Altered MicroRNA Expression in Response to Vinclozolin Exposure

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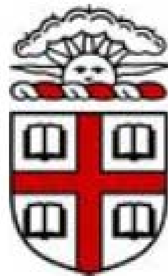

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Executive Summary

In recent decades, infertility has been rising, particularly in industrialized nations. At least 50% of infertility cases are due to male factors, such as lowered sperm counts and quality and cryptorchidism. It is thought that these symptoms, as well as others like testicular cancer, may share a common etiology; late-onset diseases may be the result of early exposure. This rise in infertility is correlated with an increased load of chemicals in the environment, particularly "endocrine disruptors," chemicals which mimic endogenous compounds and bind to endocrine receptors. The altered phenotypes leading could result from environmental exposures to pesticides and other endocrine-disrupting chemicals.

Exposure to one anti-androgenic compound, vinclozolin, a fungicide used in viticulture, produces anti-androgenic effects (lowered sperm counts, lowered sperm motility, incomplete tight junctions between Sertoli cells) for four generations in males, at a frequency high enough to suggest an epigenetic mechanism of action. To assess what, if any, role microRNAs (miRNAs) may play in mediating the effects of vinclozolin, two testicular cell lines from mice (TM3—Leydig, TM4—Sertoli) were cultured and exposed to vinclozolin or a control medium. Subsequently the cells were lysed, the total RNA was extracted and samples were sent for miRNA microarray analysis.

No miRNAs differed significantly in expression between treatment and controls in the TM3s, while the TM4s showed differential expression of 11 microRNAs. This suggests, perhaps, that vinclozolin targets the Sertoli cells in particular, and implies that miRNAs may be involved in eliciting the anti-androgenic effects.

Chapter I: Introduction

The environmental consequences associated with the modern lifestyle are receiving increased attention, both from the media and from researchers.

Many of these environmental burdens affect people directly: in recent decades, the industrialized world has seen a dramatic increase in the incidence of infertility, as well as reproductive tract cancers and developmental disorders. These phenomena, noticed as early as 1992 by Carlsen, et al (1993), have since become the focus of toxicological research aiming to determine whether the recent emergence of these problems, in conjunction with their widespread prevalence, could result from environmental exposures. Fittingly, there are high degrees of variation across countries and even between regions of the same countries, with more industrialized areas showing higher rates of infertility.

Nearly half of all cases of infertility result from male factors, such as low sperm count or poor sperm motility (Irvine, 1998). These, and other symptoms of demasculinization or femininization, are known to be caused by a variety of factors including environmental exposures. Heavy metals, such as lead and cadmium, can damage the chromosomes of sperm and cause testicular necrosis (Stine and Brown, 2006: 126). While heavy metal pollution is a concern, particularly in industrial or former industrial areas, a great deal of attention is now focused on organic pollutants. These toxicants are numerous and their uses varied: phthalates, used as plasticizing agents, seem to decrease the anogenital distance in baby boys (Swan, et al, 2005), while the various classes of pesticides and fungicides (organochlorine, organophosphate, dicarboximide) can lead to a cascade of effects including cleft phallus and hypospadias (Ostby, et al,

1999: 48). These symptoms seem to be linked with others, including testicular cancer and lowered sperm counts; it is common for the symptoms to be concurrent. It has been proposed that they may all be manifestations of an underlying condition, called testicular dysgenesis syndrome. The testicular dysgenesis syndrome (TDS) hypothesis attributes the rise of these symptoms to lifestyle changes and environmental exposures, though the etiology is not fully understood. The disease, even late-onset diseases like cancer, likely results from fetal origins. Epidemiological evidence has linked TDS, particularly hypospadias and cryptorchidism, with exposures to pesticides and industrial chemicals in several countries studied (Bay et al, 2006).

Environmental Exposures: Organic Pesticides

Due to their lipophilic nature and high degree of chemical stability, organic pesticides can accumulate in fat deposits and fat-rich organs. This may mean that they could bio-accumulate up the food chain. The organic pesticides can also enter breast milk, in turn causing neonates, whose systems are largely under-developed and vulnerable, to be exposed (Pant, et al, 2007: 135). Chemicals such as polychlorintated biphenyls (PCBs), pesticides, PCDDs, PBDEs, metals, and solvents have been found in breast milk (Soloman and Weiss, 2002).

In many cases, these organic compounds exhibit structures similar to hormones of the endocrine system, allowing them to mimic endogenous compounds. Endocrine disruptors bind to receptors and either act as hormone agonists or antagonists while preventing the hormones from binding to their target receptors. Anti-androgenic effects can result from compounds that block or prevent the action of sex hormones testosterone and 5α-dihydrotestosterone, or DHT (Ostby, et al, 1999: 49). Other chemicals, such as

phthalates, bisphenol A, or diethylstilbestrol, function by mimicking estrogen and bind to estrogenic receptors. Both classes of mechanisms can lead to an altered phenotype, which may be very similar. Some toxicants can affect both receptors. This is evidenced by DDT, which has a number of metabolites, some of which function as androgenic antagonists, while others act as estrogenic agonists (Tyler, et al, 1998).

