to the lifestyle, nutrition and activity level of Schizophrenics contribute to the metabolic dysfunction of Schizophrenics. Metabolic side effects though are still seen when treating diagnoses other than schizophrenia with antipsychotics [32], [5], [6]. In particular, children and adolescents treated with antipsychotics for autism, ADHD, or bi-polar disorder or more likely to become obese than adults treated with antipsychotics for schizophrenia [33], [34], [35].

Some have proposed the wide range of metabolic side effects associated with antipsychotics use stem solely from the adipogenic properties of the drugs. Antipsychotics have been shown to induce appetite increases, weight gain, hyperlipidemia, and obesity, which are themselves established risk factors for insulin resistance and hyperglycemia [27], [13]. In agreement with this hypothesis, the two antipsychotics which are not associated with diabetes, ziprasidone and molindone, induce little to no weight gain [32], [36], [37].

While certainly a factor, obesity alone cannot account for all metabolic complications since a direct correlation between weight gain and diabetes in antipsychotic treated patients is not always seen [38], [31], [32], [39]. Also, insulin resistance is induced in animal models of antipsychotic-induced metabolic dysfunction unrelated to changes in feeding behavior or fat accumulation, suggesting other
mechanisms for the metabolic consequences involving direct effects on glucose metabolism [40]. It has therefore been proposed that antipsychotics have separate adipogenic and diabetogenic properties that act together to induce metabolic dysfunction [23], [24], [27], [21], [13].

1.5 Potential molecular targets responsible for the metabolic side effects

For novel antipsychotics to be designed that retain beneficial CNS effects while no longer inducing metabolic dysfunction, the direct binding partner(s) of the drug responsible for the adipogenic properties and effects on glucose metabolism need(s) to be identified. Since antipsychotics are quite promiscuous and known to act on multiple targets in and out of the central nervous system (discussed in detail below), it has been unclear whether their metabolic side effects are due solely to the modulation of the neurotransmitter receptors that are responsible for their favorable therapeutic effects or whether off-target binding might be responsible. [23], [24], [25], [26], [27], [21]. This is an important distinction; novel antipsychotics with fewer metabolic side effects can only be produced if the binding partner responsible for the metabolic side effects of current antipsychotics is not related to their beneficial CNS effects.

1.5.1 Histamine receptors

The CNS target of antipsychotics most highly associated with obesity is the histamine receptor, H1. In fact, studies correlating the receptor binding affinities of antipsychotics at neurotransmitter receptors with metabolic derangement in
antipsychotics treated patients have found that H1 binding affinities of antipsychotics are significantly correlated to weight gain and type II morbidity rates [41], [42]. It has been proposed that, similar to mice with deletion of the H1 receptor, antipsychotic blockade of the H1 receptor attenuates leptin signaling, leading to increased food intake and decreases caloric output [43], [41]. Many antipsychotics though, such as haloperidol, fluphenazine, and sulpride, have low H1 receptor affinities yet are highly correlated with weight gain and diabetes in patients [44], [32]. Also, specific H1 receptor antagonists (antihistamines) used to treat allergies do not have metabolic side effects.

1.5.2 Serotonin receptors

The serotonergic 5-HT$_{2C}$ receptor has also been linked to metabolic derangement. Mice lacking the 5-HT$_{2C}$ gene develop obesity and insulin resistance and genetic variants of the 5-HT$_{2C}$ gene have been shown to protect from antipsychotic induced weight gain and metabolic dysfunction [45], [46]. Conflicting reports conducted with these genetic variants do however exist [47], [48].

Roles for neurotransmitter receptor blockade in antipsychotic induced metabolic dysfunction have also been proposed outside the central nervous system. Pancreatic β-cells express 5HT$_{1a}$ receptors which have been shown to modulate insulin secretion in response to increases in blood glucose levels [49], [27]. Since many antipsychotics are antagonists of the 5HT$_{1a}$ receptor some have suggested they may impair glucose homeostasis by decreasing the glucose responsiveness of the β-cell [50], [51]. Many typical antipsychotics though are not strong antagonists at 5HT$_{1a}$ or
5HT₂c receptors yet are still diabetogenic [44], [32].

1.5.3 Muscarinic receptors

The muscarinic M3 receptor is also expressed in the β-cell where it facilitates cholinergic-stimulated insulin secretion [52]. The β-cell specific knockout of M3 receptors results in reduced insulin secretion [53], and the atypical antipsychotics olanzapine and clozapine are strong inhibitors of M3 activity. They have been shown to markedly impair cholinergic-stimulated insulin secretion in vitro [54], suggesting blockade of M3 receptors might contribute to the impaired glucose tolerance associated with antipsychotic use. However, specific M3 antagonists are used to treat urinary incontinence and have not been associated with alterations in glucose metabolism [52].

1.5.4 Adrenergic receptors

The adrenergic system, responsible for the fight or flight response, regulates plasma glucose levels as well as food intake when activated by the neurotransmitter norepinephrine. It has been proposed that antipsychotics, through inhibition of presynaptic α2a receptors, increase norepinephrine levels through a self-feedback loop, and thereby increase blood glucose levels, as well as food intake [27]. In addition, α2 receptors are known to be expressed on the β-cell, where their inhibition can result in reduced insulin secretion [55].

The adrenergic α1a and β3 receptors have also been linked to antipsychotic induced metabolic dysfunction since SNPs in the genes encoding the receptors are
associated with a higher body mass index (BMI) following chronic exposure to antipsychotics [56], [57]. Similar associations have been made between adrenergic receptor polymorphisms and obesity in studies unrelated to antipsychotics so it is unclear whether these are specifically related to antipsychotic-induced increases in BMI [58], [59], [60], [19].

1.5.5 Non-neurotransmitter receptor targets

Antipsychotics have been linked to metabolic derangement through targets aside from neurotransmitter receptors as well. The atypical antipsychotics risperidone and clozapine can directly inhibit glucose transporters in muscle cell lines and thereby induce insulin resistance [61]. In addition, it has been shown that clozapine can inhibit glucose-dependant insulin release through membrane hyperpolarization [62], and risperidone can directly bind the $K_{ATP}$ channels involved in insulin secretion from $\beta$-cells [63].

Although through modulation of unidentified targets, it has been shown that clozapine increases glucagon levels in rats treated with the drug. Glucagon is a hormone secreted by the pancreatic $\alpha$-cells with the opposite of effect of insulin, to raise blood glucose levels [64].

Also through unidentified targets it has been shown that antipsychotics modulate secretion of TNF$\alpha$, adiponectin, and leptin from adipocytes, all three of which are adipocytokines with altered secretion in type II diabetes [65], [66]. More work is needed in this area since it is unclear whether antipsychotics directly affect adipocytokine secretion or whether the effects are indirect consequences of increases
in BMI [67], [27].

Overall there is no consensus on a mechanism that can explain the array of deleterious metabolic side effects associated with antipsychotic use. [23], [24], [27], [21], [13]. Either a cumulative effect on any number of the targets describe above is responsible, or a key receptor or pathway modulated by antipsychotics relevant to obesity and diabetes has yet to be uncovered. So, given the widespread use of the drugs, there is a pressing need for more work aimed at adding clarity to the field.

1.6 Summary of findings

The dissertation described here proposes modulation of the TGFβ pathway as a novel mechanism for antipsychotic-induced metabolic dysfunction. The work began with a high-throughput screen (HTS) conducted with an assay created in our lab to identify novel inputs into the insulin promoter. As described in chapter 2, we screened the T6PNE insulin promoter assay against a library of known drugs, identifying phenothiazine-based antipsychotics as modulators of insulin promoter activity, and showing the drugs to potently inhibit the promoter following chronic exposure. The observation was then extended to an array of structurally diverse typical and atypical antipsychotics, finding the majority to modulate the insulin promoter.

The work detailed in chapter 3 aims to uncover the mechanism by which antipsychotics signal to the insulin promoter in the hopes of shedding light on the metabolic side effects of the drugs. We found that the known targets of the drugs, the neurotransmitter receptors responsible for their therapeutic benefits as well as a number of intracellular signaling molecules, failed to connect antipsychotics to their
effects on the insulin promoter. Next, an unbiased pathway-based approach revealed antipsychotics were signaling in the TGFβ pathway downstream of TGFβ type I receptors, specifically through SMAD3, to modulate activity at the E-box sequences in the insulin promoter. This finding is significant since the TGFβ pathway has been linked with obesity, insulin resistance, and diabetes, all features of the metabolic side effects of antipsychotics.

Chapter 3 correlates this mechanism identified in vitro to gene expression data generated in vivo, finding the expression of genes with SMAD3 binding sites in their promoters to be altered in the brains of antipsychotic-treated schizophrenics when compared to healthy controls. Efforts were then made to identify the direct target of the antipsychotics that acts on SMAD3 through a comparative cell line analysis which found only a subset of cell lines to show SMAD3 activation following antipsychotic treatment. By comparing gene expression data in these cell lines a list of candidate genes was generated. If this target is identified, since it not one of the neurotransmitter receptors responsible for the therapeutic effects of the drugs, more selective antipsychotics may be designed that retain their therapeutic benefits while no longer modulating the TGFβ pathway and therefore hopefully inducing fewer metabolic side effects.
CHAPTER 2:

IDENTIFICATION OF ANTIPSYCHOTICS AS MODULATORS OF THE
HUMAN INSULIN PROMOTER

2.1 Background and significance

Diabetes currently affects 8.3% of the US population and is the 7th leading cause of death [68]. A main goal in our lab is to gain a better understanding of the pathology of this devastating disease as well as identify novel therapeutic targets through studying the control of the insulin gene.

2.1.1 Cis and trans acting factors at the human insulin promoter

The human insulin promoter is tightly regulated by a well defined network of transcription factors. These include both islet-specific factors (NeuroD, PDX-1, and MafA) as well as ubiquitous proteins such as E47 [69]. Figure 1 shows these trans-acting factors and the cis-elements to which they bind. Several conserved motifs make up these cis-elements and are widely believed to be responsible for restricting the expression of the insulin gene to β-cells. These include the E, A, and C boxes. The E boxes share the consensus sequence CANNTG and serve as binding sites for heterodimers of the basic helix-loop-helix family (bHLH) [70], [71]. In particular,
heterodimerization occurs between the β-cell specific factor, NeuroD, and ubiquitous product of the E2A gene E47 [69]. A boxes are regions of the promoter that are rich in AT sequences, where homeodomain family proteins bind. In particular, PDX-1 (pancreatic duodenal homeobox-1), which is important in the development of the pancreas and in β-cell maturation, binds at this location [72]. Additional factors include the C box which binds transcription factor MafA, and HNF1α which binds the A box and is regulated by HNF4α [73], [69].

![Figure 1. Several insulin promoter transcription factors and their cis-acting elements.](image)

### 2.1.2 Rationale for studying control of insulin promoter trans-factors

A number of the pathways that play a role in the β-cell dysfunction which contributes to disease progression in both type I and type II diabetes affect insulin gene transcription [74]. These include pathways downstream of fatty acids and glucose in type II diabetes as well as the inflammatory mediators that are present early in the course of type I diabetes.

