UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA
CÂMPUS ARAÇATUBA

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Substâncias obesogênicas alteram a expressão de RNAs longos não-codificadores de maneira transgeracional

Araçatuba
2021

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Dissertação apresentada à Faculdade de Medicina Veterinária de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Mestre em Ciência Animal (Área de Medicina Veterinária Preventiva e Produção Animal).

Orientadora: Pesquisadora Flávia Lombardi Lopes

Araçatuba

2021

L864s

Lopes, Maria Fernanda da Silva
Substâncias obesogênicas alteram a expressão de RNAs longos não-codificadores de maneira transgeracional / Maria Fernanda da Silva Lopes. -- Araçatuba, 2021
47 p.

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Faculdade de Medicina Veterinária, Araçatuba
Orientadora: Flávia Lombardi Lopes

1. Epigenética. 2. Substâncias obesogênicas. 3. Camundongo. 4. LncRNA. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Medicina Veterinária, Araçatuba. Dados fornecidos pelo autor(a).

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CERTIFICADO DE APROVAÇÃO

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Araçatuba, 14 de janeiro de 2021.

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Araçatuba, 14 de janeiro de 2021.

À minha família, com muito amor e carinho,
por sua intensa dedicação e apoio durante
a elaboração deste trabalho.

AGRADECIMENTOS

Agradeço à minha família, em especial aos meus pais, à minha irmã e ao meu namorado Lucas, que sempre acreditaram em mim e me deram todo o apoio e incentivo nos momentos em que mais precisei, principalmente nos mais difíceis. Sou imensamente grata por tudo que fizeram por mim e por aceitarem compartilhar esta jornada ao meu lado. Amo vocês, serão sempre a minha base de tudo!

Às minhas amigas que a ciência me presenteou: Amanda Furlan, Beatriz Trigo, Juliana Felix, Jéssica Troiano, Mariana Cordeiro, Natália Scaramelle e Nayra Herrera. Obrigada pelo companheirismo, pelas conversas, conselhos, alegrias e tristezas compartilhadas durante essa caminhada.

À minha orientadora, Profa. Dra. Flávia Lombardi Lopes, que me deu a oportunidade de fazer parte da equipe do seu laboratório de Epigenômica, confiando inteiramente a mim a condução deste trabalho. Palavras não são suficientes para descrever minha imensa gratidão.

À UNESP – Universidade Estadual Paulista "Júlio de Mesquita Filho", em especial ao Programa de Pós-Graduação em Ciência Animal da Faculdade de Medicina Veterinária de Araçatuba (UNESP/FMVA), pela oportunidade de realização do mestrado.

A todos os professores e colaboradores do Departamento de Produção e Saúde Animal.

À minha banca de qualificação, Prof. Dr. Yuri Tani Utsunomyia e a Profa. Dra. Joselaine de Oliveira, que me servem de exemplo profissional pela dedicação e comprometimento, obrigado pelas considerações dadas ao meu trabalho.

À Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior - CAPES, pela concessão da bolsa de mestrado.

“O que é escrito sem esforço, em geral, é lido sem prazer”.

(Samuel Johnson)

LOPES, M.F.S. **Substâncias obesogênicas alteram a expressão de RNAs longos não-codificadores de maneira transgeracional.** 2021. 47 f. Dissertação (Mestrado) – Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2021.

RESUMO

A epidemia da obesidade é considerada uma crise mundial de Saúde Pública, tendo como fatores contribuintes o aumento da ingestão calórica, estilos de vida sedentários e/ou predisposições genéticas. Ainda que o balanço energético positivo seja uma das causas mais significativas da obesidade, pesquisas recentes relacionam a epidemia da doença à exposição precoce a produtos químicos desreguladores endócrinos (DEs) (do inglês, *Endocrine-Disrupting Chemicals* - *EDCs*). A Sociedade Endócrina define DEs como "substâncias químicas exógenas, ou misturas de substâncias químicas, que interferem em qualquer aspecto da ação hormonal". Substâncias obesogênicas compõem um subconjunto de DEs que estimulam inadequadamente a adipogênese e o armazenamento de gordura direta ou indiretamente, aumentando o número e/ou o tamanho das células adiposas, ou interferindo na regulação hormonal do metabolismo, apetite e saciedade. A exposição a essas substâncias químicas durante o período intrauterino e/ou de lactação pode influenciar fortemente a predisposição tardia à obesidade. Os RNAs longos não-codificadores (lncRNAs) são caracterizados por desempenhar relevantes papéis regulatórios em diversos processos biológicos. Considerando a importância da nutrição materna para a regulação dos padrões epigenéticos da prole, o presente estudo visa elucidar os efeitos da exposição ancestral a substâncias obesogênicas na expressão de lncRNAs no tecido adiposo gonadal de camundongos. Os dados de RNA-Seq de 8 amostras de tecido adiposo gonadal de camundongos F4 adultos expostos ancestralmente a dimetilsulfóxido (DMSO) (0,1%) como grupo controle, ou ao DE TBT (50 nM) durante o desenvolvimento uterino e lactação, foram obtidos de estudo previamente realizado (NCBI - GEO Datasets - GSE105051). Após alinhamento e contagem das *reads*, foi realizada análise de expressão diferencial, e identificamos 74 lncRNAs diferencialmente expressos ($p<0.05$), visto que 22 apresentaram expressão aumentada no grupo TBT e 52 estavam mais expressos no grupo DMSO em relação ao TBT. As análises dos termos de ontologia gênica (do inglês *Gene Ontology* - GO) revelaram o enriquecimento dos processos relacionados ao metabolismo da glicose e

à atividade do receptor ativado por Wnt. Sendo assim, nossos resultados sugerem que a regulação dos lncRNAs e de seus genes parceiros podem contribuir para o controle do processo de adipogênese.