Structure and Function of the Mammalian Testis

The mammalian testes are comprised of many compacted seminiferous tubules and an interstitium. The interstitial Leydig cells produce testosterone, while the seminiferous tubules contain both germ cells and the somatic Sertoli cells. Sertoli cells undergo many morphological changes during puberty, becoming elongated, and forming tight junctions between neighboring Sertoli cells. These establish the blood-testis barrier, which creates an internal environment which serves to protect spermatozoans as they mature into sperm.

The number of Sertoli cells becomes set and the cells become mitotically inactive as they fully differentiate. Sertoli cells then provide nutrition and growth factors to the developing germ cells. A single Sertoli cell is associated with many germ cells and these germ cells develop by mitotic and meiotic divisions into sperm. It is important, then, that an individual develop an adequate amount of Sertoli cells before the onset of puberty. These cells must be healthy and able to respond to all developmental signals, such as follicle-stimulating hormone (FSH), the major endocrine regulator of Sertoli cell function.

Some toxicants can cross this barrier, or act before its full development (Stine and Brown, 2006: 125). As there has been evidence that hypospadias, cryptorchidism,

lowered sperm counts and testicular cancer all may result from events during early development, the symptoms of testicular dysgenesis syndrome may well result from prenatal or prepubertal exposures (Petersen and Söder 2006: 148). Studies in rats have linked exposures to the synthetic estrogen diethylstilbestrol to decreased numbers of Sertoli cells. In turn, this can lead to decreased sperm counts and malformations of the blood-testis barrier. Other toxicants may target the Sertoli cells directly. Phthalates, a widely-used group of plasticizing agents, seem to cause effects similar to the symptoms of TDS in rats by acting on Sertoli cells (Fisher, et al, 2003).

Exposure to one particular compound, vinclozolin, a dicarboximide fungicide used primarily in viticulture, causes a wide array of symptoms in the male reproductive tract of the rat. These symptoms, including reduced fertility, delayed puberty, decreased anogenital distance, hypospadias, cleft phallus, and even more subtle alterations in the differentiation of external genitalia, suggest that vinclozolin acts as an anti-androgen (Ostby, 1999: 49). Even when exposed *in utero* at low doses, male rats will exhibit these symptoms. These effects seem due primarily to the inhibition of DHT-induced transcriptional activity on behalf of vinclozolin’s two primary metabolites (M1 and M2). Vinclozolin has no affinity for estrogenic receptors and does not seem to affect female rats, nor does it decrease the viability of either sex (49). While ethical concerns have dictated that these tests be conducted almost exclusively in rats, human androgenic receptors bind M1 and M2 nearly the same way that rat androgenic receptors bind these metabolites. Additionally, since the androgens have a high degree of conservation of function across the mammals, similar effects should be expected (61). Thus there is reason for concern about human exposures.

Perhaps most interestingly, symptoms such as reduced fertility continue in subsequent generations. A male rat exposed to vinclozolin *in utero* during the period of sexual differentiation (between embryonic days E8 and E14) will exhibit apoptosis of spermatogenic cells (Anway, et al, 2005: 1466). This leads, in turn, to somewhat diminished sperm counts and sperm motility. These effects continue for multiple generations, being observed through the F4 generation, only passed on by males (Anway, et al 2008: 30). There do not seem to be any genotoxic mechanisms operating due to the high prevalence of the effect, a prevalence much greater than what would be expected due to mutation frequency.

Epigenetic Mechanisms and Disease

In cases such as exhibited by vinclozolin, when a toxicant does not seem to cause mutation or show genotoxicity, it has been reasonably proposed that epigenetic mechanisms underlie the mechanism of action. An area of great interest, epigenetics are implicated in the mechanisms of many environmental pollutants that lack overt genotoxicity yet exhibit a relative permanence in their effect (Anway, et al 2008: 30). Epigenetic inheritance is defined as the passing on, or inheritance, of cellular information through cell division beyond what is encoded in the DNA sequence. Most research in epigenetics so far has focused primarily on DNA methylation, histone modifications and genomic imprinting, the silencing of one allele in a parent-of-origin specific fashion. These all work to control and alter gene expression and may be transmitted to daughter cells (Feinberg and Tycko, 2004). More recently, it has been recognized that gene expression control by small non-coding RNA species also can serve as a mode of

epigenetic regulation with important implications in human health and development (Szyf, 2007).