Initially the β-cell mounts a compensatory response to obesity and insulin resistance that involves increases in β-cell replication as well as insulin biosynthesis
and secretion. This eventually becomes insufficient though and β-cell dysfunction develops on the way to outright loss of β-cell mass. During this stage of type II diabetes, excess glucose and fatty acids (glucolipotoxicity) contribute to β-cell dysfunction through inhibition of a number of the insulin promoter transcription factors [75]. High glucose and fatty acid concentrations have been shown to decrease activity of PDX-1 MafA, and NeuroD [76], [77], [78], and we have found an array of fatty acids to inhibit E47 activity in vitro (unpublished). Similarly, in the beginning stages of type I diabetes, immune cytokines induce β-cell dysfunction through reduction of PDX-1, MafA, and NFAT activity before apoptosis occurs [79].

These same insulin promoter transcription factors targeted in β-cell dysfunction also act globally to maintain many aspects of proper β-cell function in healthy islets and promote neogenesis in β-cell precursors. Mice with a β-cell specific PDX-1 knock-out, for example, develop diabetes due to a deficit of mature β-cells [80]. Also, when delivered directly to the pancreas, PDX-1 combined with MafA and Ngn3, induce pancreatic exocrine cells to trans-differentiate into functional β-cells [81]. Therapies that activate the insulin promoter transcription factors therefore have the potential to prevent disease progression by maintaining promoter activity during β-cell dysfunction, as well as cure later stages of the disease by promoting neogenesis in β-cell stem/progenitors.

2.1.3 Development of T6PNE

To discover novel targets and pathways which signal to the insulin promoter
transcription factors, our lab has developed a novel human cell line that mimics several important aspects of β-cell biology [82]. Originally derived from human fetal islets, T6PNE was engineered to express the homeodomain transcription factor PDX-1, the bHLH factor NeuroD1, and the NeuroD1 dimerization partner E47 fused to a modified estrogen receptor ligand binding domain (MER), rendering it inducible by tamoxifen [83]. E47 induction in T6PNE results in strong upregulation of insulin gene expression (Fig. 2A) and other β-cell specific genes (Fig. 2C) while also inducing cell cycle arrest (Figure 2D) through upregulation of the cyclin-dependent kinase inhibitor (CDKI) p57kip2, (Fig. 2E) [82]. The controlled activation of the E47-MER fusion with an intermediate dose of tamoxifen allows for the upregulation of β-cell specific genes such as insulin without inducing growth arrest [82]. This human insulin expressing cell line presents a unique assay tool; its ease of culture sets it apart from primary human islets and T6PNE is superior to rodent cell lines due to both its sensitivity to modulation of insulin promoter activity, as well as the documented differences between the rodent and human insulin promoters [69].

2.1.4 Adaptation of T6PNE for high-throughput screening

The ability to precisely modulate insulin gene expression with tamoxifen as shown in Figure 1A suggested that T6PNE cells could be used as a platform for High-Throughput Screening (HTS) to detect compounds that affect insulin promoter activity. To adapt the cell line for HTS assays, we infected T6PNE cells with a lentiviral vector expressing the eGFP gene from a 1.4-kb fragment containing the human insulin promoter [84], [85], providing for direct visualization of changes in
Figure 2. Development of T6PNE as a model of human β-cell growth and differentiation, and adaptation of the T6PNE insulin promoter assay for HTS. (A) Dose response of tamoxifen-induced E47 activation on endogenous insulin mRNA levels by QPCR (n=3). Error bars are SEM. * indicates p<0.05 relative to DMSO control. (B) T6PNE cells infected with a lentiviral vector expressing an insulin promoter-eGFP cassette exhibited a tamoxifen dose-dependent increase in green fluorescence similar to that seen with endogenous insulin mRNA levels in panel A. (C) E47 induction by tamoxifen resulted in upregulation of glucokinase, SUR-1, and MafA determined by RT-PCR. Assay was done twice, and GAPDH control was equivalent between samples. (D) Cells cultured in the absence or presence of tamoxifen (4 µM) were counted on the indicated days, demonstrating substantial growth arrest with the addition of tamoxifen. (n=3). Error bars are SEM. *p < 0.05, **p < 0.001. (E) Tamoxifen-induced E47 activity increases Kip2 mRNA content measured by QPCR. (n=6). Error bars are SEM. * indicates p<0.05 relative to DMSO control.

insulin promoter activity (T6PNE Insulin Promoter Assay). The specificity of the insulin promoter eGFP transgene has been demonstrated previously [85]. Tamoxifen dose-dependent eGFP fluorescence, precisely in concert with endogenous insulin
mRNA level, was observed in T6PNE cells infected with the transgene construct (Fig. 2B) [82]. Therefore, we were able to prime T6PNE with an intermediate level of insulin promoter activity by administering low levels of tamoxifen, allowing for detection of insulin promoter activators and inhibitors. Assay optimization of T6PNE cells in 384-well plates was performed using increasing doses of tamoxifen from 0.5 to 4 µM, yielding Z’ scores that ranged between 0.2 and 0.6. The variation in the Z’ occurred because the extent to which GFP expression was induced by tamoxifen varied in different pilot studies for reasons that are unclear but appear to be related to growth characteristics of the T6PNE cells [82].

2.2 Chapter 2 Summary

This chapter details a screen conducted on the T6PNE insulin promoter assay with a known drug library, identifying phenothiazine antipsychotics as modulators of insulin promoter activity. It is shown that an array of other typical antipsychotics as well as the more commonly used atypical antipsychotics also modulate the insulin promoter in T6PNE. Chronic exposure inhibits the insulin promoter, a finding with potential relevance to the metabolic side effects of antipsychotics given the essential role the insulin promoter plays in proper β-cell function.
2.3 Results

2.3.1 HTS conducted on the T6PNE insulin promoter assay with a known drug library identifies phenothiazine antipsychotics as modulators of insulin promoter activity

As an initial screen, we chose to study a library of known drugs for those that activated the insulin promoter. This had the advantage that many of the compounds have known mechanisms of action, unlike commonly used large and chemically diverse compound libraries. Moreover, many commonly used drugs act on pathways that are active in β-cells, but little is known about how those drugs affect β-cell function. So, we conducted a HTS on the T6PNE Insulin Promoter Assay with a library of 1040 known drugs from the NIH/JDRF Custom Collection (NJCC) constructed by MicroSource Discovery Systems, Inc. (Gaylordsville, CT). Because of the variability in Z’ calculations discussed in section 2.1.3, the primary screen was done in duplicate in separate plates and the results are shown in Figure 3A [82]. A number of compounds, including merbromin, acriflavinium hydrochloride, calcein, aklavine hydrochloride, isoreserpine, and pyrvinium pamoate, were found to exhibit autofluorescence and thus were not pursued further. Others were cytotoxic and increased GFP by creating fluorescent cell aggregates. As expected, known estrogenic compounds such as estrone, clomiphene citrate, estradiol diacetate, estrone acetate,
diethylstilbestrol, estradiol cypionate, estriol, and estradiol acetate were positive in the assay through their ability to activate the E47\textsuperscript{MER} transgene.

Figure 3. HTS of T6PNE insulin promoter assay with known drug library reveals antipsychotics as modulators of insulin promoter activity. (A) Scatter plot of the high-throughput screen conducted on the T6PNE insulin promoter assay with 1040 known drugs from the NIH/JDRF Custom Collection. The screen was run in duplicate. Each set of replicates is depicted as either red squares or blue triangles. Duplicate values for the 3 compounds that passed the primary confirmatory assays (berbamine, chlorpromazine, and ethopropazine) are circled. (B-C) (B) Ethopropazine and Chlorpromazine increase activity on the endogenous insulin promoter and (C) Ethopropazine has no effect on the insulin promoter at 0\textmu M Tamoxifen, both assayed by QPCR (n=6). Error bars are SEM. * indicates p<0.05 relative to DMSO control.
Of the remaining compounds, 3 were identified as increasing the number of GFP-positive cells in both wells from the primary screen. These compounds—chlorpromazine, ethopropazine, and berbamine—were subjected to a primary confirmatory assay, consisting of a repeat of the initial assay but with a dose response and 8 replicates for each dose. Image acquisition and analysis was performed as for the primary screen. All 3 of these compounds demonstrated dose-responsive increases in the percentage of GFP-positive cells [82].

The 3 compounds that passed the primary counterscreens—ethopropazine, chlorpromazine, and berbamine—were subjected to a secondary assay consisting of quantitative RT-PCR for GFP and endogenous insulin mRNA. Berbamine increased GFP but not endogenous insulin mRNA and thus was not studied further. However, ethopropazine stimulated an increase in both GFP and insulin mRNA above 0.1% DMSO by $3.86 \pm 0.33$–fold and $2.89 \pm 0.13$–fold, respectively (Fig. 3B)[82]. Chlorpromazine acted similarly, stimulating an increase in GFP and insulin mRNA of $2.33 \pm 0.14$–fold and $2.45 \pm 0.19$–fold, respectively (Fig. 3B)[82]. To ensure that the phenothiazines were not acting in a manner similar to the estrogenic compounds that were true, but biologically uninteresting positives in the initial screen, we tested their ability to activate the insulin promoter in the absence of added tamoxifen, finding that there was no effect under that condition (Fig. 3C) [82].

2.3.2 **Chronic exposure to ethopropazine induces β-cell dysfunction**
A puzzling aspect of the finding that some phenothiazines increase insulin expression in our assay is that this class of drugs has been reported to be diabetogenic [20], [21], [28]. Since patients take these drugs for longer periods of time though before seeing metabolic side effects, we investigated the effect of chronic exposure of Ethopropazine in the T6PNE insulin promoter assay. Although the positive effect on insulin expression was confirmed early in the course of treatment, chronic exposure to the drug led not only to the loss of stimulatory activity but also to repression of insulin promoter activity (Fig. 4) [82].

Figure 4. Chronic exposure to ethopropazine induces b-cell dysfunction.
Ethopropazine (red), Vehicle (blue). Ethopropazine (10 µM) was administered to T6PNE cells for 12 days. Media were changed every 3 days. A lower concentration of drug was used than in acute treatment to minimize toxic effects on the cells. Values are expressed as fold change in GFP-positive cells relative to vehicle-treated cells ($n = 3$; error bars are SEM; *$p < 0.05$).

2.3.3 A subset of phenothiazines modulate insulin promoter activity

Given that the NJCC library contained many phenothiazines, it was interesting that the only members of the class that were detected as hits in the primary screen were chlorpromazine and ethopropazine. To further examine the structural specificity
of phenothiazines on insulin gene expression, we conducted a structure activity relationship (SAR) of the phenothiazines present in the original screen using the methodology employed for the primary confirmatory screen described above. The results, displayed in figure 5A, showed many phenothiazines in the NJCC library were weakly positive but fell below the threshold value used in the high-throughput screen to detect hits (Fig. 5A)[82]. Examining the structures of the positive and negative phenothiazines revealed that the most active compound was chlorpromazine, which differs from promazine only by the addition of a chlorine atom onto the carbon at the 3 position (Fig 6A). This suggests that adding an electron-withdrawing group at that position increases activity. The increasing activity from promazine to ethopropazine suggests that increasing the hydrophobicity at the terminal amine by adding alkyl substituents also increases activity (Fig 6A). The presence of a piperazine rather than an alkyl group at the terminal amine had a strong negative effect on activity. This suggests the possibility that this side group is limiting interaction with a specific receptor by steric hindrance (Fig. 6A)[82].