Palavras-chave: Epigenética. Substâncias obesogênicas. Camundongo. LncRNA.

LOPES, M. F. S. **Obesogenic substances alter the expression of long non-coding RNAs in a transgerational manner.** 2021. 47 f. Dissertação (Mestrado) – Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2021.

ABSTRACT

The obesity epidemic is considered a global public health crisis. Increased caloric intake, sedentary lifestyles and/or genetic predisposition are all factors contributing to the epidemic. Although the positive energy balance is one of the most significant causes of obesity, recent research has linked early exposure to Endocrine-Disrupting Chemicals (EDCs) to the disease. The Endocrine Society defines EDCs as "exogenous chemicals, or mixtures of chemicals, that interfere with any aspect of hormonal action". Obesogenic substances make up a subset of EDCs that inadequately stimulate adipogenesis and fat storage directly or indirectly, increasing the number and/or size of fat cells, or interfering with the hormonal regulation of metabolism, appetite and satiety. Exposure to these chemicals during intrauterine and/or lactation periods can strongly influence the late predisposition to obesity. Long noncoding RNAs (lncRNAs) are characterized by playing important regulatory roles in several biological processes. Considering the importance of maternal nutrition to the regulation of epigenetic patterns in the offspring, the present study aimed at investigating the effects of ancestral exposure to obesogenic substances on the expression of lncRNAs in the gonadal adipose tissue of mice. RNA-seq data from 8 gonadal adipose tissue samples from adult F4 mice ancestrally exposed to dimethyl sulfoxide (DMSO) (0.1%) as a control group, or to EDC TBT (50 nM) during uterine development and lactation, were obtained from a previously conducted study (NCBI - GEO Datasets - GSE105051). After reading alignment and counting, differential expression analysis was performed, and we identified 74 differentially expressed lncRNAs were identified ($p < 0.05$), as 22 showed increased expression in the TBT group and 52 were more expressed in the TBT group. The analysis of the GO terms revealed an enrichment for glucose metabolism and the activity of the receptor activated by Wnt. Therefore, our results suggest that the regulation of lncRNAs and their partner genes may contribute to the control of the adipogenesis process.

Keywords: Epigenetics. Obesogenic substances. Mouse. LncRNA.

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1 INTRODUÇÃO GERAL

A epidemia da obesidade é considerada uma crise mundial de Saúde Pública, tendo como fatores contribuintes o aumento da ingestão calórica, estilos de vida sedentários e/ou predisposições genéticas. Ainda que o balanço energético positivo seja uma das causas mais significativas da obesidade, pesquisas recentes relacionam a exposição precoce a produtos químicos desreguladores endócrinos (DEs) (do inglês, *Endocrine-Disrupting Chemicals* - *EDCs*) à doença. A Sociedade Endócrina (*The Endocrine Society*) e a Organização Mundial da Saúde definem DEs como "substâncias químicas exógenas, ou misturas de substâncias químicas, que alteram funções do sistema endócrino e, por consequência, causam efeitos adversos em um organismo, em sua prole ou em populações" (1).

Substâncias obesogênicas compõem um subconjunto de DEs que estimulam inadequadamente a adipogênese e o armazenamento de gordura direta ou indiretamente, aumentando o número e/ou o tamanho das células adiposas, ou interferindo na regulação hormonal do metabolismo, apetite e saciedade. O tecido adiposo é, particularmente, um reservatório significativo dos DEs e sua presença nos tecidos corporais refletem não apenas ao contato recente, mas também a exposições anteriores a essas substâncias químicas (2,3).

Efeitos transgeracionais foram observados com alguns tipos de DEs, como: Bisfenol A, também conhecido como BPA - composto químico que está presente na composição do plásticos policarbonato; Dicloro-difenil-tricloroetano ou DDT, pesticida utilizado para controlar pestes agrícolas; Dibutilftalato (DBP) – plastificante utilizado como aditivo em adesivos e tintas de impressão; Trifenilestanho (TPT) e Tributilestanho (TBT) - compostos orgânicos de estanho, comumente utilizados em tintas anti-incrustante de navios (4). O TBT apresenta baixa solubilidade em água e perfil lipofílico, podendo causar efeitos no sistema endócrino e cardiovascular. Estudos recentes mostram que a exposição pré-natal a obesogênicos ambientais pode produzir efeitos permanentes nos animais expostos (F0) e, transgeracionalmente, em seus descendentes. A exposição a essas substâncias químicas durante o período intrauterino e/ou de lactação pode influenciar fortemente a predisposição tardia à obesidade (1).

Estudos epidemiológicos sobre a nutrição materna durante diversas fases da gestação revelam que nesta fase da vida as fêmeas podem induzir transformações

permanentes na estrutura, na fisiologia e/ou no metabolismo da prole (5). A nutrição materna pode ocasionar alterações epigenéticas no genoma fetal (6) como, por exemplo, no perfil de metilação do DNA, podendo levar a mudanças fenotípicas permanentes na prole (7).

Fetal programming ou programação fetal é o efeito do ambiente uterino, através de mecanismos epigenéticos, no desenvolvimento fetal, sendo capaz de resultar em alterações permanentes na fisiologia e metabolismo dos descendentes (8). Em epigenética, as principais modificações incluem a metilação do DNA, modificações químicas na cauda das histonas e a ação dos RNAs não-codificadores (ncRNAs) (9). O termo epigenética foi usado por Conrad Waddington na década de 1940, para elucidar as interações de genes com o meio ambiente e as possíveis alternativas que cada célula pode desempenhar durante o desenvolvimento (10). A mesma é definida como um processo capaz de regular a expressão gênica sem alteração no código genético, um exemplo clássico são as células de um mesmo organismo que, apesar de geneticamente idênticas, são capazes de se distinguir fenotipicamente, dependendo de sua localização e/ou função, mediante o controle da expressão gênica (11).