Epigenetic alterations underlie many diseases and cancers. Methylation, the covalent addition of a methyl group to a cytosine nucleotide, provides a good example of this. All cancers are characterized by global hypomethylation of the genome causing the expression of tumor-promoting oncogenes, which would normally be methylated and leading to enhanced genomic instability (Szyf, 2007: 15). Additionally, some environmental agents, such as cigarette smoke have been linked to gene-specific hypermethylation (Marsit et al Carcinogenesis 2007, Marsit et al Int J Cancer 2005). If tumor suppressor genes are hypermethylated, as is the case in many cancers, this acts to silence their transcription, while the genomic coding sequence of these genes remains unaltered, the production of proteins is thus blocked, leading to disease.

A recent study, conducted by Anway, et al, further underscores the belief that vinclozolin acts via an epigenetic mechanism. Aside from the deleterious reproductive outcomes for male rats, vinclozolin seems to cause late-onset diseases such as breast tumors and kidney and prostate diseases, transgenerationally (30). Females may exhibit disease phenotypes, but, as with reproductive phenotypes, the effects of vinclozolin are transmitted solely by males. Using microarray analysis, Anway and colleagues aimed to study RNA expression profiles across generations, starting with male rats exposed to vinclozolin *in utero* (F1). These rats exhibited the highest number of genes expressed differentially relative to the control group, around 2000. The F2 generation saw about 1375 altered, while the F3 showed only 566 and the F4 results were inconclusive (33). Of these genes, 196 were shown to be similarly altered across the generations (35). About

90% of the genes exhibited decreased expression, or down-regulation. It seems, then, that some portion of the altered epigenome is heritable and responsible for vinclozolin's transgenerational action (37).

MicroRNA: A Possible Epigenetic Mechanism

The control of gene expression is, then, an area of interest. MicroRNAs (miRNAs) provide a possible mechanism for this epigenetic mode of gene expression control. Reported first in 1993 by Lee, et al, in *C. elegans,* microRNAs are small, non-coding RNAs typically around 22 nucleotides in length. MicroRNAs have been found in metazoans. Between species related even distantly, a given miRNA will be very similar. In comparing mice and human miRNAs, for instance, most differ by no more than a couple nucleotides. This conservation, despite millennia of evolution, suggests that the miRNAs perform important functions, and did so for a distant common ancestor.

These miRNA function through a complicated series of interactions, with a long precursor gene transcript first folding into a hairpin loop of double-stranded RNA (dsRNA). The protein complex Dicer then acts to help process this miRNA precursor, yielding a single strand. The miRNA then associates with the RISC complex of proteins, which serves to help mediate the binding between an miRNA and a partially complementary mRNA transcript. This binding results in either the translational repression of the mRNA or its direct degradation, if there is direct complementarity. Both of these processes allow miRNAs to silence the translation of mRNA, preventing the expression of particular genes. The requirement of only partial complementarity allows a single miRNA to bind with and regulate hundreds of cognate genes. MicroRNA function catalytically, since they are not consumed by their reactions. Thus, once

transcribed and processed, a miRNA may degrade many copies of several different transcripts (Bartel, 2004).

MicroRNAs have been found to play a critical regulatory role in protein expression (Bartel and Chen, 2004). It seems that different developmental stages may have distinct miRNA expression patterns (Bartel, 2004), and this may be true of all metazoan cell types. The recent discovery of miRNAs means that their functions are only now beginning to be studied. Aside from developmental roles, many miRNAs are associated with cancers (Harfe, 2005). Particular tumors have been shown to be characterized by altered miRNA expression profiles. Their distribution in the genome appears to be non-random; many microRNAs are located near genomic regions targeted for alteration in cancer, such as repetitive regions, fragile sites, and viral integration sites (Calin, et al, 2004).

It is yet largely unknown exactly how miRNA are regulated and how environmental agents can alter the expression of miRNAs. Recent studies have started to analyze the effects of various exposures. For instance, gamma radiation does not appear to alter miRNA expression, while both folate deficiency and exposure to arsenic were shown to globally increase miRNA expression (Marsit, et al, 2006). Specific miRNA also appear to be expressed in response to inflammatory signals (O'Connell RM et al, PNAS 2007; Moschos SA et al BMC Genomics 2007), tamoxifen exposure (Pogribny Ip et al Mutat Res 2007), ethanol exposure (Sathyan P et al J Neuroscience 2007), and chemotherapeutics (Rossi L et al Pharmacol Res 2007). Since vinclozolin has been demonstrated to exert its toxicity via an epigenetic pathway, our experiment is part of the emerging field of "environmental epigenetics," a term coined by Stella Reamon-Buttler

(2008). To examine if the endocrine disrupting effects of vinclozolin could be due to alterations in microRNA expression, our *in vitro* study looked at two *Mus musculus* testicular cell lines: Sertoli cells (TM-4) and Leydig cells (TM-3). These cells, as described above, are the somatic cells responsible for sperm development and secondary sexual characteristics. By altering microRNA expression profiles, vinclozolin could silence particular genes, leading to the observed phenotypes typical of anti-androgens and estrogen imitators.