2.3.4 Both typical and atypical antipsychotics modulate the insulin promoter

Because typical antipsychotics such as the phenothiazines have been supplanted to a large degree by the structurally diverse atypical antipsychotics, we extended our analysis of antipsychotic activity beyond the phenothiazines that were the initial hits in our high-throughput screen for insulin promoter modulators. Despite
Figure 5. Both typical and atypical antipsychotics modulate the insulin promoter. 
(A) Retesting of all phenothiazines in the NIH/JDRF library for effect in the T6PNE Ins-GFP insulin promoter assay. All compounds are shown at their maximum tolerated dose (n=12). Error bars are SEM. * indicates p<0.05 relative to DMSO control. (B) SAR by QPCR of the effect of typical and atypical antipsychotics on endogenous insulin gene expression. (n≥6). Error bars are SEM. * indicates, p≤0.05 relative to DMSO control.

the structural diversity of the antipsychotics tested, almost all modulated insulin promoter activity (Fig. 5B). There was no clear relationship between the classification
of a drug as typical or atypical and its activity in the assay, as the potency of the active atypical antipsychotics tested fell between 1.5-2.5 fold over control, ranking them in the middle of the 1.8-4 fold range of the typical antipsychotics which tested positive in the assay. Molindone and Ziprasidone, which are not associated with strong metabolic side effects in patients [32], [36], [37], did not significantly enhance insulin promoter activity in T6PNE, which is to be expected if the effects of antipsychotics on the insulin promoter in T6PNE are relevant to the metabolic side effects of the drugs.
Figure 6. Antipsychotics show dose-responsive activation in the T6PNE Insulin Promoter Assay. (A) SAR of antipsychotic effects on the endogenous insulin promoter, values listed are from experiment in Figure 5. (B) Effects of antipsychotics in the T6PNE Ins-GFP insulin promoter assay. Ziprasidone is autofluorescent and therefore not included. All antipsychotics were cytotoxic at 40uM. Fluphenazine, perphenazine, and trifluoperazine were cytotoxic at 20uM. (n=12).
2.4 Methods

Cell culture

T6PNE was maintained in RPMI-5.5 mM glucose supplemented with 10% fetal bovine serum and grown at 5% CO₂, 37°C. To induce E47 activity in cells under experimental conditions, 0.5, .6, or 1µM Tamoxifen (Sigma) was added to culture media.

Compounds

All antipsychotics were purchased from Sigma, except for olanzapine (Toronto Research Chemicals). All antipsychotics were used at 10 or 20 mM, the concentration at which dose-response analysis revealed maximum activity on the human insulin promoter-eGFP transgene was evident without substantial cytotoxicity (Fig. 6B).

T6PNE Ins-GFP insulin promoter assay

T6PNE cells were seeded at 1750 cells per well in clear bottom black 384 well plates (Greiner) with 0.6 or 1µM Tamoxifen. Twenty-four hours later, compound or vehicle was added. Forty-eight hours after compound addition, cells were fixed with 4% formaldehyde. DAPI (0.167 µg/ml) was added to visualize nuclei. The plates were imaged on an IC 100 high-throughput microscope in the blue and green channels. Cytoshop software was then used to determine the percent of cells in each well that exhibited green fluorescence above a threshold determined by a MATLAB
algorithm, which is then expressed as the fold change from vehicle treated control (Fold Change GFP+ cells) as described in Image Acquisition and Analysis below.

**Compound library screening**

T6PNE Ins-GFP insulin promoter assay was run as described above. Compound addition was performed 24 hours after tamoxifen administration with the BiomekFX (Beckman Coulter). This was done by adding a single compound from the NIH/JDRF compound set. The plates were stored in 50% glycerol and read on the GE/Amersham InCell 1000 high throughput microscopy system [82].

**Image acquisition and analysis**

Images were obtained on a Beckman Coulter IC 100 instrument equipped with a Hamamatsu ORCA-ER scientific camera using a Nikon S Flour 10x NA 0.5 objective. Each 384-well plate was scanned and DAPI (40 ms integration time), GFP (200 ms integration time) and RFP channels were collected at one 3-color, 1280 x 1024 image per well. Images are captured of each well in the blue channel (to evaluate cell number), the green channel (readout for insulin gene expression) and the red channel (to eliminate autofluorescent compounds). DAPI, a fluorescent stain that binds tightly to DNA, produces a punctate nuclear mask that allows the microscope to focus from well to well on the plate.

Image processing (Cytoshop, Beckman Coulter, USA) was used to perform shade correction, nuclear segmentation and cytometry. Due to lamp variations throughout the imaging process, shade correction and plate-to-plate normalization
techniques were utilized by equilibrating plate and global medians. Cell-by-cell analysis was performed by tessellation and pixel intensity measurements. The segmentation protocol used performed the following steps: The nucleus of each cell was identified using Cytoshop’s nuclear segmentation algorithm that included an “open” morphological operation, the equidistance tessellation lines were drawn between the centroids of the identified nuclei, effectively breaking up the images into cellular regions, then an object extraction correlation radius of 30 pixels was set, inside which pixels were assumed to belong to the cytoplasm of the cell. Hits were then determined by applying a threshold intensity gates on the green channel and surveying the number of cells above or below that gate in a given well by analyzing the average green pixel intensity under the cytoplasmic mask. These counts were then normalized to the total number of cells per well to give a percent GFP positive cells quotient. To construct the final assay-wide database from the individual plate databases, a Matlab algorithm was utilized to collate the individual plate data [82].

RT PCR analysis

Following 48 hours of compound addition (unless otherwise stated), RNA Purification (Qiagen), and cDNA synthesis (Quanta Master Mix, MJ Research PTC-200) Quantitative PCR was performed on cDNA corresponding to 100 ng of RNA using the Opticon Real-Time System (MJ Research) and BioPioneer QPCR Super Mix. QPCR for 18s was used for normalization. All mRNA values are normalized to 18s mRNA and are expressed as fold change from vehicle treated control.
Primers:

**Insulin**
FWD: CTACCTAGTGTGCGGGGAAC  
REV: GCTGGTAGAGGAGCAGATGI

**Kip2**
FW:GGCGATCAAGAAGCTGTCC  
REV: GGGCTCTTTGGGCTCTAAA

**18S**
FW:GATATGCTCATGTGGTTG  
REV:AATCTTCTTCAGTGCCTCCA

**Chronic ethopropazine treatment.**

T6PNE cells were seeded in a 10cm dish with .5uM Tamoxifen. At 24 hours either 10uM Ethopropazine or a Vehicle Control was added. At 96 hours the cells were passed into either a 384 well clear bottom black well plate for imaging or a 10cm dish for continued culture along with fresh Ethopropazine or vehicle. The same passing procedure was performed every 3 days for the remainder of the 12-day experiment. The 384 well plates seeded for analysis as conducted in the T6PNE Ins-GFP insulin promoter assay.
2.5 Conclusions

The principal finding here is that a subset of phenothiazine neuroleptic drugs were able to modulate insulin promoter activity. It is significant that the only class of drugs within the 1,040 compound library that influenced insulin gene expression were phenothiazines since they are known to predispose to diabetes upon chronic treatment [86]. Prolonged treatment of T6PNE with the phenothiazine antipsychotic ethopropazine led to repression of insulin promoter activity. Thus, as with other diabetogenic molecules such as fatty acids, acute exposure to antipsychotics can stimulate aspects of β-cell function, while chronic exposure is deleterious [75]. This could result from desensitization of a pathway and might play a role in the deleterious effects of antipsychotics on β-cell function that are found in published studies showing the drugs to inhibit glucose-responsive insulin secretion [87], [61], [62], [63].

We were able extend the results from the screen to include an array of other typical and atypical antipsychotics, the majority of which are known to be diabetogenic, finding almost all of them to have some activity in the insulin promoter assay aside from molindone and ziprasidone. This strongly suggests that our findings in T6PNE are relevant to the metabolic side effects of the drugs since molindone and ziprasidone are the only antipsychotics tested to show weak or no association with weight gain and diabetes in patients [32], [36], [37]. These two antipsychotics fall into a structurally distinct class containing dihydroindolone and indolinone cores, respectively, which are not found in the phenothiazines or any of the other typical or atypical antipsychotics. Our efforts to extend this SAR of antipsychotics activity in the insulin promoter assay to the propensity of a given drug to cause metabolic side
effects were unsuccessful though due to inconsistencies in the literature on this issue [23], [24], [25], [26], [27], [21].

Of note is the over 3X difference in ability to modulate the insulin promoter between the antipsychotics, and in particular between the structurally similar phenothiazines. When comparing prochlorperazine to chlorpromazine once can see that a specific structural feature of the terminal amine determined the level of activity. This suggests that a specific molecule is being targeted by the antipsychotics in T6PNE that leads to modulation of insulin promoter activity.

The targets of antipsychotics are diverse, having been shown to affect a variety of proteins, including dopamine receptors, protein kinase C, calmodulin, and others [88]. Chapter 3 will move on to describe our efforts to identify the targets of the antipsychotics relevant to their effects on the insulin promoter. The identification of this target will hopefully shed light on the mechanisms responsible for the metabolic side effects of the drugs.

2.6 Acknowledgements

Chapter 2, in part, is a reprint of the material as it appears in the Journal of Biomolecular Screening, 2010. It was co-authored by Alice Kiselyuk, Suzette Farber-Katz, Tom Cohen, Seung-Hee Lee, Ifat Geron, Behrat Azimi, Suzanne, Heynen-Genel, Oded Singer, Jefferey Price, Mark Mercola, Pamela Itkin-Ansari, and Fred Levine.
CHAPTER 3:

ANTIPSYCHOTICS ACT ON THE INSULIN PROMOTER AT E-BOXES DOWNSTREAM OF A NON-CANONICAL TGFβ PATHWAY AND NOT THROUGH THE NEUROTRANSMITTER RECEPTORS RESPONSIBLE FOR THEIR THERAPEUTIC EFFECT

3.1 Summary

Given the central role of insulin expression in the β-cell, the findings detailed in chapter 2 raise the possibility that insights into the metabolic side effects of antipsychotics could be achieved through uncovering the mechanism by which they affect the insulin promoter. Since antipsychotics are known to modulate the activity of a wide range of targets, including the GPCR neurotransmitter receptors responsible for their therapeutic effects, and a number of intracellular signaling molecules [88], chapter 2 begins with experiments testing the hypothesis that the drugs act on the insulin promoter through a previously identified target. This hypothesis was tested by assaying other chemical inhibitors of the neurotransmitter receptors and intracellular signaling molecules known to be targeted by antipsychotics for activity in the T6PNE Ins-GFP insulin promoter assay. Since none of these chemical inhibitors modulated the insulin promoter, we concluded the antipsychotics were acting on the insulin promoter through a previously unidentified target in T6PNE, and therefore moved to an unbiased approach.
A high-throughput antipsychotic primed counterscreen conducted with a library of kinase inhibitors demonstrated that antipsychotics modulate the insulin promoter through the TGFβ pathway. Further studies found that antipsychotics activated SMAD3, an important downstream effector of the TGFβ pathway, which ultimately acts on E-box sequences within the insulin promoter. Also, antipsychotic-mediated SMAD3 activation occurred through a receptor distinct from those acted upon by TGFβ itself. Such non-canonical activation of SMADs is documented, with signaling molecules such as AKT and MAPK family members known to directly activate SMAD3 unrelated to the kinase activity of the TGFβ receptor family [89].