A condição vital para o desenvolvimento de um novo organismo é a programação epigenética dos gametas e embriões em início de desenvolvimento. Esta programação engloba a integração dos três processos epigenéticos, interferindo na transcrição de genes (12). Tais eventos epigenéticos equilibram a expressão gênica através do controle da transcrição e/ou tradução, e são altamente dependentes das condições uterinas no momento do desenvolvimento de embriões ou gametas da prole.

Aproximadamente 62% do DNA funcional é transcrito em ncRNAs que apresentam amplas funções, como: regulação da expressão gênica, regulação da síntese de proteínas, inativação de cromossomos, modulação da cromatina, dentre outras (13–15). Os ncRNAs são classificados de acordo com o tamanho: ncRNAs longos (acima de 200 nucleotídeos) ou ncRNAs pequenos (abaixo de 200 nucleotídeos) (13,14,16,17).

Análises recentes sobre os RNAs longos não-codificadores (lncRNAs) apontam seu importante papel na regulação transcricional, pós-transcricional e epigenética da expressão gênica, podendo assim, silenciar ou ativar genes e loci cromossômicos

específicos (18). A inativação do cromossomo X e o *imprinting* gênomico são importantes exemplos da ação de lncRNAs (19–21).

Considerando a importância da nutrição materna para regulação dos padrões epigenéticos da prole e os diversos papéis que os lncRNAs desempenham, o presente estudo visa elucidar os efeitos da exposição ancestral a substâncias obesogênicas na expressão de lncRNAs no tecido adiposo gonadal de camundongos.

1.1 Objetivos

1.1.1 Objetivo Geral

Analisar os efeitos da exposição ancestral a substâncias obesogênicas na expressão de RNAs longos não-codificadores no tecido adiposo gonadal de camundongos descendentes F4 (efeito transgeracional).

1.1.2 Objetivos Específicos

I. Caracterizar, *in silico,* os lncRNAs diferencialmente expressos no tecido adiposo gonadal de camundongos, transgeracionalmente expostos a substâncias obesogênicas;
II. Analisar o perfil de co-expressão de lncRNAs–mRNA diferencialmente expressos.

2 CAPÍTULO 1 - CORRELATION ANALYSIS OF LNCRNA AND MRNA IN MICE ANCESTRALLY EXPOSED TO OBESOGENIC SUBSTANCES IDENTIFIES POTENTIAL ADIPOGENESIS REGULATORY GENES

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2.1 Resumo

A epidemia de obesidade é considerada uma crise global de saúde pública, com aumento da ingestão calórica, sedentarismo e / ou predisposições genéticas como fatores contribuintes. Embora o balanço energético positivo seja uma das causas mais significativas da obesidade, pesquisas recentes relacionaram a exposição precoce a Produtos Químicos Desreguladores Endócrinos (EDCs) à doença. Além de suas ações no perfil hormonal, os EDCs podem induzir alterações de longo prazo na expressão gênica, possivelmente devido a alterações nos padrões epigenéticos. Os longos RNAs não codificantes (lncRNAs) são um mediador epigenético que desempenha importantes papéis regulatórios em diversos processos biológicos. Considerando a importância da nutrição materna na regulação do padrão epigenético da prole, o presente estudo tem como objetivo elucidar os efeitos da exposição ancestral a substâncias obesogênicas sobre a expressão de lncRNAs no tecido adiposo gonadal de camundongos. Neste estudo, exploramos a expressão diferencial de lncRNAs em amostras de tecido adiposo branco gonadal de camundongos F4 adultos expostos ancestralmente a EDC TBT durante o desenvolvimento uterino e a lactação, analisando dados de RNA-seq do conjunto de dados público GSE105051. Um total de 74 lncRNAs foram expressos diferencialmente, 22 foram regulados positivamente e 52 regulados negativamente no grupo exposto ao TBT quando comparado aos controles (DMSO). Seguindo o gene parceiro do mRNA para a predição dos lncRNAs de ação cis, a análise dos termos do GO revelou o enriquecimento dos processos relacionados ao metabolismo da glicose e à atividade do receptor ativado por Wnt. Em conclusão, nossos resultados indicam que a regulação de lncRNAs e seus genes-parceiros no tecido adiposo gonadal branco de camundongos ancestralmente expostos ao EDC TBT pode estar diretamente relacionada ao controle do processo de adipogênese, sugerindo que esta regulação pode ser hereditária epigeneticamente.

Palavras-chave: Epigenética. Substâncias obesogênicas. Camundongo. LncRNA.

2.2 Abstract

The obesity epidemic is considered a global public health crisis, with an increase in caloric intake, sedentary lifestyles and/or genetic predispositions as contributing factors. Although the positive energy balance is one of the most significant causes of obesity, recent research has linked early exposure to Endocrine-Disrupting Chemicals (EDCs) to the disease. In addition to their actions on the hormonal profile, EDCs can induce long-term changes in gene expression, possibly due to changes in epigenetic patterns. The long non-coding RNAs (lncRNAs) is an epigenetic mediator that play important regulatory roles in several biological processes. Considering the importance of maternal nutrition for regulating epigenetic patterning in the offspring, the present study aims to elucidate the effects of ancestral exposure to obesogenic substances on the expression of lncRNAs in the gonadal adipose tissue of mice. In this study, we explored the differential expression of lncRNAs in gonadal white adipose tissue samples from adult F4 mice ancestrally exposed to EDC TBT during uterine development and lactation, by analyzing RNA-seq data the public dataset GSE105051. A total of 74 lncRNAs were diferentially expressed, 22 were up-regulated and 52 were down-regulated in the group exposed to TBT when compared to controls (DMSO). Following mRNA gene-partner to *cis*-acting lncRNAs prediction, analysis of GO terms unveiled enrichment of processes related to glucose metabolism and Wnt-activated receptor activity. In conclusion, our results indicate that regulation of lncRNAs and their gene-partners in the white gonadal adipose tissue of mice ancestrally exposed to EDC TBT may be directly related to the control of the adipogenesis process, suggesting that this regulation may be epigenetically inherited.