Chapter II: Materials and Methods

Cells of both lines (TM3 and TM4) were obtained from American Type Culture Collection (ATCC) and were grown in Dulbecco's Modified Eagle Medium with F12, further supplemented with fetal bovine serum, horse serum and a penicillin/streptomycin mixture. Vinclozolin doses were suspended in DMSO and added into this medium. Dosages of 0µM (containing DMSO, however, for use as a control), 100µM, 200µM, 300µM and 400µM of vinclozolin were determined based on results of a 2005 study which cultured similar cell lines in vinclozolin to assess its carcinogenicity (Wu, et al, 2005). The range of doses used in the Wu, et al study were chosen because they were determined to be physiologically relevant.

Assays: Colony Formation

Two assays were conducted to study the dosage range. First, cells were studied in a colony formation assay. In this study, approximately 1,000 cells were exposed for 24 hours, after which the vinclozolin-containing medium was removed. Cells were then left in normal DMEM/F12 medium for approximately a week, after which point the medium was removed and the dishes were stained to show the colonies which had formed over the course of the week. From this first experiment, the 400 µM dose was eliminated, as too few colonies formed.

Trypan Blue Assay

Next the trypan blue assay was conducted with the TM3 cells to confirm the dosage range and identify a single dose to be used in the final experiment. Cells were seeded in 6-well plates with 100,000 cells per well. After a day of growth in normal

medium, the cells were given medium containing vinclozolin. There were three groups of cells, 24-, 72- and 120-hour exposures. In the first group of cells, the vinclozolin-containing was left on for 24 hours, then removed. In the other treatment groups, we replaced the medium and left it on for two more days. Triplicates of each dose (0, 100, 200 and 300μM) were used, totaling 12 wells of cells for each day group. When each treatment period ended, the medium was removed, and cells splashed with trypsin to remove them from the wells. Cells were stained with the trypan blue and counted using a hemacytometer to see both how many had grown and died in each dose group based on a trypan blue exclusion assay.

The use of the trypan blue stain enabled differentiation between living and dead cells, though, overall, few dead cells were observed, suggesting that vinclozolin did not kill cells but rather retarded, or limited, their growth. The 100μM dose did not seem to have much effect on either cell line and showed results similar to the control, while 300 was effective at preventing growth. Furthermore, both increasing doses and lengths of exposure proved to be more effective at preventing growth. The trypan assay was repeated in TM4s, with only one well for each dose and length of treatment. Results were similar to those seen in TM3s. On the basis of the results of the colony formation and the trypan blue assay, we decided on a dose of 250μM for the final experiment.

Sustained Exposure Growth Experiment

The final experiment began by plating cells in 75mL flasks. The medium was changed every two days, with the first exposure (Day 0) occurring one day after the cells were plated. The media was changed and replaced on Days 2 and 4. On Day 6, the

medium was removed from the plates and the cells were washed with PBS before the total RNA was collected from each plate.

RNA Collection and Processing

RNA collection was performed using the Ambion miRvana kit (Austin, Tex) following the manufacturer's protocol for total RNA isolation. This protocol involved several steps; ultimately, cells were lysed and the total RNA was collected and washed to remove other macromolecules such as DNA. Following elution, the final step of the miRvana process, the RNA was tested on the NanoDrop to analyze its concentration and absorbance. This provided a degree of quality control. An Agilent Bioanalyzer analysis was also conducted to further verify the quality of the RNA. Using information about the concentrations obtained from the NanoDrop tests, 5μg aliquots of the total RNA were sent out to Invitrogen's lab, where the miRNA expression profile was analyzed.

miRNA Microarray Processing

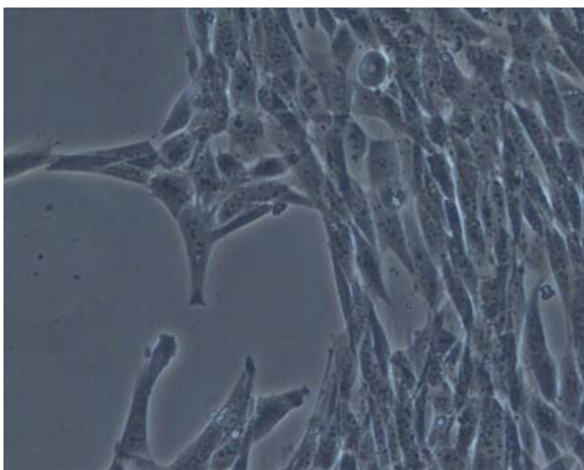
At Invitrogen (Carlsbad, Calif.), samples were processed using the NCode Rapid miRNA Expression Profiling Service. The triplicate samples for each cell line were tailed with Poly (A) tailing reactions in accordance with the company's protocol. These were ligated to labeled DNA polymers and hybridized to an Invitrogen multi-species microarray. Scanning enabled the collection of fluorescence data. These data were returned to our lab.