The TGFβ pathway and SMAD3 in particular are highly associated with obesity, insulin resistance, and diabetes [90], [91], [92], [93], [94]. The findings in this chapter that antipsychotics activate this pathway through a mechanism distinct from that responsible for the neurological effects of those drugs raises the possibility that antipsychotics could be designed that retain the beneficial CNS activity while lacking the deleterious metabolic side effects.

3.2 Results

3.2.1 Neurotransmitter receptors and intracellular signaling molecules targeted by antipsychotics are inactive in the insulin promoter assay

Since it is known that antipsychotics activate multiple neurotransmitter receptors, [95], [11] and that many of those are expressed in islets [96] where they
play important roles in β-cell function [97], we hypothesized that the effect of antipsychotics on the insulin promoter was through a pathway involving the known neurotransmitter receptor targets of antipsychotics. To test this, we first analyzed gene expression data (GSE18821)[82], to determine which neurotransmitter receptor targets of antipsychotics were expressed in T6PNE cells, finding that multiple receptors were expressed at approximately equal levels in T6PNE and primary human islets (Fig. 7A). Specific antagonists of these receptors were then tested for activity on the human insulin promoter-eGFP transgene in T6PNE cells. None of the antagonists tested altered insulin promoter activity (Fig. 7B), leading us to conclude that the target of the antipsychotics responsible for insulin promoter modulation was not one of the known neurotransmitter receptor targets responsible for their therapeutic effects.

A small number of intracellular signaling molecules unrelated to their neuroleptic properties are also known to be inhibited by antipsychotics including protein kinase C, PI3K/Akt, CaM kinase/calmodulin, clathrin-mediated endocytosis, and p38 [98], [99], [100], [101], [102]. In a similar approach we tested other inhibitors of these intracellular targets in the T6PNE Insulin promoter assay and found two to be active (Figure 8A), the p38 inhibitor SB203580 and the clathrin mediated-endocytosis inhibitor dynasore. Since ethopropazine signaled in the opposite polarity from dynasore and other clathrin inhibitors tested, they were all very toxic, and ethopropazine was still able to activate the insulin promoter in the presence of dynasore (Figure 8A), we do not believe antipsychotics act on the insulin promoter through modulation of clathrin-mediated endocytosis. The MAPK p38 was also ruled out since Ethopropazine did not enhance phosphorylation of p38 (Figure 8B) and was
Figure 7. Neurotransmitter Receptors Targeted by Antipsychotics are Inactive in the Insulin Promoter Assay. (A) Neurotransmitter receptors acted on by antipsychotics were expressed at comparable levels in the T6PNE and primary human islets. Microarray analysis using Illumina BeadArrays was performed on T6PNE cells and primary human islets. The y-axis represents the level of hybridization to the oligonucleotide on the array, an indirect measure of mRNA level. (B) Pharmacologic antagonists of neurotransmitter acted on by antipsychotics were tested for activity in the T6PNE Ins-GFP insulin promoter assay (n=12). Error bars are SEM. * indicates p<0.05 relative to DMSO control.
still able to modulate the insulin promoter in the presence of siRNAs directed at p38 α and β, the targets of SB203580 (Figure 8C-E).
3.2.2 Antipsychotics signal to the insulin promoter through the TGF\(\beta\) pathway downstream of the TGF\(\beta\) type I receptor kinase.

Given the negative results with known antipsychotic targets, we switched to an unbiased approach, screening the T6PNE insulin promoter assay with a library of kinase inhibitors that act on many different pathways. This library (Calbiochem Inhibitor) has been used previously in high-throughput screens [103] and contains 80 inhibitors against tyrosine, AGC, and atypical families of kinases, 80 inhibitors against CMGC and CaMK families of kinases, and 84 Ser/Thr kinase inhibitors. The inhibitors were selected to ensure structural diversity, cell permeability, reversibility, and potency, and are therefore ideal for screening. We screened this library in the presence and absence of Ethopropazine, used because of its chemical stability and high level of activity, to ascertain kinase inhibitors with activity that was selectively affected by antipsychotics (Figure 9A,B).

Ten compounds repressed the insulin promoter in the absence of the phenothiazine antipsychotic Ethopropazine (Fig. 9B, \(\uparrow\) and \(\bullet\)); other apparent repressors were cytotoxic false-positives. One of the ten, SB-505124, an inhibitor of transforming growth factor beta (TGF\(\beta\)) type I receptors [104], completely lost activity in the presence of ethopropazine (Fig. 9A \(\bullet\)). This indicates that ethopropazine is epistatic to the TGF\(\beta\) type I receptor kinase, signaling to the insulin promoter through the TGF\(\beta\) pathway at a point downstream of the type I kinase. A dose-response study of the effect of SB-505124 demonstrated potent repression of the human insulin promoter-eGFP transgene (Fig. 9D). It also potently repressed the endogenous insulin gene in the absence, but not in the presence of ethopropazine,
Figure 9. Antipsychotics Signals To the Insulin Promoter Downstream of type I TGFβ receptors. (A-B) The T6PNE Ins-GFP insulin promoter assay was screened against the Calbiochem 242 compound kinase Inhibitor library in the presence (A) or absence (B) of ethopropazine (20mM) in duplicate. A total of 10 inhibitors repressed the insulin promoter (▲). Of the ten, one, the TGFβ type I receptor inhibitor SB-505124 (●), was a potent insulin promoter inhibitor in the absence but not the presence of ethopropazine. (C). QPCR analysis of endogenous insulin gene expression (n=9). Error bars are SEM. * indicates p<0.05 relative to DMSO control. (D) SB-505124 shows dose responsive inhibition in the 48 hour T6PNE Ins-GFP insulin promoter assay. (n=12), error bars are SEM, * indicates p<0.05 relative to DMSO control. (E) Microarray analysis shows TGFβ receptor family members were also expressed at comparable levels in the T6PNE and primary human islets, with ALK4/5/7, targets of SB-505124, expressed at detectable levels in both.
supporting the results with the transgene (Fig. 9C).

The specificity of SB-505124 for different TGFβ type I receptors has been studied [104]. It exhibits a strong preference for ALK 4/5/7 over ALK 1/2/3/6, and has no or minimal activity on a panel of 27 other protein kinases [104]. We examined the expression of the TGFβ receptor family in T6PNE using gene expression microarray data (Fig. 9E)(GSE18821)[82]. Similar to the neurotransmitter receptors, there was good concordance between the level of expression in T6PNE and primary human islets (GSE18821)[82]. While ALK2 was the most highly expressed, ALK4, ALK5, and ALK7 were also present, consistent with the preferential activity against ALK4/5/7 exhibited by SB-505214.

3.2.3 Antipsychotics activate the TGFβ pathway

We next tested if the major downstream mediators of the TGFβ pathway, the SMAD transcription factors, were activated by antipsychotics. In the classic TGFβ pathway receptor-regulated SMADs (RSMADs) are phosphorylated by TGFβ type I receptors, promoting association with coSMADs, nuclear translocation, and then binding to specific sequence elements in promoters to modulate gene expression [105]. However, non-canonical RSMAD activation is well described [89]. As expected, TGFβ1 activated and SB-505124 inhibited the RSMAD reporter SBE4-Luc (Fig. 10A), which contains multimerized sequence elements responsive to the RSMADs SMAD2 and SMAD3 driving a luciferase reporter [106]. In the same assay,
Figure 10. Antipsychotics activate the TGFβ pathway. (A-B. Ethopropazine increases activity on the SMAD2/3 responsive SBE4-luc reporter (A), SAR of typical and atypical antipsychotics on the SBE4-luc reporter (B), both measured by luciferase activity (n≥6). Error bars are SEM. * indicates p<0.05 relative to DMSO control. (C) SBE4 reporter activity of antipsychotics from 2C is plotted against the insulin promoter activity from Figure 1A. Each point represents a single antipsychotic (Pearson correlation coefficient = 0.93, p=.00012).
ethopropazine activated the SBE4-Luc reporter, but to a lesser extent than TGFβ1 (Fig. 10A).

Next, we performed an SAR on the SBE4-Luc reporter with the array of typical and atypical antipsychotics used previously (Fig. 10B). Strikingly, the two SARs (insulin promoter versus SBE4-Luc reporter) were highly correlated (pearson correlation coefficient \( r = 0.93, p=0.00012 \), Fig. 10C). This high correlation coefficient strongly supports a model in which antipsychotics signal to the insulin promoter through activation of the TGFβ pathway. The insulin promoter does appear to be more sensitive than the SMAD reporter to the effects of antipsychotics since perphenazine, fluphenazine, and trifluoperazine were active on the insulin promoter but did not induce statistically significant increases in SBE4 reporter activity.

### 3.2.4 Antipsychotics promote phosphorylation of SMAD3 but not SMAD2

Since it is well established that RSMADs are activated by phosphorylation \[107\], and antipsychotics activated a SMAD reporter, we tested if they promoted increased phosphorylation of the RSMADs SMAD2 and SMAD3, assayed by immunoblot with phospho-specific antibodies. As expected, TGFβ1 increased the levels of both phosphorylated RSMADs (Fig. 11A-D) and SB-505124 decreased the levels of both phosphorylated RSMADs (Fig. 11A-D). However, ethopropazine increased the level of phospho-SMAD3 (Fig. 11A,C) but not phospho-SMAD2 (Fig. 11B,D). Also, ethopropazine antagonized the effect of SB-505124 on SMAD3 (Fig. 11A,C) but not on SMAD2 (Fig. 11B,D).
Consistent with the specific effect of ethopropazine on SMAD3 phosphorylation, we found that ethopropazine activated CAGA12-Luc [108], a reporter construct specific to SMAD3 activity (Figure 12). Interestingly, while ethopropazine was less effective than TGFβ1 in the SBE4-luc assay (Fig. 10A), it was as effective as TGFβ1 at activating the CAGA12 reporter (Fig. 12), consistent with TGFβ1 activation of both SMAD2 and SMAD3, while antipsychotics activate only

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Figure 11. Antipsychotics enhance phosphorylation of SMAD3 but not SMAD2. (A-B) Immunoblot analysis of phospho-SMAD levels. T6PNE cells were treated for 1hr with the indicated condition. (C-D) Quantification of pSMAD3 (C) and pSMAD2 (D) protein levels, demonstrated that antipsychotics induced increased pSMAD3 but not pSMAD2. The pSMAD intensity was divided by the total SMAD intensity. (n=3, Blot shown is representative of 3 independent experiments). Error bars are SEM. * indicates p<0.05 relative to DMSO control.
SMAD3. Ethopropazine was also somewhat more efficient at antagonizing the effect of SB-505124 on the CAGA12-Luc reporter than it was on SBE4-Luc activity. Taken together, these experiments lead us to conclude that antipsychotics induce SMAD3 phosphorylation downstream of TGFβ type I receptors. Since TGFβ and antipsychotics differentially activate SMAD3 and SMAD2, there must be distinct pathways by which antipsychotics and TGFβ promote phosphorylation of RSMADs.