Keywords: Epigenetics. Obesogenic substances. Mouse. LncRNA.

2.3 Introduction

Exposure to environmental factors during embryonic development has been linked to the risk of diseases such as obesity and type 2 diabetes mellitus later in life. The obesity epidemic is considered a global public health crisis, having as contributing factors increased caloric intake, sedentary lifestyles and/or genetic predispositions. Although the positive energy balance is one of the most significant causes of obesity, recent research has linked early exposure to endocrine disrupting chemicals (EDCs) to the disease (Chamorro-Garcia et al., 2017).

EDCs are a group of different chemical compounds that interfere with the production, release, transport, metabolism, action or elimination of endogenous hormones responsible for maintaining homeostasis and regulating developmental processes (Shahnazaryan et al., 2019). Obesogenic substances comprise a subset of EDCs, which can generate an increase in the accumulation of lipids, inadequately stimulating adipogenesis, hypertrophy or hyperplasia of adipocytes or interfering with the hormonal regulation of metabolism, appetite and satiety (Gupta et al., 2020). There is a growing range of evidence showing that exposure to these chemicals during intrauterine development or lactation can strongly influence offspring's late predisposition to obesity (Street et al., 2018).

In addition to their actions on the hormonal profile, EDCs can induce long-term changes in gene expression, possibly due to changes in epigenetic patterns (Alavian-Ghavanini and Rüegg, 2018). Shoucri et al. (2017) demonstrated that the obesogenic effects of prenatal tributyltin (TBT) exposure are propagated transgenerationally to unexposed generations, presumably through epigenetic changes. In fact, transgenerational effects have been observed with some types of EDCs, such as bisphenol A (BPA), dichlorodiphenyltrichloroethane (DDT), dibutyl phthalate (DBP), triphenyl tin (TPT) and TBT. Obesogens were defined as chemical substances that lead to an increase in the accumulation of white adipose tissue (WAT). Egusquiza and Blumberg (2020) reported that TBT induces obesity by promoting the differentiation of adipocytes in the body while stimulating the activity of the RXR-PPARγ complex. Interestingly, Chamorro-Garcia and his collaborators (2017) showed in their study that prenatal exposure to environmental obesogens, especially EDC TBT, can produce permanent effects on exposed animals (F0) and, transgenerationally, on their

*Text according to the Guidelines for Authors of the journal Molecular and Cellular Endocrinology – Available in ANEXO A

descendants of the F4 generation, such as global changes in DNA methylation, altered expression of genes relevant to metabolism and obesity consistent with leptin resistance.

A considerable body of studies within the past two decades have shown that maternal nutrition can cause changes in the fetal epigenome, i.e, the DNA methylation profile, post-translational histone modifications and regulation of and by non-coding RNAs (ncRNAs), which can lead to permanent phenotypic changes in the offspring reviewed by Greco et al. (2019). Epigenetic mechanisms play essential roles in the processes that determine adult phenotypes, through epigenetic programming. While research has shown that exposure to EDC during periods of development can disrupt epigenetic programming, it is not clear whether these changes are actually causing adverse outcomes (Jacobs et al., 2017). Geisler and Coller (2016) proposed that exposure to environmental toxins like bisphenol A, benzo(a)pyrene, tributyltin, and phthalates produces alterations in the expression of long noncoding RNAs (lncRNAs) that may be associated with a range of diseases.

Long non-coding RNAs (lncRNAs) are ncRNAs composed of more than 200 nucleotides and play an important role in the transcriptional, post-transcriptional and epigenetic regulation of gene expression, thus being able to silence or activate specific genes or loci (Charles Richard and Eichhorn, 2018). LncRNAs are classified according to their location in the genome as: 1) sense or antisense, when they overlap one or more exons of coding genes, on the same strand or on the opposite, respectively; 2) bidirectional, when the transcription of lncRNA and a coding transcript on the opposite strand are located nearby; 3) intronic, when it is derived from the intronic region of a protein-coding gene; 4) intergenic, when it is located between coding genes (Kopp and Mendell, 2018).

Considering the importance of maternal nutrition for regulating epigenetic patterning on the offspring (and transgenerationally on their descendents), the diverse roles that lncRNAs play on gene expression control, and the altered expression of genes relevant to metabolism (Chamorro-Garcia et al., 2017), the present study aimed at evaluating the effects of ancestral exposure to obesogenic substances on the expression of lncRNAs in the gonadal white adipose tissue (gWAT) of mice.

2.4 Methods

2.4.1 RNA-Seq data sets

RNA-seq data were obtained from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/) under the bioproject PRJNA414476, with accession number GSE105051. This dataset was produced by Chamorro-Garcia et al. (2017) in simultaneous experiments using samples of gWAT from C57BL/6J male F4 mice (7 weeks of age) ancestrally exposed via drinking water to EDC TBT (50 nM) (n = 4) or to the vehicle dimethylsulfoxide (DMSO) (0.1%) as a control group (n = 4) (both dilute in 0.5% carboxymethyl cellulose in water for solubility) during pregnancy and lactation. In total, 8 samples were used, consisting of 4 samples from the TBT exposed group and 4 samples from the control group.