Statistical Analysis

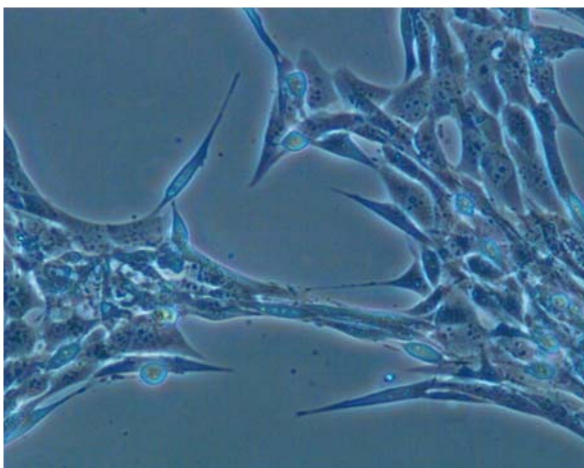
A latin squares or loop design for pairing of samples on each array was utilized, to allow for appropriate controlling of dye labeling and fluorescence efficiency, and to

allow for immediate comparison across treatments (Kerr, et al, 2000). This model-based analysis allows for normalization and differential marker detection simultaneously. P-values examining the difference in miRNA expression between treated and control groups were calculated through bootstrapping of the residuals of the model. To control for the multiple comparisons performed in each analysis, false discovery rate (FDR) analysis was applied to these P-values, following the methods of (Benjamini Y and Hochberg Y. (1995) and Storey JD and Tibshirani R. (2003). FDR was implemented using the QValue software (Storey JD and Tibshirani R. (2003) and Storey JD, Taylor JE, and Siegmund D. (2004) in the R statistical package, setting the lambda range from 0 to 0.95, and the smoother methods of (Storey and Tibshirani 2003) for π_o determination. miRNA with an FDR of less than 20% (q-value <0.2) were considered statistically significant in their differential expression.

Chapter III: Results

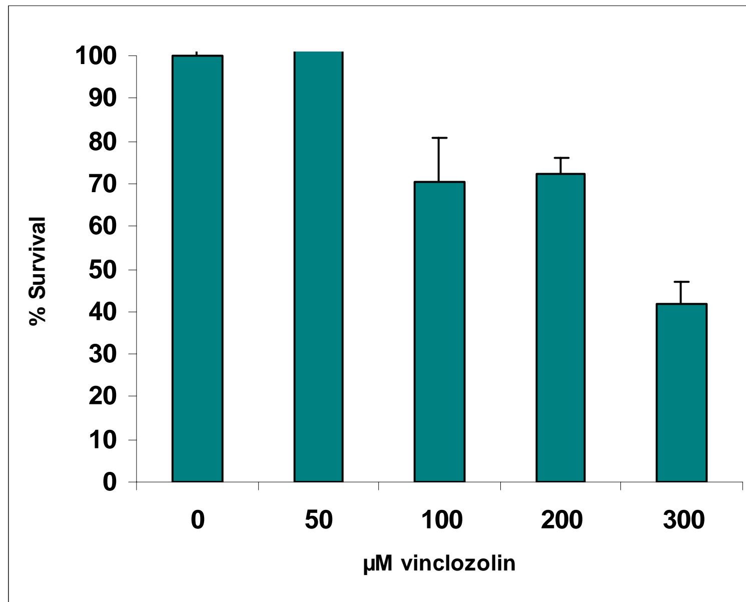


TM4 Control, Day 5

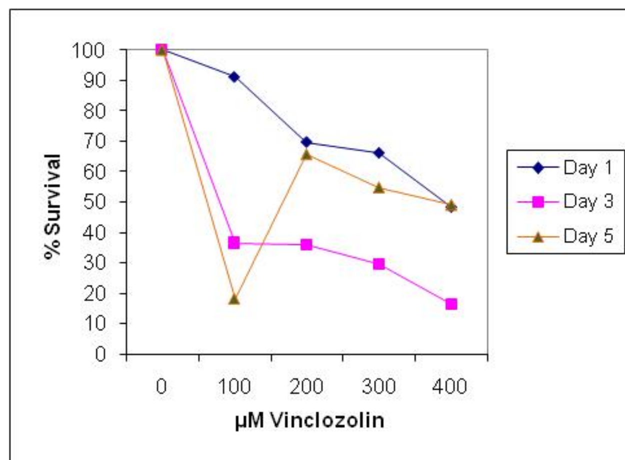


TM4 Treatment, Day 5

By the fifth day, there were noticeable differences between cells in the two dosing groups. The cells exposed to vinclozolin were more sparse. Their morphology was significantly different; many were multi-nucleated. The control cells, by comparison, looked fairly healthy and had grown to be confluent.