### 3.2.5 SMAD3, but not SMAD2, is required for the effect of antipsychotics on the insulin promoter

If antipsychotics signal to the insulin promoter solely through SMAD3, then downregulation of SMAD3, but not SMAD2, by siRNA should ablate the effect of antipsychotics on the insulin promoter. We found this to be true; SMAD2 siRNAs did
not affect antipsychotic-induced effects on the insulin promoters, while those effects were almost completely ablated by a SMAD3 siRNA (Fig. 13A). We also found that SMAD3 overexpression, confirmed by immunoblot to result in a 2.1 fold increase in SMAD3 protein, enhanced insulin transcription (Fig. 14), while SMAD3 siRNA potently inhibited the insulin promoter (Fig. 13A).

### 3.2.6 TGFβ inhibits the insulin promoter through downregulation of SMAD3 transcription

The fact that SB-505124 repressed insulin gene expression in T6PNE cells implies that there is a basal level of TGFβ pathway activity in those cells, acting to stimulate promoter activity. This is consistent with the effects of SMAD3 siRNA (Fig. 13A) and overexpression (Fig. 14), and with previous data on TGFβ pathway activation in β-cells [92], [109], [110]. However, TGFβ1, TGFβ2, and Activin A all exhibited dose-dependent inhibition of the human insulin promoter-eGFP transgene (Fig. 15B-D) and the endogenous insulin promoter (Figure 15A), with TGFβ1 being the most potent. The inhibition of the insulin promoter by TGFβ1 was unaffected by siRNA to SMAD2 (Fig. 13A), suggesting that TGFβ1 was acting through a dominant, non-canonical pathway. Since it has been reported previously that TGFβ1 can inhibit SMAD3 gene expression [111], [112], we tested the effect of TGFβ1 on SMAD mRNA levels in T6PNE cells. TGFβ1 induced a dramatic decrease in SMAD3 but not SMAD2 mRNA levels, while ethopropazine had no effect on either SMAD3 or SMAD2 mRNA (Fig. 13B,C). In addition, 48 hours of treatment with TGFβ but not
ethopropazine decreased SMAD3 protein levels (Fig. 16). Thus, in the acute 24 hour setting of a transient transfection assay, the effect of TGFβ1 on SMAD

Figure 13. SMAD3, but not SMAD2, is required for the effect of antipsychotics on the insulin promoter. (A) siRNA to SMAD3 but not SMAD2 inhibited insulin gene expression in the absence and presence of antipsychotics. (B, C) SMAD3 (B) and SMAD2 (C) siRNAs demonstrate efficacy and specificity. Insulin (A), SMAD3 (B), or SMAD2 (C) mRNA content was measured by quantitative RT-PCR (n=6, this is a representative of 2 independent experiments). Error bars are SEM. * indicates p<0.05 relative to DMSO control.
**Figure 14.** SMAD3 overexpression enhances insulin promoter activity.
SMAD3 overexpression enhanced insulin promoter activity. Insulin mRNA was measured by quantitative RT-PCR (n=6), Error bars are SEM. * indicates p<0.05 relative to DMSO control.

**Figure 15.** TGFβ family members inhibit the insulin promoter (A) RT-PCR analysis of endogenous insulin gene expression. (n=9). Error bars are SEM. * indicates p<0.05 relative to DMSO control. (B-D) TGFβ1 (B), TGFβ2 (C), and Activin A (D) all showed dose responsive inhibition in the T6PNE Ins-GFP Insulin Promoter Assay. (n=12), error bars are SEM, * indicates p<0.05 relative to DMSO control.
phosphorylation is dominant, while in the more chronic 48 hour assay on insulin promoter activity, its effect on SMAD3 gene expression predominates. The complexity of the feedback loops in the TGFβ pathway is further exemplified by the increase in SMAD2 mRNA induced by SMAD3 siRNA (Fig. 13C). However, a reciprocal increase in SMAD3 mRNA by SMAD2 siRNA was not found (Fig. 13B).

To determine the generality of the effect of TGFβ on SMAD gene expression, we used the Pubmed Gene Expression Omnibus (GEO) database to examine the large number of previous reports of the effect of TGFβ on a variety of different cells. The vast majority of those studies found substantial effects of TGFβ on SMAD3 (average fold change = -2.15), but not SMAD2 mRNA expression (average fold change = -.31), (Table 1).

**Figure 16. TGFβ1 decreases SMAD3 protein levels.** SMAD3 protein levels decrease following 48 hour TGFβ1 exposure. (n=3, Blot shown is representative of 3 independent experiments). Error bars are SEM. * indicates p<0.05 relative to DMSO control.
Table 1. TGFβ treatment decreases SMAD3 mRNA content across gene expression studies. Data from the GEO database was utilized to determine the generality of the effect of TGFβ on SMAD gene expression. The vast majority the studies found substantial repression of SMAD3 but not SMAD2 mRNA by TGFβ.

<table>
<thead>
<tr>
<th>PMID/ GEO Reference Series</th>
<th>Title</th>
<th>Description</th>
<th>Species</th>
<th>SMAD3 Fold Change</th>
<th>SMAD2 Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>unpublished/ GSE6676</td>
<td>TGFβ overexpression effect on the cornea</td>
<td>TGFβ expressed under Strong lens-specific promoter (Transgenic vs. Control)</td>
<td>Mouse</td>
<td>-1.40</td>
<td>1.38</td>
</tr>
<tr>
<td>19615063/ GSE6653</td>
<td>TGFβ effect on immortalized ovarian surface epithelial cell line</td>
<td>IOSE cells (TGFβ1 12hr vs untreated)</td>
<td>Human</td>
<td>-2.43</td>
<td>-1.00</td>
</tr>
<tr>
<td>15477587/ GSE1805</td>
<td>TGFβ effect on acute myelogenous leukemia cells</td>
<td>M091 Cells (200 pM TGFβ 4hr vs untreated)</td>
<td>Human</td>
<td>-1.30</td>
<td>-1.14</td>
</tr>
<tr>
<td>15477587/ GSE1802</td>
<td>TGFβ effect on CD34+ hematopoietic stem cells</td>
<td>CB-CD34 CD34+ hematopoietic stem cells (200 pM TGFβ 4hr vs untreated)</td>
<td>Human</td>
<td>-1.52</td>
<td>1.04</td>
</tr>
<tr>
<td>16078232/ GSE2705</td>
<td>GFAP-negative lamina cribrosa cell response to TGFβ</td>
<td>GFAP-negative lamina cribrosa glial cell (10 ng/ml TGFβ1 24 hours vs untreated)</td>
<td>Human</td>
<td>-2.07</td>
<td>1.08</td>
</tr>
<tr>
<td>20197308/ GSE20247</td>
<td>Proinsulin C-peptide and TGFβ effect on proximal tubular cell line: time course</td>
<td>HK2 cells (TGFβ 48hr vs untreated)</td>
<td>Human</td>
<td>-1.49</td>
<td>-1.12</td>
</tr>
<tr>
<td>20007254/ GSE17708</td>
<td>TGFβ-induced epithelial-mesenchymal transition model</td>
<td>A549 Cells (TGFβ 72hr vs. untreated)</td>
<td>Human</td>
<td>-1.51</td>
<td>1.08</td>
</tr>
<tr>
<td>17178593/ GSE2558</td>
<td>TGFβ effect on renal mesangial cells</td>
<td>MES-13 Cells (100ng/ml TGFβ 24hr vs untreated)</td>
<td>Mouse</td>
<td>-1.23</td>
<td>-1.09</td>
</tr>
<tr>
<td>15571627/ GSE1724</td>
<td>Idiopathic and scleroderma-associated pulmonary fibrosis derived fibroblasts response-TGFβ</td>
<td>Adult Lung Fibroblasts, Normal Tissue (4ng/ml 4hr vs untreated)</td>
<td>Human</td>
<td>-1.93</td>
<td>-1.23</td>
</tr>
<tr>
<td>19701206/ GSE17518</td>
<td>mRNA expression profile in IMR-90 cells in response to TGFβ</td>
<td>IMR90 fetal lung mesenchymal cells (2ng/ml TGF-beta1 48hr vs untreated)</td>
<td>Human</td>
<td>-6.82</td>
<td>-1.05</td>
</tr>
<tr>
<td>Unpublished/ GSE23952</td>
<td>Expression data from TGFβ treated Panc-1 pancreatic adenocarcinoma cell line</td>
<td>Panc-1 cells (serum-starved, 24 h, 5 ng/mL TGFbeta1 48hr vs untreated)</td>
<td>Human</td>
<td>-1.98</td>
<td>-1.42</td>
</tr>
</tbody>
</table>
3.2.7 Antipsychotics signal to the insulin promoter through modulation of E-box activity downstream of SMAD3.

The effects of the TGFβ pathway on insulin expression are complex, with both activation and repression having been reported [110], [92], [113]. Reflecting this complexity, there are multiple mechanisms by which SMAD proteins could affect promoter activity. The human insulin promoter contains a SMAD binding site [92]. In addition, the TGFβ pathway has been shown to modulate E-box activity [114], and SMAD proteins have been shown to interact directly with bHLH factors [115], [116], suggesting that the effect of antipsychotics on the insulin promoter could be mediated at E-boxes. We showed previously that the p57Kip2 gene, encoding a cyclin-dependent kinase inhibitor, is regulated in T6PNE by a specific E-box in the promoter, resulting in co-regulation with the insulin gene in those cells [82]. Since no SMAD binding element is present in the Kip2 promoter, we tested the effect of antipsychotics on E-box activity through Kip2 gene expression levels. Ethopropazine potently activated Kip2 transcription in T6PNE cells (Fig. 17A), as well as in T6PN cells (Fig.17B), which express PDX1 and NeuroD1 and low levels of endogenous E47, but not E47MER [117], [82]. T6PN was used to rule out the possibility that antipsychotics could be acting as agonists for the modified estrogen receptor (MER), leading to activation of E47 by a physiologically irrelevant mechanism. Of significance, the SAR for the antipsychotics on Kip2 expression was highly correlated with their effect on insulin expression, as one would expect if the mechanisms by which the drugs affect the two promoters were the same (Fig. 17C).
Similar to its effect on the insulin promoter, SB-505124 decreased the level of Kip2 mRNA in the absence of ethopropazine, and this effect was blocked in the presence of...
the drug (Fig. 17A). Also in accordance with its effect on the insulin promoter, TGFβ1 decreased Kip2 mRNA, as expected due to the downregulation of SMAD3 levels by TGFβ1 at 48 hours. SMAD3 siRNA potently inhibited Kip2 gene expression while SMAD2 siRNA had no effect on Kip2 mRNA levels (Figure 18A), demonstrating the specificity of regulation of E-box activity by SMAD3.