2.4.2 Bioinformatic identification of lncRNAs in RNA-Seq datasets

Quality of the extracted RNA-seq readings was evaluated with FastQC on the Galaxy web plataform and we used the public server at www.usegalaxy.org to analyse data (Afgan et al., 2016). The data were then aligned to the latest mouse genome reference sequence (GRCm38.p6, as provided by GENCODE) using HISAT2 version 2.1.0+galaxy5 (Kim et al., 2015) with the Burrows-Wheeler Transformation (BWT) and the Ferragina-Manzini (FM) indexing algorithms. The resulting BAM file was then passed to FeatureCounts version 1.6.4+galaxy1 (Liao et al., 2014) to perform read counts using the GENCODE M25 (mouse) annotation as reference. Quality control of all steps was carried out with MultiQC.

Next, DESeq2 (version 2.11.40.6 + galaxy1) was used to perform statistical analyses of differential expression of lncRNA between samples from the TBT exposed group and the control group. This tool estimates the average variance in the count data and tests the differential expression using a binomial distribution model as basis. By default, the DESeq2 tool in Galaxy uses the Wald test to identify genes that are differentially expressed between two sample classes (Love et al., 2014). Using the Biomart tool, the biotypes “processed_transcript”, “pseudogene”, “lincRNA”, “3prime_overlapping_ncrna”, “antisense”, “sense_intronic” and sense_overlapping” were classified as lncRNAs (http://www.ensembl.org/info/genome/genebuild/biotypes.html).

Differentially expressed (DE) lncRNAs (p-value<0.05) were clustered with the heatmap3 package in R using “complete linkage” method and Euclidian distance as

parameters (https://www.rdocumentation.org/packages/heatmap3/versions/1.1.7/topics/heatmap3).

2.4.3 Prediction analysis of lncRNAs-mRNAs with cis action

The lncRNAs can act in the regulation of genes in c*is* or *trans* forms. The *cis* function refers to the action of lncRNAs in target genes neighboring its transcription site, whereas lncRNAs that act in trans exercise their function in different location to which they were transcribed. Differentially expressed lncRNAs (*P*-value <0.05) were selected for the prediction of the *cis*- and *trans*-target genes. We used the FEELnc tool (FlExible Extraction of Long non-coding RNAs) to predict nearby partner genes (*cis*-genes) of DE lncRNAs (within the window size of 10.000 - 100.000 nt). For each lncRNA-mRNA interaction, a best lncRNA:RNA-partner interaction was identified (isBest score = 1). Next, Pearson correlation coefficient method and Student`s t-test were used to analyze correlations between all DE mRNAs and DE lncRNAs. The lncRNA-mRNA interaction was considered significant when Pearson`s correlation $|r| \geq 0.8$ and $p<0.05$ assuming that r is normally-distributed under the null hypothesis of true $r = 0$.

2.4.4 Functional Enrichment Analysis

We used the g:GOSt (Function Profiling) tool within gProfiler (https://biit.cs.ut.ee/gprofiler/gost) for analysis of the Gene Ontology (GO) annotation of all significant lncRNAs and partner mRNA pairs (https://biit.cs.ut.ee/gprofiler/gost). All *p*-values were adjusted using the Benjamini-Hochberg (FDR) method (FDR-adjusted p-value<0.05).