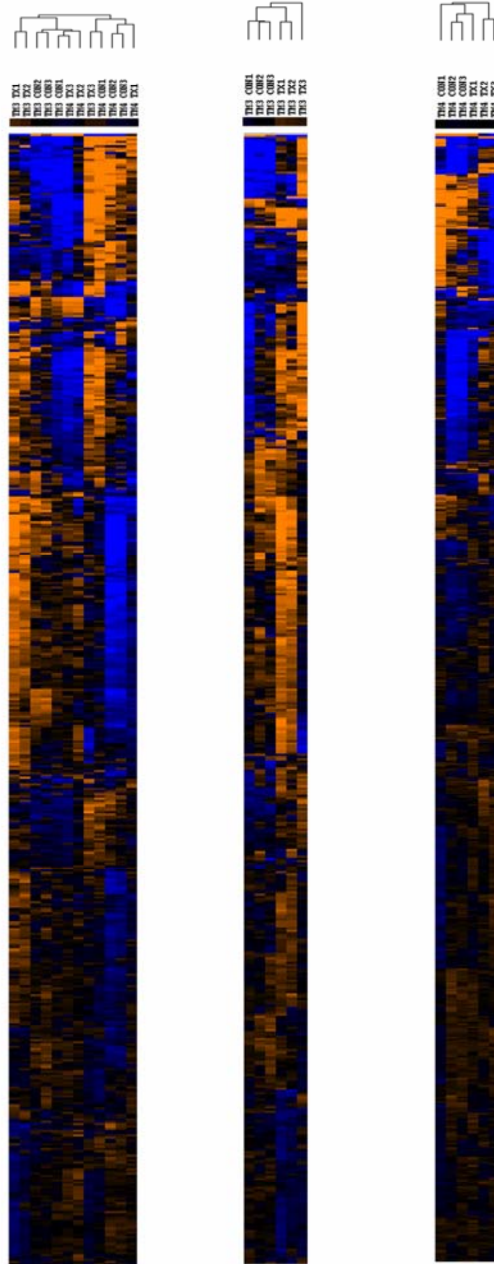


Colony formation data for TM4 colony formation. Low doses of vinclozolin did not inhibit survival, while a significant drop in survival was observed between the 200 and 300 µM groups.



Graph showing results for the trypan blue assay in TM3 cells. Increasing doses generally decreased survival more than increasing the length of exposure.

Column A



From left: Heat map for both cell lines, heat map for TM3s, heat map for TM4s.

Heat maps, showing the miRNA expression. Each column represents a sample; each row represents a microRNA. Orange represents high relative expression, while blue represents low relative expression. The phylogenetic tree diagrams at the top show how

the computer program clusters the samples, using unsupervised hierarchical clustering—the program groups samples exclusively on the basis of similarities in miRNA expression. The heat map at left shows TM3s and TM4s grouping separately, implying that the two cell types have significantly different miRNA expression profiles. Furthermore, TM4 samples will group with other TM4 samples; TM3 samples group with other TM3 samples. In the heat map for the TM3s alone (middle heat map), there is a clear separation between treatment and controls, meaning that the cells responded to treatment and control relatively differently. This is not as clear in the TM4s (right), evidenced by the more-complicated phylogenetic tree. This suggests that the response of these cells to the treatment may not have been as clear.

mmu-miR-	Fold Change	q value
21	2.732479941	0.174532
22	2.752032371	0.174532
30c	2.735858075	0.174532
92	3.115912162	0.174532
155	2.539803606	0.174532
378	-4.526295006	0.174532
92	2.943044373	0.174532
289	2.497731328	0.187951
494	3.304568075	0.174532
23a	2.523197291	0.19377
493	-3.369336984	0.174532

The specific miRNA identified as differentially expressed in TM4 are shown above in Table 1. Consistent with the clustering analysis, there were not any significant changes in miRNA expression between the control and exposed groups in TM3s. As these are Leydig cells, this could mean that the Leydig cells are relatively less susceptible to vinclozolin. In the TM4s, however, eleven miRNAs were identified as showing significantly different patterns of expression between controls and exposed. The murine

specific miRNA detected as differentially expressed were mmu-miR-21, 22, 30c, 92, 155 and 378. In addition, probes identifying the highly conserved miRNAs miR-92, miR-289, miR-494, miR-23a, and miR-493 were also detected. These miRNA exhibitied expression differences ranging from -4.5 to 3.3, demonstrating both up- and down-modulation in response to exposure.

Chapter IV: Discussion and Implications for Environmental Health

Vinclozolin Exposure Altered miRNA Expression in TM4 Cells

Our results showed a clear divide in response to vinclozolin between the two cell groups. The differences in microRNA expression in TM3s were not statistically significant comparing between treatments and controls. In TM4s, however, we observed that eleven microRNAs were differentially expressed between the dosing groups. This could suggest that vinclozolin's pathological effects could be due to the action of the chemical solely on TM4s, which are Sertoli cells. These cells are perhaps the most crucial in the development and maturation of sperm. MicroRNA-mediated interference with the Sertoli cells on the part may underlie, or at least be involved with, the altered phenotypes observed after pre-natal vinclozolin exposure.