To confirm that the effects of antipsychotics and the TGFβ pathway were directly through the E-box and not any other sequence elements in the Kip2 promoter, we tested its effect on the 4RTK-Luc reporter, in which the luciferase reporter is under the control of a promoter consisting of multimerized E-box elements 5’ of a thymidine kinase minimal promoter [118]. Antipsychotics activated 4RTK-Luc, while TGFβ1 and SB-505124 repressed reporter activity (Fig. 18B), with antipsychotics being epistatic to SB-505124 on the E-box reporter. Thus, all of the results are consistent with a model in which antipsychotic effects on the insulin promoter are mediated by activation of SMAD3, which then acts through E-boxes to affect gene expression.
Figure 18. Antipsychotics signal to the insulin promoter through modulation of E-box activity downstream of SMAD3. (A) siRNA to SMAD3, but not SMAD2 or scrambled, inhibit Kip2 transcription measured by QPCR and block antipsychotic-induced effects on the Kip2 promoter. (n≥6). Error bars are SEM. * indicates p<0.05 relative to DMSO control. (B) Antipsychotics enhance while TGFβ1 and SB-505124 inhibit activity on an E-box reporter driving luciferase (4RTK-luc) in T6PNE cells. Cells were treated for 48 hr (n=9), Error bars are SEM. * indicates p<0.05 relative to DMSO control.
3.3 Methods

Compounds

All antipsychotics and neurotransmitter receptor antagonists were purchased from Sigma, except for olanzapine (Toronto Research Chemicals). SB-505124 was purchased from Calbiochem and recombinant Activin, TGFβ1, and TGFβ2 from Thermo Scientific. All antipsychotics were used at 10 or 20 mM, the concentration at which dose-response analysis revealed maximum activity on the human insulin promoter-eGFP transgene was evident without substantial cytotoxicity (Suppl. Fig. 1). SB-505124 was used at 2.5 uM. TGFβ1, TGFβ2, and Activin A were used at 10 ng/ml.

Microarray Analysis

Microarray analysis using Illumina BeadArrays was performed on T6PNE cells and primary human islets (GSE18821)[82]. The y-axis represents the level of hybridization to the oligonucleotide on the array, an indirect measure of mRNA level.

Transfection studies

T6PNE cells were transfected in serum free media with Lipofectamine 2000 (1.2ul per 100ul media, Invitrogen) and .5ug plasmid DNA ((4RTK-luc) [118] reporter, SBE4-Luc reporter [106], CAGA-Luc reporter [108] or SMAD3 overexpression plasmid [119]), media was changed and .6uM Tamoxifen was added at 4 hours, compound was added at 24 hours, and then at either 48 or 72 hours cells
were harvested with lysis buffer. For experiments using the reporter plasmids, activity was measured by Luciferase Reporter gene assay kit (Roche) and data was normalized to protein content as determined by BCA Assay (Thermo Scientific), and are expressed as fold change from vehicle treated control. For the SMAD3 overexpression experiments, cell lysates were subjected to Immunoblot or RNA purification (Qiagen), cDNA synthesis, and QPCR as described below.

**siRNA studies**

T6PNE cells were transfected with Lipofectamine RNAiMax Reagent (Invitrogen), OptiMEM, and 8 mMoles siRNA. Tamoxifen was added at 24 hours, compound was added at 48 hours, and then at 96 hours the cells were harvested for RNA isolation (Qiagen) cDNA synthesis, and QPCR as described below. Applied Biosystems Silencer Select siRNA's were used (s8402 SMAD3, s8398 SMAD2). Scrambled was a mix of 48 non targeting siRNAs

**RT PCR analysis**

Following RNA Purification (Qiagen) and cDNA synthesis (Quanta Master Mix, MJ Research PTC-200) Quantitative PCR was performed on cDNA corresponding to 100 ng of RNA using the Opticon Real-Time System (MJ Research) and BioPioneer QPCR Super Mix. All mRNA values are normalized to 18s mRNA and are expressed as fold change from vehicle treated control.
Primers:

Insulin
FWD: CTACCTAGTGTGCGGGGAAC
REV: GCTGGTAGAGGGAGCAGATG1

Kip2
FW:GGCGATCAAGAAGCTGTCC
REV: GGGCTCTTTGGGCTCTAAAT

18S
FW:GATATGCTCATGTGGGTGTG
REV:AATCTTCTTTCAGTCGCTCCA

SMAD3
FW: GAGAAATGGGTGACAGACGGGC
REV: TTCCGATGGGACACCTGCAACC

SMAD2
FW: CCTCCAATCGCCCATTTCCCCTCT
REV: CAAAGGAGCAGCAAGCCACGCTAG

Immunoblot

Whole-cell extracts were prepared by incubation in Cell Extraction Buffer (Invitrogen) supplemented with protease/phosphatase inhibitor cocktail (Thermo Scientific). Protein (20µg) was separated on 4–20% Longlife gels (Invitrogen) and transferred to Invitrolon Membrane (Invitrogen). After overnight blocking in PBS-
Tween (PBST) with 3% milk, membrane was incubated with p38, phospho-p38, phospho-SMAD3 (#9502S), phospho-SMAD2(#310S), total SMAD3/SMAD2 (#3102) antibody (all Cell Signaling), or Loading Control HSP-90 (#610410 BD) followed by secondary antibody conjugated to horseradish peroxidase (Amersham/GE, Buckinghamshire, UK), and signal revealed by ECL (Amersham/GE). Blot Intensity was quantified using Image J as described in Gassmann et al. [120], all protein levels are normalized to HSP90 levels and expressed as fold change from vehicle treated control.

3.4 Conclusions

Chapter 3 demonstrates that the effect of antipsychotics on the insulin promoter occurs through a pathway involving SMAD3 activation, leading to enhancement of transcriptional activity at E-boxes in the insulin promoter. It has previously been reported that SMAD3 modulates insulin gene expression [110], [92], [113], although the polarity of its effects vary. The human insulin promoter does contain a full SMAD binding element (SBE, CAGAC) at position -217 to -221 bp [113], [92]. SMAD3 has been shown to interact at this SBE with the insulin promoter trans-activator PDX-1, which has an adjacent binding site at -216 bp, to activate [92] and also to inhibit [113] insulin transcription. However, there is an E-box nearby at -233 bp [69], which we found to be the promoter element where SMAD3 mediated insulin promoter activation in T6PNE occurred.

It has been previously reported that E-boxes adjacent to SBEs are essential for TGFβ mediated SMAD3 activation at promoters [121]. Also, both activation and
repression have been reported of bHLH factors by the SMAD proteins downstream of TGFβ [114], [116], [121], [122]. A consistent finding is that the effect occurs through SMAD3, similar to the pattern of SMAD activation induced by antipsychotics in T6PNE. In fact, SMAD3, and not SMAD2, has been shown to directly interact with the bHLH factor MyoD, affecting its E-box-dependent transcriptional activity in muscle cells [115].

The finding that antipsychotics activate SMAD3 is significant since the TGFβ pathway is highly associated with obesity, insulin resistance, and diabetes [123], [93], [94]. Obese individuals and type II diabetics have higher serum levels of TGFβ than normal controls, and healthy individuals with high serum TGFβ are more likely to develop Type II diabetes [123], [90], [93]. SMAD3 has been shown to play a direct role, as mice with homozygous inactivating mutations of SMAD3 show enhanced glucose tolerance, reduced adiposity, and are resistant to high fat diet-induced obesity and insulin resistance [113], [94]. However, the effects of TGFβ signaling are complex. It has been reported that SMAD3 both enhances and inhibits insulin gene expression [110], [92], and studies with transgenic models of TGFβ inhibition in mouse β-cells suggest that TGFβ signaling is required for insulin production and proper β-cell function, but studies of TGFβ overexpression lead to β-cell dysfunction and hyperglycemia [109], [113], [91].

To demonstrate our findings in T6PNE, that antipsychotics modulate the TGFβ pathway, are relevant to antipsychotic treated patients chapter 4 will correlate this mechanism identified in vitro to in vivo data generated from the brains of human patients treated with antipsychotics. It will then go on to describe our efforts to
identify the direct binding partner of antipsychotics relevant to their effects on SMAD activity. The work presented in chapter 3 demonstrates that the direct binding partner is not one of the known targets of antipsychotics. Also, since TGFβ and the drugs differentially activate SMAD2 and effect SMAD3 transcription they appear to signal to SMAD3 through distinct mechanisms and the target is therefore not part of the TGFβ receptor complex.

3.5 Acknowledgements

Chapter 3, in part, has been submitted for publication of the material as it may appear in Nature Medicine, with co-authors Suman Sundaresh and Fred Levine. The dissertation author was the primary investigator and author of this paper.
4.1 Background and Significance

The amount of data from biological research available in the public domain has increased exponentially in the past ten years [124]. Finding ways to leverage this publically available data presents opportunities to further the scope of a study inexpensively and without the need for access to precious tissues. To prove the mechanism we identified in T6PNE was relevant in primary tissue we mined the Pubmed Gene Expression Omnibus (GEO) to find gene expression studies conducted with tissue from human schizophrenics taking antipsychotics. We found a number of studies, and while none had published a link between genes altered in antipsychotic treated schizophrenics and the TGFβ pathway, we reanalyzed the data with the web based system biology tool Nextbio in light of the SAR we uncovered in T6PNE.

Nextbio has taken thousands of publically available gene expression studies and created a database of differentially expressed gene lists from those studies, each list designated as having a unique gene signature e.g., altered by a particular experimental condition or sharing a response element for a particular transcription factor. The Nextbio program compares those lists with gene lists provided by the user
and generates a statistical measure of the association between the two, expressed as a p-value calculated using a rank-based enrichment algorithm they have deemed a “running Fisher’s test” (Kupershmidt et al., 2010). As detailed in this Chapter, we obtained lists of genes altered in antipsychotic-treated patients as compared to healthy controls, and then used the Nextbio software to calculate the level of association of those lists with lists of genes that contain SMAD3 binding sites, finding that antipsychotic treatment lead to modulation of SMAD3 responsive genes in vivo. The list of genes with SMAD3 binding sites was determined by a genome-wide analysis in promoters and 3’ untranslated regions for known transcription factor regulatory motifs conducted by the Broad Institute [125].

We then moved forward with this approach to analyze SMAD3 responsiveness to antipsychotic treatment in diverse cell lines. Starting with the Connectivity Map (CMAP 2.0), a database created with gene expression data from human cells treated with drugs, including 13 antipsychotics [126], our Nextbio analysis showed differential activation of SMAD3 responsive genes by antipsychotics among the cell lines used in the CMAP 2.0. Further analysis in vitro with an array of cell lines confirmed these results, raising the possibility that the direct target of antipsychotics relevant to their effects on SMAD3 is only expressed in a subset of cell lines, and could be revealed through analysis of gene expression data from those cell lines.
4.2 Results

4.2.1 Brain tissue from antipsychotic-treated schizophrenics shows gene expression patterns consistent with activated SMAD3

To determine whether the finding that antipsychotics activated the TGFβ pathway \textit{in vitro} was relevant to effects of antipsychotics in patients, we used a bioinformatic approach, taking advantage of published transcriptome data from human schizophrenics treated with antipsychotics. In 2008, Mudge et al. used RNA-Seq technology to study the pattern of gene expression in the brains of 14 schizophrenics treated with antipsychotics and 6 healthy controls. They concluded that genes involved in vesicle-mediated transport and Golgi function were differentially expressed in the schizophrenic samples when compared to the healthy controls [127]. We utilized this same dataset and the web-based systems biology software NextBio to reanalyze their data with a focus on the effect of specific antipsychotics on SMAD-responsive genes.