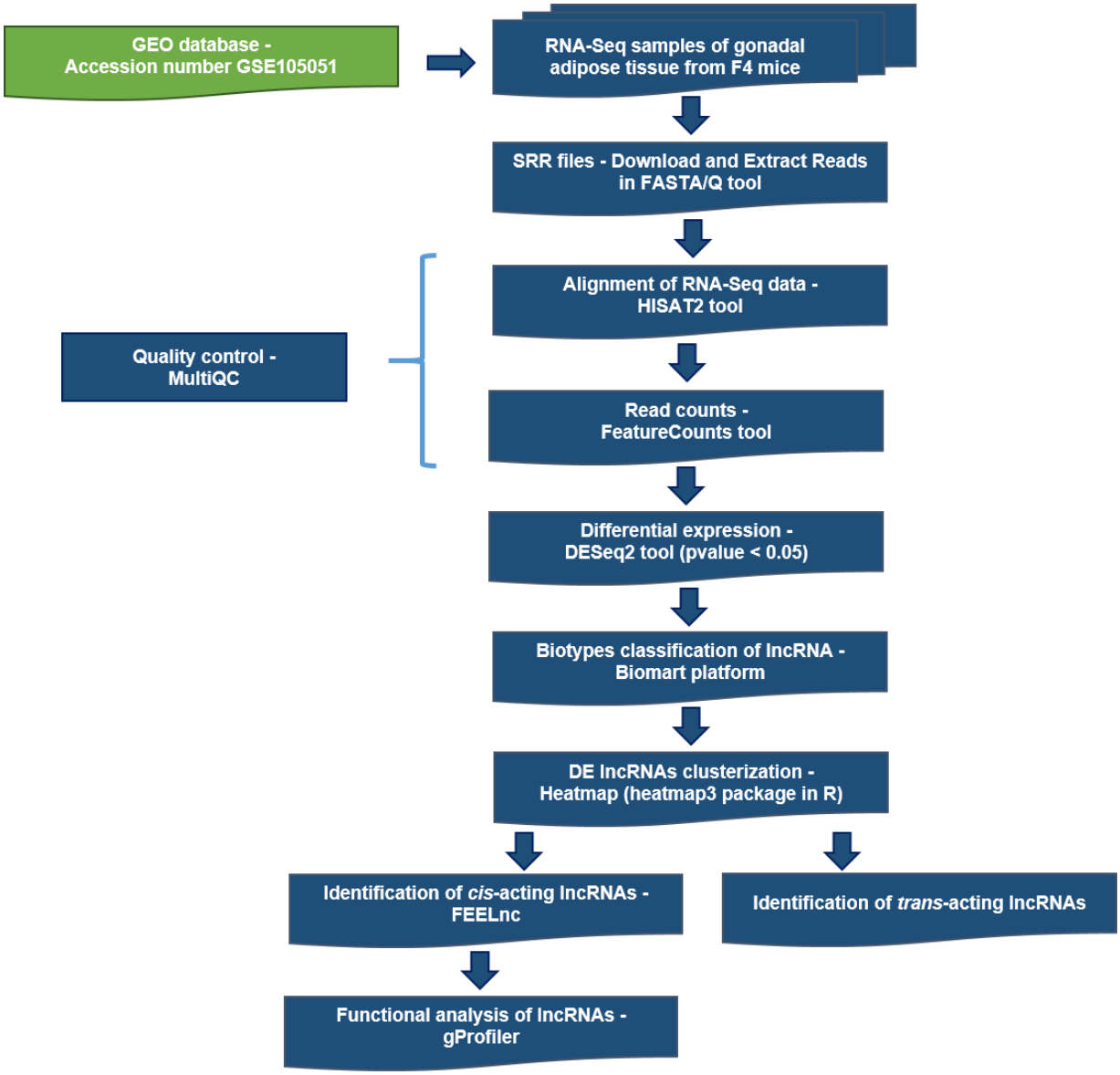

Figure 1 - Workflow of lncRNA analysis and functional predictions.

2.5 Results

Out of 1324 differentially expressed transcripts, 74 were considered to have a lncRNA biotype, according to the classification available on the Ensembl Biomart tool (Table 1).

Table 1 – Biomart classification of biotype, counts and percentage of total transcripts.

Biotype	Counts	% of total
Antisense	20	1.51
bidirectional_promoter_lncRNA	4	0.30
lincRNA	29	2.19
processed_transcript	6	0.45
sense_intronic	1	0.08

TEC	14	1.06
IG_C_gene	6	0.45
IG_J_gene	1	0.08
IG_V_gene	23	1.74
miRNA	1	0.08
Mt_tRNA	2	0.15
processed_pseudogene	16	1.21
protein_coding	1194	90.18
pseudogene	1	0.08
snoRNA	2	0.15
transcribed_processed_pseudogene	1	0.08
transcribed_unprocessed_pseudogene	2	0.15
unprocessed_pseudogene	1	0.08
Total	1324	100
Total lncRNAs	74	5.59

Of these 74 DE lncRNAs in the contrast of the EDC TBT and DMSO control groups (filtered using p-value <0.05 for the Wald test for log2FoldChange difference between groups, after adjusting for the rate of false discoveries, 22 showed increased expression in the group exposed to the EDC TBT when compared to controls (DMSO). The other 52 lncRNAs were downregulated in the EDC TBT ancestrally exposed group (Figure 2).

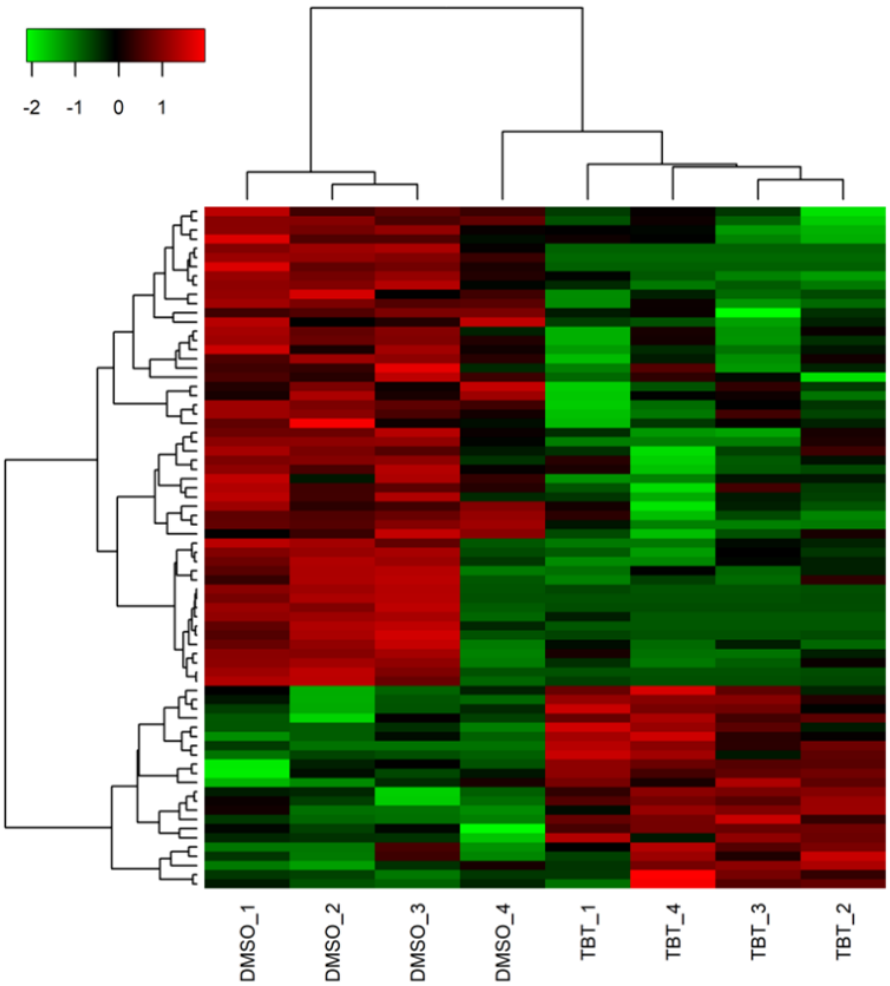

Figure 2 – Heatmap of 74 differentially expressed lncRNAs between samples of gWAT ancestrally exposed to EDC TBT and samples of the control group (DMSO). Expression of lncRNA is represented according to the colour scale shown at the top, corresponding to the z-score. Red represents high expression, and green represents low expression.