Discussion of Specific miRNAs

In humans, miR-21 has been observed to be over-expressed in tumors. It targets the mRNA transcript that codes for the cancer suppressing protein Programmed cell death 4 (PDCD4). Ordinarily PCDC4 serves to prevent metastasis and tumor growth. This has been observed in breast cancer as well as colorectal cancer (Frankel, et al, 2008; Asangani, et al, 2008). Another miRNA with a related function is miR-378, which has been shown to promote cell survival, tumor growth and angiogenesis by targeting another suppressor protein. However, in our study, miR-21 expression more than doubled, while miR-378 was under-expressed by a factor of nearly five times. Despite studies showing their functions to be similar, we found that the two microRNAs responded differently to vinclozolin treatment.

Ibarra, et al (2007) found that miR-22 was consistently expressed in abundance in mammary precursor cells in mice. This typical function, coupled with our finding that miR-22 was expressed more than two-fold in the treated TM4s when compared with the controls, could, perhaps, be responsible for some of the symptoms of femininization that result from exposure to vinclozolin. This is, however, speculative.

Another microRNA our study identified as being over-expressed, miR-155 has been implicated to have an important role in the development of B cells and B cell leukemia. Deletions in the genes encoding miR-155 result in abnormal B cell development, while leukemia is characterized by an over-abundance of miR-155 (Calame, 2007).

Our findings indicated that miR-30c was over-expressed. This miRNA seems to be involved in the development of the excretory system in zebrafish (Wienhold, et al, 2005). Expression of miR-92 more than tripled. This miR seems to be carried on the X chromosome in humans and is found in the fetal livers of both mice and humans (Fu, et al, 2005). With the exception of mir-493, all of the newly-identified microRNAs (92, 289, 494, and 23a) were over-expressed in the controls, usually by a factor between two- and three-fold. In contrast, expression of miR-493 decreased by a factor of more than three-fold.

In light of our results, it is important to consider how applicable data from mouse cell lines may be to humans. It is reasonable to assume that vinclozolin will alter miRNA expression in human cells in a manner similar to its activity in mouse cells. Furthermore, all of the mmu-miRNAs our study identified have human homologues, some of which (miR-21, miR-155) have been studied in detail.

Moving Towards a Risk Analysis: Dose and Exposure

We must consider whether our results are obtained from a dose within a feasible range of human exposures. This is difficult for a few reasons. First, it is not always certain whether human cells will respond to a toxicant in the same way at similar doses. Human cells may not react to the same toxicants as mouse or rat cells. This is not a major concern with vinclozolin, however; our selected doses were based on a study that looked at human cells. Furthermore, vinclozolin's metabolites bind to extremely similar androgenic receptors in both mice and humans.

There is a high degree of uncertainty when trying to estimate dietary exposures. It is very difficult to monitor pesticide concentrations in foods. Methods are being developed to study, for instance, the amount of pesticides in leafy vegetables by means of gas chromatography (Gonzalez-Rodriguez, 2008). Even with more powerful detection methods, there is a wide range of variation as different farmers may use varying amounts of chemicals and crops may not always absorb the pesticides the same way. There is also a great deal of variability related to the preparation of the food prior to consumption. The thoroughness of washing, as well as any cooking steps can drastically alter the dose and potency of pesticides or contaminating toxicants ingested.

Nevertheless, it is worthwhile to estimate a range of exposures. Unfortunately, few studies have looked at vinclozolin in terms that directly estimate dietary intake. Vinclozolin is commonly used in viticulture, so some studies have looked at residual concentrations in raw wines and grape juice. A study by George Soleas and David Goldberg, published in 2000, analyzed more than 3000 wines and grape juices, finding significant, detectable amounts in only four wines and three grape juices (Soleas and

Goldberg, 2000). While this study did not take into account the primary metabolites of vinclozolin, it is consistent with their general findings: few of the wines contained any pesticides in detectable quantities. Since many wines are filtered, looking at raw wines may be a conservative estimate, looking at the higher end of human exposures. Actual exposures to pesticides in wine may be lower.

Populations at Risk?

If this study is considered to be indicative of the proportion of food products with appreciable amounts of vinclozolin, it seems that vinclozolin residues are not much of a concern. Nevertheless, vinclozolin can be hazardous to the health of those who work with it; pesticide producers and farmers represent populations which may be at significant risk. Vinclozolin is commonly used in growing sod for turf—it may seem absurd, but even frequent golfers may be exposed to high amounts of vinclozolin (EPA, 2002).

Other Sources of Exposure

There is also the potential for vinclozolin to enter into ground- and surface-water supplies via runoff. The EPA's survey of vinclozolin literature found reason to believe that vinclozolin is highly mobile in water (EPA, 2000). The migration of pesticides in this manner may help explain the regional distribution of symptoms like reduced fertility, discussed earlier. Indeed, studies have shown that pesticides, particularly fungicides like vinclozolin, have been found in rivers (Haith and Rossi, 2003) and other drinking water sources. Drinking water sources have been known to be contaminated by pesticides for decades, yet little action has been taken.