NextBio is a database comprising lists of genes sharing a common property. It compares those lists with gene lists provided by the user and generates a statistical measure of the association between the two lists, expressed as a p-value calculated using their “running Fisher’s test” algorithm [128]. To validate the NextBio algorithm with the Mudge et al., dataset we analyzed a list comprising genes altered in all the antipsychotic-treated patients as compared to the healthy, untreated controls. As to be expected, the Neurotransmitter Receptor Activity gene list was associated with the genes altered in the antipsychotic treated schizophrenics (Table 2, p=0.023). The
Golgi Apparatus and Vesicle-Mediated Transport gene lists were also associated to a statistically significant degree (Table 2, p=0.00037 and 0.0089, respectively), consistent with the conclusions in Mudge et al [127].

Table 2. Antipsychotics alter SMAD3 responsive genes in Mudge et al. dataset.
Listed in the table are p-values calculated by the Nextbio algorithm for the significance of the association between the Experimental/Random Gene list (columns) and the Biogroup gene lists (rows).

<table>
<thead>
<tr>
<th></th>
<th>Genes Altered in AP Treated Patients (1291 genes)</th>
<th>Random Gene Lists (1291 genes)</th>
<th>Genes Altered in Patients Taking APs Most Potent in T6PNE (819 genes)</th>
<th>Random Gene Lists (819 genes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golgi Apparatus</td>
<td>0.00037</td>
<td>0.19</td>
<td>0.11</td>
<td>0.25</td>
</tr>
<tr>
<td>Post-Golgi Vesicle-Mediated Transport</td>
<td>0.0089</td>
<td>0.38</td>
<td>0.03</td>
<td>0.79</td>
</tr>
<tr>
<td>Neurotransmitter Receptor Activity</td>
<td>0.023</td>
<td>0.14</td>
<td>0.019</td>
<td>0.12</td>
</tr>
<tr>
<td>Gene Promoters with SMAD1 Binding Sites</td>
<td>0.11</td>
<td>0.16</td>
<td>0.18</td>
<td>0.28</td>
</tr>
<tr>
<td>Gene Promoters with SMAD3 Binding Sites</td>
<td>0.054</td>
<td>0.28</td>
<td>0.0096</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Next, we examined the Mudge et al., dataset for an association between genes altered in the brains of schizophrenics treated with antipsychotics and genes which contain SMAD binding sites in their promoter as determined by a genome-wide analysis of transcription factor regulatory motifs [125]. When the data from all patients were considered together, the significance of the association between the list of genes altered in antipsychotic treated patients and the list of genes with SMAD3 binding sites in their promoters was marginal, but greater than a randomly generated
list of genes of the same size \( (p=0.054 \text{ vs } 0.28, \text{ Table 2}) \). However, when we restricted the analysis to the patients taking the four antipsychotics (chlorpromazine, haloperidol, risperidone, and thioridazine) that were most active in the insulin promoter and SMAD reporter assays (Fig. 5B, 10B), there was a highly significant association \( (p=0.0096, \text{ Table 2}) \). To test the specificity of the association between our \textit{in vitro} SAR and the \textit{in vivo} effects of the drugs on genes containing SMAD3 sites in their promoters, we examined the effect of antipsychotics on genes containing SMAD1 sites, finding no association regardless of whether all antipsychotics or the 4 most potent in the \textit{in vitro} assays were considered \( (p=0.11 \text{ and } 0.18 \text{ respectively, Table 2}) \). This is important since, while SMAD3 and SMAD1 are highly related, SMAD1 is an effector of BMP and not TGF\( \beta \) signaling\[129\].

To test further whether the activation of SMAD3 by antipsychotics was clinically relevant, we applied this analysis to a second set of gene expression data from the brains of schizophrenics treated with antipsychotics compared to non-schizophrenics\[130\]. In this study, using microarrays rather than RNA-Seq, they found that the number of altered genes declined almost to zero with increasing duration of illness. Focusing therefore on patients with shorter durations of illness, we found an increase in the statistical significance of the association between the genes with SMAD3 binding sites and the genes altered by antipsychotics compared with random gene lists \( (0.071 \text{ vs } 0.26 \text{ Table 3}) \). This increase was not found with SMAD1 \( (0.18 \text{ vs. } 0.12 \text{ for the random gene lists, Table 3}) \). The association with SMAD3-responsive genes increased when only the antipsychotics that were most potent in vitro were considered \( (0.043 \text{ vs. } 0.071 \text{ for the entire group of drugs, Table 3}) \). Once again,
there was no statically significant association with SMAD1-responsive genes (p=.16 vs. 0.23 for random gene lists, Table 3).

Having shown in two independent sets of gene expression data from antipsychotic-treated patients a correlation between the most active drugs in vitro with effects on SMAD3-responsive genes in vivo, we increased the granularity of the study by analyzing each antipsychotic individually, calculating the statistical association between the genes altered in each antipsychotic treated patient brain with SMAD3 or SMAD1-responsive genes. The values for each antipsychotic were averaged and plotted against the data from the SMAD reporter SAR (Fig. 10B). For SMAD3-regulated genes (Fig. 19A), the correlation of these two parameters was highly significant, with a Pearson correlation coefficient of 0.89 (p=.0013 ), while for

| Table 3. Antipsychotics alter SMAD3 responsive genes in Narayan et al. dataset. Listed in the table are p-values calculated by the Nextbio algorithm for the significance of the association between the Experimental/Random Gene list (columns) and the Biogroup gene lists (rows). |
|---|---|---|---|
| Gene Promoters with SMAD1 Binding Sites | 0.18 | 0.12 | 0.16 | 0.23 |
| Gene Promoters with SMAD3 Binding Sites | 0.071 | 0.26 | 0.043 | 0.30 |
Figure 19. Antipsychotics modulate SMAD3-responsive genes in brains from Antipsychotic-treated patients. (A,B) SBE4 SMAD reporter SAR data in T6PNE (Y-axis, Figure 2C) correlates with the magnitude of the association between genes altered in human brains of antipsychotic treated Schizophrenics and genes containing SMAD3 (A) or SMAD1 (B) binding sites in their promoters (X-axis) for each antipsychotic. Data from Mudge et al. are in black and data from Narayan et al. are in red. For A, correlation coefficient=$0.89$ (p=$0.0013$) and for B, correlation coefficient=$0.42$ (p=$0.24$)
SMAD1 (Fig. 19B) there was poor correlation (Pearson correlation coefficient (r) = .42, p = .24). The striking and highly significant correlation between the insulin promoter and SMAD reporter SAR data generated in vitro in T6PNE cells and transcriptome data generated by two independent groups in schizophrenic patient samples provides strong evidence that our finding of TGFβ pathway activation by antipsychotics is clinically relevant.

### 4.2.2 Antipsychotics activate the TGFβ pathway in only a subset of cell lines

Since antipsychotics and TGFβ appear to act through distinct pathways that converge on SMAD3, we speculated there might be cell lines in which antipsychotics and TGFβ differ in their ability to activate SMAD3, due to differential expression of proteins that act in the distinct pathways. If such cell lines could be found, they would be of great value in identifying pathway-specific components, facilitating the design of novel, non-diabetogenic, antipsychotics. Thus, we examined published data on patterns of gene expression in cell lines treated with antipsychotics in vitro.

The Broad Connectivity Map (CMAP 2.0) is a database consisting of gene expression data from three cultured human cells treated with bioactive small molecules, including 13 antipsychotics. [126]. Using this dataset, we performed the same Nextbio analysis used previously for each of the 3 cell lines, calculating the significance of the association between genes altered by treatment with a specific antipsychotic and genes with SMAD3 binding sites in their promoters (a measure of whether antipsychotics are modulating SMAD3-responsive genes) and plotting this by the in vitro SMAD reporter SAR (Fig. 20A-C). No correlation was seen for the
leukemic cell line HL-60 (Fig. 20B, Pearson correlation coefficient (r)=-.19 p=.53) or breast cancer line MCF7

Figure 20. Antipsychotics modulate SMAD3-responsive genes a subset of cell lines. (A-C). Same analysis used in Figure 19 was performed on data from the Broad Connectivity Map (CMAP 2.0) compound database. The SBE4 reporter SAR (Figure 5C) was correlated with SMAD3 responsiveness in PC3 cells (r=.90, p=.00028) (A), but not MCF7 cells (r=-.16, p=.60) (C) or HL-60 cells (r=-.19 p=.53) (B). MCF7 (Fig 20C, r=-.16, p=.60), but a significant correlation was seen for the prostate cancer line PC3 (20A, r=.90, p=.00028).
(Fig 5E, Pearson correlation coefficient (r)= -0.16, p=0.60), but a significant correlation was seen for the prostate cancer line PC3 (Fig. 5C, Pearson correlation coefficient (r)=0.90, p=0.00028).

The statistical analysis predicts that in PC3, but not MCF7 or HL-60 cells, antipsychotics should activate the SBE4 SMAD reporter. This proved true, ethopropazine activated the SMAD-responsive SBE4 reporter in PC3 but not in MCF7 cells (Fig. 21). However, TGFβ1 exhibited the same pattern, suggesting that the defect in MCF7 was in a pathway leading to SMAD3 activation shared by antipsychotics and TGFβ1 (Fig. 21). MCF7 has been reported to be unresponsive to TGFβ1 due to the direct binding and inhibition of SMAD3 by the intracellular domain of Notch4 [131].

To determine whether cell lines with defects in a pathway specific to antipsychotic-mediated SMAD3 activation existed, we studied a panel of 7 other cell lines. Similar to T6PNE and PC3, both ethopropazine and TGFβ1 activated the SMAD reporter in the melanoma cell line LU-1205 (Fig. 21). Similar to MCF7, WM35 was refractory to both ethopropazine and TGFβ1 (Fig. 21). However, in HepG2, H157, Panc-1, and Hela cells, TGFβ1 potently activated the SMAD reporter but antipsychotics were completely inactive, suggesting that a component unique to the pathway linking antipsychotics to SMAD3 activation is defective in those cell lines (Fig. 21).
Figure 21. Antipsychotics activate an R-SMAD reporter in only a subset of cell lines. Ethopropazine increased activity on the SBE4 reporter in PC3, LU1205, and T6PNE, but not MCF7, WM35, HepG2, H157, Panc-1, or Hela cells. (n≥3), Error bars are SEM. * indicates p<0.05 relative to DMSO control.
4.3 Methods

Cell culture

T6PNE was maintained in RPMI-5.5 mM glucose supplemented with 10% fetal bovine serum and grown at 5% CO₂, 37°C. To induce E47 activity in cells under experimental conditions, 0.6 or 1uM Tamoxifen (Sigma) was added to culture media. PC3 Cells were cultured in F-12 media with 10% FBS, at 5% CO₂, 37°C. MCF7, HepG2, Panc-1, H157, WM35, LU 1205, and Hela Cells were cultured in DMEM with 10% fetal bovine serum and grown at 5% CO₂, 37°C.

Compounds

Ethopropazine was purchased from Sigma, TGFβ1, from Thermo Scientific. Ethopropazine was used 20 mM, the concentration at which dose-response analysis revealed maximum activity on the human insulin promoter-eGFP transgene was evident without substantial cytotoxicity (Suppl. Fig. 1). TGFβ1 was used at 10 ng/ml.