From the correlation matrix containing 11.685 interactions between lncRNA-mRNA and from the prediction analysis of the cis lncRNAs-mRNAs pairs performed on the FEELnc tool, we observed that, of a total of 12 cis-lncRNA-mRNAs considered as best (isBest=1), only 3 DE mRNAs (Figure 3) were found to have a significant correlation of expression with their lncRNA predicted cis-partner (Pearson`s correlation $|r| \geq 0.8$ and p-value <0.05; Table 2).

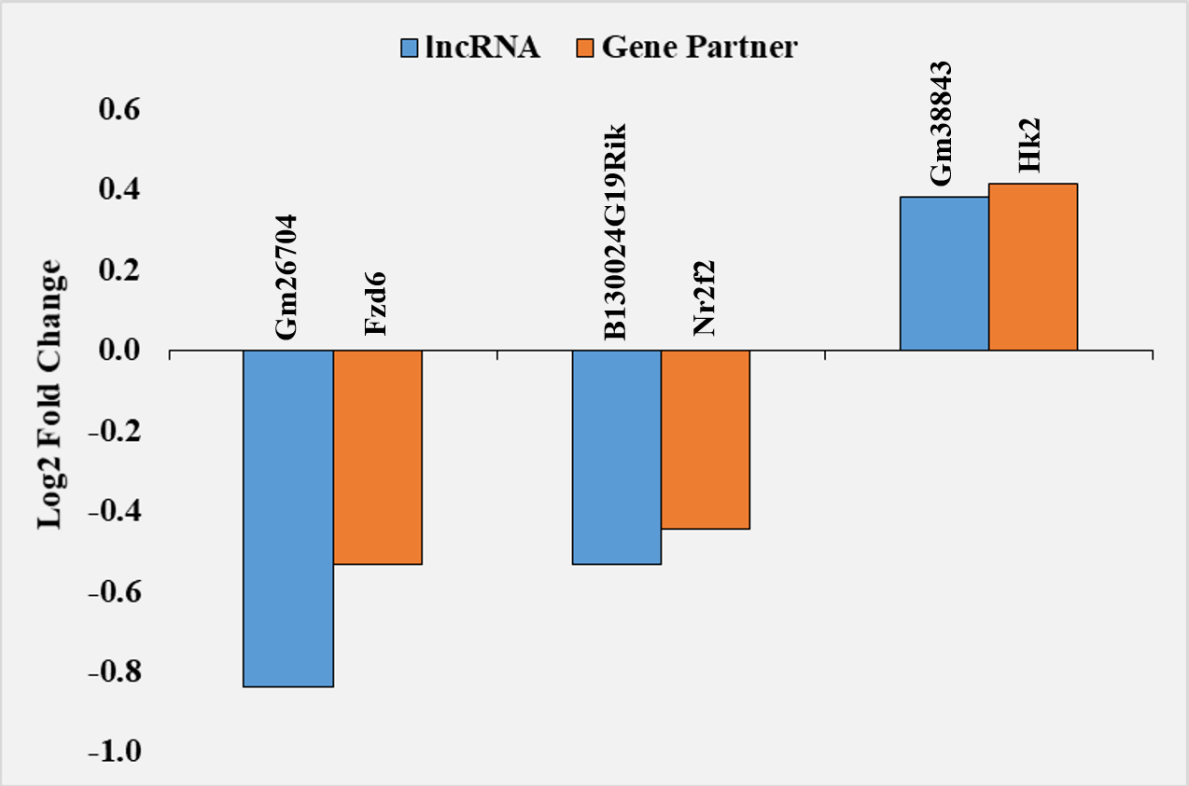

Figure 3 - *Cis*-acting lncRNAs and their differentially expressed gene-partners between samples of gWAT ancestrally exposed to EDC TBT and samples of the control group (DMSO). Y-axis shows expression (log2 fold change) of DE lncRNAs and gene-partners. On the x-axis lncRNAs are shown in blue and gene-partners in orange.

Table 2 – Top *cis*-acting Pearson's correlation between lncRNAs-gene partners

LncRNA		**mRNA**		
Name	Biotype	Name	*P*-value	Correlation
Gm26704	Bidirectional promoter lncRNA	Fzd6	4.33×10^{-6}	0.87
B130024G19Rik	Processed transcript	NR2F2	1.18×10^{-8}	0.86
Gm38843	lincRNA	HK2	1.29×10^{-8}	0.93

GO analysis was performed to obtain information about the function of the identified target genes of all DE lncRNAs. In our analysis of enrichment of GO terms, we identified that the lncRNAs partner genes were significantly enriched for a total 384 GO terms, 27 for molecular function, 332 for biological processes and 25 for cellular

component. The top 10 ranked GO terms in molecular function of the EDC TBT group, are shown in Table 3.

Table 3 – Top 10 GO terms enrichment analysis for Molecular Function related to glucose metabolism.