Other Considerations

While, overwhelmingly, research has focused on vinclozolin's endocrine disrupting effects, consideration should be given to its other target organs and organ systems. Few studies have looked at other effects, mainly because the metabolites are androgen antagonists. Androgens are involved in the development of the brain, particularly in the male brain (André and Markowski, 2006). Fittingly, then, vinclozolin, an anti-androgen, can affect the nervous system's development. Early (prenatal or juvenile) exposure to vinclozolin can suppress important sexual behaviors, like courtship, in male guppies. Learning deficits have also been shown to occur in male rats exposed to vinclozolin early in life. (André and Markowski, 2006). The study did not demonstrate if these deficits might be heritable, nor did the study analyze miRNA. We also do not know how this might affect more complex, uniquely human cognitive processes.

Additionally, there is the potential that vinclozolin may work in concert with other chemicals, or that its effects may be exacerbated by other endocrine disrupting chemicals. These potential synergies are unfortunately hard to test for or predict. For instance, a recent Associated Press survey identified many pharmaceuticals including synthetic estrogens in drinking water samples from cities around the United States. We are potentially faced with an incredibly complex system involving exposures to a wide range of chemicals in drinking water and food products.

Policy and Implications

The United States Environmental Protection Agency (EPA) first registered vinclozolin for use in 1981. It was approved then for use in a number of agricultural products. When the manufacturer, BASF, requested permission for vinclozolin to be used for growing sugar snap peas, the EPA, unable to fully substantiate the "reasonable

certainty of no harm" required by Section 408(b)(2) of the Federal Food, Drug, and Cosmetic Act (FFCDA), started to phase out is usage after re-scheduling the pesticide in 2000. This is similar to a ban in the United Kingdom, though the efficacy of this ban has been doubted, since residues have been found in lettuces for at least six years after the ban was put in place (Shaw, 2000). This could be due to farmers having stockpiled the chemical, its sale continuing.

Furthermore, domestic policy regarding pesticides does not always correspond with international policy. Latin American produce, for instance, which is imported in winter months, is not held up to the same regulatory standards governing pesticide use in the United States or Europe. It may be grown with pesticides that are banned in the importing country. Testing imported produce would be difficult and probably would require ports to be equipped with the necessary testing materials.

Current Limitations and Potential Future Studies

It is not yet fully understood how toxicants can alter miRNA expression. The mechanisms that lead to increased or decreased expression of a given miRNA have not been discovered. It is likely that there will soon be research in this field. It is also highly likely that other exposures will be found to operate via epigenetic changes. It remains unknown even how many chemicals that are widely accepted as carcinogens operate. This is true of arsenic; the toxic effects of arsenic have been documented for millennia but only recently did studies begin to link exposure to arsenic with altered miRNA expression (Marsit, et al, 2006).

Often a mix of pesticides are applied to crops. The process of risk analysis may be complicated by considering possible synergistic interactions between chemicals. Even

more complicated, these chemicals could interact with other exposures or lifestyles (ie, cigarette smoke). This may be a concern particularly if two or more exposures have similar miRNA expression profiles. Perhaps the risk analyses will suggest altering usage guidelines.

Pesticides are, by design, toxic substances. Researchers have lately been trying to develop pesticides which, though still toxic towards insects, bacteria or fungi which would harm the crops, would be less toxic to other, higher organisms. The conceptual more specialized, less toxic pesticides have been termed biopesticides. These vary widely. Some biopesticides are common household products such as baking soda and canola oil. Microbes may, themselves, be used to target organisms which would feed on crops. Genetically-modified crops, where plants produce their own defenses, are included in this category by the EPA. Somewhat of a controversial measure themselves, genetically-modified (GM) crops have attracted a stigma. There are legal issues that need to be resolved with genetically modified crops; some farmers have been sued when their corn hybridized with Monsanto-developed corn. Further research needs to verify the efficacy and the safety of these measures. Nevertheless, biopesticides are motivated by a desire to practice less harmful agriculture.

Conclusion

Vinclozolin has been shown to alter miRNA expression profiles. The significance of this effect has to be determined, but it suggests that the transgenerational, anti-androgenic effects observed in rats could result from some sort of miRNA-mediated interference with the development of the male reproductive tract. It is very likely that other chemicals act in a similar manner.

Studies show us that early exposures can lead to pronounced phenotypic variation, with some phenotypes not becoming manifest until adult life. MicroRNA may provide a mechanism for these exposures to exert their effects, since we have seen a marked difference in miRNA expression between exposed and control groups. Studies on chemicals, particularly pesticides of economic importance, must consider epigenetic modes of action and the potential for trans-generational toxicology.

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