Transfection studies

T6PNE cells were transfected in serum free media with Lipofectamine 2000 (1.2ul per 100ul media, Invitrogen) and .5ug SBE4-Luc reporter [106], media was changed and .6uM Tamoxifen was added at 4 hours, compound was added at 24 hours, and then at either 48 hours cells were harvested with lysis buffer. Luciferase activity was measured by Luciferase Reporter gene assay kit (Roche) and data was normalized
to protein content determined by BCA Assay (Thermo Scientific), and are expressed as fold change from vehicle treated control.

**Nextbio analysis**

The gene expression data used from Mudge et al. [127], was downloaded from GEO (GSE12297), and a list of differentially expressed genes was created by calculating the fold change of the average gene expression value for all 14 treated subjects, or that for those taking the 4 most potent antipsychotics, from the average value for that gene in the 6 control subjects. The two lists were then filtered with a +/- 1.8X fold change cutoff, and 5 random gene lists were created of the same size as each of the two experimental gene lists. This was done using a function in the statistical software R which samples the given number of genes from the NCGR Human RefSeq 7 08 platform used in the original experiment and assigns a random fold change between -5 and 5 to each. All genes lists (random and experimental) were then loaded into Nextbio, allowing its “Running-Fisher” algorithm [128] to generate the p-values shown in Table 2 for the significance of the association between our uploaded gene lists and the Nextbio gene lists (SMAD3 Binding Sites, SMAD1 Binding Sites, Neurotransmitter Receptor Activity, Golgi Apparatus, Vesicle-Mediated Transport). The p-values used from Nextbio in all instances are the minimum p-value across that calculated for the up-regulated genes, down-regulated genes and both lists considered together. The p-values listed for the random gene lists in Table 2 are the average of the p-values across the 5 random gene lists used for each experimental list.
The data plotted on the x-axis in figure 5 were produced by calculating the fold change of the gene expression data from each of the 14 patient samples considered individually from the average of the 6 control samples, creating 14 lists of differentially expressed genes. These lists were then filtered with a +/-1.8X fold cutoff and 5 random gene lists of the same size as each of the 14 experimental gene lists were created. All genes lists (random and experimental) were then analyzed by the Nextbio algorithm, generating a p-value for each individual gene list. The p-value was -log transformed to normally distribute and equalize the variance between the treatment groups and then normalized to the average of the –log transformed p-values for the 5 random gene list associated with that individual. This value was then averaged across each group of individuals taking the same antipsychotic.

For Narayan et al. [130] data was downloaded from GEO (GSE21138), and analyzed with RMA algorithm (quantile normalization) to generate log2 expression value for each array. The quality controls of the hybridization for each array were assessed by the percent present calls and GAPDH 3’/5’ ratios. Hierarchical clustering methods were next performed based on the gene expression patterns for those patients taking the same antipsychotic and the results, combined with the QC results, lead to the removal of two outliers and a batch effect adjustment, which is consistent with the data processing conducted in Narayan et al. Of the remaining 57 samples, only the short duration of illness samples were used because the low number of differentially expressed genes among the individuals with longer durations of illness precluded analysis. This left 8 patients and 8 age and sex matched healthy controls. These were then treated identically to the data from Mudge et al. except fold change was
calculated for each patient compared to its matched control, not the average across all controls, for the data in Figure 10.

### 4.4 Conclusions

Chapter 3 began by extending the mechanism identified *in vitro* in chapter 2 to tissue from human patients by utilizing publically available expression data. Gene expression datasets from the brains of antipsychotic-treated patients and normal controls were analyzed using the NextBio systems biology software [128]. Analyzing the data while taking into consideration the SAR of antipsychotic effects on the SMAD reporter revealed a highly significant correlation between the effects of particular antipsychotics on SMAD3-responsive genes in patients and their effect on SMAD and insulin promoter activity *in vitro* (Fig. 10A). This finding confirms the effects of antipsychotics in T6PNE are relevant in primary tissue and could in fact play a role in the metabolic side effects of the drugs.

Because of its importance to the impact of our findings, significant effort was made part to validate the Nextbio approach since we were applying the previously reported Nextbio algorithm to answer a new, more specific question [128]. The Gene Ontology groups reported as associated with the data in Mudge et al., were found to significantly associate in our Nextbio analysis (Table 2), and random gene lists were used to normalize all associations found with experimental lists (Table 2, Figure 10). We also used two distinct datasets to confirm our findings, the data from Mudge et al., was generated with RNA-seq technology and loaded into Nextbio significantly less
processed than the Microarray-based gene expression data from Narayan et al., which was processed as detailed in the methods section of this chapter.

The ultimate goal of this work is to facilitate the design of novel antipsychotics that retain their clinical benefits while inducing fewer deleterious metabolic side effects. Such drug design efforts would be significantly aided by the discovery of the direct target of antipsychotics responsible for signaling to SMAD3, which itself should be aided by the identification of cell lines in this chapter that differ in their responsiveness to SMAD3. Through the use of gene expression data from those cell lines, candidate genes can be identified that may be required for antipsychotic-mediated SMAD3 activation.

4.5 Acknowledgements

Chapter 4, in part, has been submitted for publication of the material as it may appear in Nature Medicine, with co-authors Suman Sundaresh and Fred Levine. The dissertation author was the primary investigator and author of this paper.
CHAPTER 5:

THERAPEUTIC IMPLICATIONS AND FUTURE DIRECTIONS

The major finding presented in this dissertation is that antipsychotics activate SMAD3, a downstream effector of TGFβ signaling, through a non-canonical pathway that involves neither the TGFβ receptor complex nor the neurotransmitter receptors that are believed to be responsible for the therapeutic effects of antipsychotics.

We identified the TGFβ pathway as being responsible for the effect of antipsychotics on the insulin promoter through a counterscreen of a kinase inhibitor library, detailed in chapter 3 (Fig. 5A,B). The screen revealed that antipsychotics were epistatic to a TGFβ type I receptor kinase inhibitor, demonstrating that the effect of antipsychotics on the TGFβ pathway occurred downstream and independently of TGFβ type I receptors. The other 9 compounds from the counterscreen that inhibited the insulin promoter were active both in the presence and absence of Ethopropazine, so they could be downstream of antipsychotics in a pathway that exhibits some level of basal activity, or they could be signaling in an unrelated pathway. Unfortunately, none of them, including inhibitors of AKT, casein kinase, PIM1K, ERK, and p38, provides a strong clue as to the direct antipsychotic target.
Our data support a model in which antipsychotics and TGFβ signal to SMAD3 through independent pathways that converge at some point to activate SMAD3. Support for this comes from the fact that TGFβ, but not antipsychotics, activate SMAD2 by phosphorylation (Fig. 11B), and repress SMAD3 gene expression (Fig. 13B). The existence of cell lines showing potent SMAD activation by TGFβ1 that were unresponsive to antipsychotics also indicates differences in the pathways acted on by TGFβ and antipsychotics (Fig. 21).

Our finding that antipsychotics activate SMAD3 is significant because, as detailed previously, metabolic side effects, including obesity, insulin resistance, and type II diabetes, are both a major clinical issue with that class of drugs and highly associated with SMAD3 [28], [20], [24]. Previously presented mechanisms for the metabolic effects of antipsychotics are diverse [23], [24], [27] [21] [13], but the majority center around the CNS effects of antipsychotics leading to increased appetite and weight gain [27], [13].

To a possibly greater extent than the neurotransmitter targets that are thought to be largely responsible for both the metabolic side effects and therapeutic benefits of antipsychotics, the TGFβ pathway, as detailed in the conclusions of chapter 3, is highly associated with obesity, insulin resistance, and diabetes. While serum levels of TGFβ have been correlated with increased risks for type II diabetes and insulin resistance, SMAD3 transgenic animals have defined a role for the TGFβ pathway in insulin resistance and obesity as well. So, whether or not the weight gain associated with antipsychotic use is the primary factor behind the metabolic side effects of the
drugs, designing antipsychotics that do not modulate SMAD3 activity has the potential to alleviate those side effects.

*In vivo* relevance of SMAD activation by antipsychotics came from analysis of two independent gene expression datasets from the brains of antipsychotic-treated patients and normal controls (Fig 19A)[127],[130]. In both, antipsychotics were analyzed as a group, and neither identified TGFβ signaling as being affected by antipsychotics. However, performing the analysis in light of the SAR of antipsychotic effects on the SMAD reporter produced a dramatically different picture, revealing a highly significant correlation between the effects of particular antipsychotics on SMAD3-responsive genes in patients and their effect on SMAD and insulin promoter activity *in vitro*.

Efforts to extend our *in vitro* antipsychotic SAR to the propensity of a given antipsychotic to cause metabolic side effects were complicated by inconsistencies in the literature on this issue [23], [24], [25], [26], [27], [21]. Studies showing small perturbations of TGFβ signaling intensity to have large physiological effects [132], [109], indicate that even antipsychotics that are at the low end of our SAR could cause substantial metabolic side effects when administered over time. Of note, there was no pattern in the insulin promoter or SMAD reporter SAR in terms of whether a drug was typical or atypical. However, two antipsychotics, molindone and ziprasidone, that have consistently been found to have a weak or no association with gain and diabetes in patients [32], [36], [37], were low or inactive in the insulin promoter and SMAD reporter assays. This strongly suggests that the activity of antipsychotics on the TGFβ
pathway is a significant factor in causing the deleterious metabolic side effects of the drugs.

The structural specificity of antipsychotics for SMAD3 activation, combined with the data on the differential responsiveness of cell lines to antipsychotics, implies that the effects of antipsychotics are mediated through a specific molecular target that is affected by structural features of particular antipsychotics. The experiments conducted in chapter 3, which show neurotransmitter receptor targets of the antipsychotics are unrelated to their modulation of the TGFβ pathway, indicate that the direct target is not related to the beneficial neurological effects of antipsychotics. This raises the possibility that antipsychotics could be designed that retain a favorable profile of activity on the neurotransmitter receptors that are responsible for their clinical benefit, while lacking effect on the target that activates SMAD3. Such drug design efforts would be aided by the identification of the direct target of antipsychotics responsible for signaling to SMAD3.

Having cell lines that both respond and lack responsiveness to antipsychotics in terms of SMAD3 activation (Fig. 21) allows for the identification of candidate genes for the direct target through comparisons of gene expression data in the cell lines. We have taken this approach, and through the use of gene expression data accessed through Pubmed GEO, created a list of differentially expressed genes between the responsive and non-responsive cell lines (present in T6PNE, PC3, Lu1205, and absent in Hela, Panc-1, H157, HepG2). Since a large portion of that list was GPCRs, and antipsychotics are known to bind multiple GPCRs [11] [133], which have precedence for signaling to SMAD3 and other kinases known to phosphorylate
future experiments will be designed to test the hypothesis that antipsychotics are signaling to SMAD3 through a GPCR only expressed in a subset of cell types.

5.1 Acknowledgements

Chapter 5, in part, has been submitted for publication of the material as it may appear in Nature Medicine, with co-authors Suman Sundaresh and Fred Levine. The dissertation author was the primary investigator and author of this paper.
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