GO - ID	Term	*P*-value
GO:0004340	glucokinase activity	4.60×10^{-3}
GO:0004396	hexokinase activity	4.60×10^{-3}
GO:0008865	fructokinase activity	4.60×10^{-3}
GO:0019158	mannokinase activity	4.60×10^{-3}
GO:0001972	retinoic acid binding	8.40×10^{-3}
GO:0042813	Wnt-activated receptor activity	8.40×10^{-3}
GO:0005536	glucose binding	8.40×10^{-3}
GO:0019200	carbohydrate kinase activity	1.01×10^{-2}
GO:0004879	nuclear receptor activity	1.13×10^{-2}
GO:0005501	retinoid binding	1.13×10^{-2}

2.6 Discussion

In the present study, we analyzed the expression profiles of lncRNAs in the gWAT of F4 mice ancestrally exposed to obesogenic substances. Based on the RNA-Seq data, we identified a total of 74 differentiatly expressed lncRNAs in the group ancestrally exposed to the EDC TBT, and analyzed the expression correlation with their respective partner genes, as lncRNAs are known to regulate neighboring protein-coding genes (Kopp and Mendell, 2018). Enrichment of GO terms revealed that the partner genes of our DE lncRNAs are primarily involved in glucose and lipid metabolism and in Wnt-activated receptor activity, all of which play an important role in regulating adipogenesis.

The B130024G19Rik and Gm26704 lncRNAs, which interact with the gene-partners NR2F2 and Fzd6, respectivelywere downregulated in our study. The nuclear receptor of subfamily 2, group F, member 2 (NR2F2, also known as COUP-TFII, Chicken ovalbumin upstream promoter-transcription factor II) is expressed in adipose tissue, and may contribute to the pathogenesis of obesity, playing a role fundamental in adipogenesis and energy homeostasis (Polvani et al., 2020). Overexpression of

NR2F2 impairs adipogenesis by suppressing the expression of pro-adipogenic factors in adipocytes, whereas downregulation of NR2F2 promotes fibroblast differentiation into fat cells, and can act on development of chronic diseases ranging from obesity to metabolic syndrome (Li et al., 2010). Okamura (2009) and collaborators demonstrated that NR2F2 provides a direct link between the Wnt/beta-catenin signaling pathway and modulation of PPARγ. Wnt activates the expression of NR2F2, which in turn, prevents the expression of PPARγ, resulting in inhibition of adipogenesis. The gene-partner to Gm26704, Fzd6, belongs to the family of 10 Frizzled receptors, which play important roles in embryonic development, regulation of stem cells and tissue homeostasis, and its stimulation can activate the canonical Wnt/β-catenin and non-canonical Wnt/Ca 2+ pathways (Kozielewicz et al., 2020). Chamorro-Garcia et al. (2017), in the study that generated the data employed in our analysis, showed that ancestral exposure to TBT stimulated fat storage in adulthood. We propose that sustained inhibition of NR2F2 expression through lncRNA modulation is allowing for the differentiation of fibroblasts into adipocytes, thus contributing to the observed development of obesity.

The lncRNA Gm38843, up regulated in the TBT group, is correlated with the HK2 gene partner. GO term analysis indicated that HK2, also up-regulated in the TBT group, is associated with glucose metabolism (GO:0019158 - mannokinase activity; GO:0008865 - fructokinase activity; GO:0004396 - hexokinase activity and GO:0004340 - glucokinase activity). Initial glucose metabolism is regulated by four main steps: I) glucose uptake; II) action of hexokinase (HK); III) action of phosphofrutokinase (PFK); and IV) lactate import and export (Tanner et al., 2018). HK2 is one of four hexokinase isoforms highly expressed in skeletal muscle and adipose tissue. It acts as a fundamental part of the glycolysis process, participating in the conversion of glucose to glucose-6-phosphate (G6P) (Rabbani and Thornalley, 2019). High concentration of glucose in stem derived adipose cells increases the accumulation of lipids and positively regulates the expression of the adipogenic factors PPARγ and LPL (Kolodziej et al., 2019). Therefore, our results suggest that the positive regulation of HK2 expression in the gonadal white adipose tissue of mice ancestrally exposed to the obesogenic substance TBT may be directed at glucose homeostasis within the adipose tissue as a response to the observed lipid accumulation in these mice (Chamorro-Garcia et al., 2017).

Our findings indicate that modulation of lncRNAs may play a fundamental role in the adipogenesis process. In fact, a recent review published by Squillaro et al.

(2020), focuses on the role of lncRNAs in the biology and metabolism of adipocytes. In this review, the authors discuss that lncRNAs such as SRA, ADINR and HOTAIR, are involved in the transcriptional silencing of genes involved in the differentiation of adipocytes in WAT. On the other hand, high expression of lncRNAs ASMER-1, ASMER-2 and Plnc1 in the adipose tissue of obese mice positively regulates the expression of adipogenic transcription factors, such as PPARγ, which play a fundamental role during adipogenesis (Squillaro et al., 2020). Identification of target genes for *cis*-acting lncRNAs provides information that can help uncover the function of lncRNAs modulated during the adipogenesis process.

Endocrine Disrupting Chemicals, e.g tributyltin (TBT), promote adipogenesis by altering the development of fat cells and/or by increasing energy storage in the adipose tissue (Papalou et al., 2019). Our results indicated that regulation of lncRNAs and their gene-partners in the white gonadal adipose tissue of mice ancestrally exposed to EDC TBT may be related to the control of the adipogenesis process, suggesting that this regulation may be epigenetically inherited.

Declaration of competing interest

The authors have declared no conflict of interest.

Acknowledgements

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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APÊNDICE A – Referências da Introdução Geral

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DESCRIPTION

Molecular and Cellular Endocrinology was established in 1974 to meet the demand for integrated publication on all aspects related to the genetic and biochemical effects, synthesis and secretions of **extracellular signals** (hormones, neurotransmitters, etc.) and to the understanding of **cellular regulatory mechanisms** involved in **hormonal control.**

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