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Gene Expression Profiling in the Aging Ovary

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Abstract

DNA microarray is an important discovery technology that allows the analysis of the expression of thousands of genes at a time. Data from DNA microarrays elucidate fundamental biological processes through discovery of differential expression of genes not previously known or predicted to be involved in a particular process. In the ovary and other hormone-responsive tissues, the technology can be used to examine the effects of gene mutations, pharmaceutical agents, disease, hormones, developmental changes, or changes in gene expression related to aging.

1. Introduction

Obesity is associated with progressive health disorders, e.g. metabolic syndrome, and has a negative impact on fertility. A major reproductive disorder related to obesity is polycystic ovary syndrome (PCOS), which is the most prevalent endocrinopathy of reproductive age women. Moreover, women with PCOS are at greater risk for developing type II diabetes and cardiovascular disease as they age (1).

Mice heterozygous for the Lethal Yellow (LY) mutation at the agouti gene locus exhibit distinct characteristics including yellow coat color, adult-onset obesity, insulin resistance (2), hyperleptinemia (3), and accelerated reproductive senescence (3,4). Declining ovarian function in aging LY mice is directly related to progressive obesity and hyperleptinemia (3). Because of the adult-onset, progressive obesity characteristic of LY mice, these mice more closely mimic typical human obesity than many other mouse models of obesity, e.g. *ob/ob* or *db/db* mice. Therefore, we have used the obese LY mouse and their lean black littermates to study the effects of aging and progressive obesity on gene expression in the ovary (3,5).

DNA microarray is a powerful technology that allows the analysis of the expression of thousands of genes at a time. It is an important discovery technology. DNA microarrays are often used to elucidate fundamental biological processes through discovery of differential expression of genes not previously known or predicted to be involved in a particular process. In the ovary, the technology can be used to examine the effects of pharmaceutical agents (5), disease, hormones, developmental changes, or changes in gene expression related to aging. Differential gene expression can be measured in whole synchronized ovaries, in isolated granulosa cells, or in isolated corpora lutea.

2. Materials

2.1 Preparation of Synchronized Mouse Ovaries for RNA Extraction

1. Lethal Yellow mice (C57BL/6J A^y/a) and their lean black littermates (C57BL/6J a/a) were obtained from the Augustana College Biology Department (Sioux Falls, SD) breeding colony; the breeding colony was founded by breeder mice obtained from Jackson Laboratory (Bar Harbor, ME, current strain number KK Cg-A^y/J). Mice were housed and fed as described (5).
2. GnRH antagonist, Antide© (Bachem, Torrance, CA).
3. Equine chorionic gonadotropin (eCG, Sigma) has primarily follicle stimulating hormone activity in the mouse. Human chorionic gonadotropin (hCG, Sigma) has luteinizing hormone activity.
4. 1.8 mL cryovial (Nunc).
5. PBS + 0.1% BSA.
6. RNAlater (Ambion, Austin, TX) is an RNA-preserving reagent.

2.2 RNA Hygiene

1. RNase ZAP (Ambion).
2. Diethylpyrocarbonate (DEPC, Sigma).

2.3 RNA Extraction

1. TRI reagent (Molecular Research Center, Cincinnati, OH)
2. Bromochloropropane (Molecular Research Center, Cincinnati, OH).
3. 3M sodium acetate (Sigma).
4. Silica gel membrane and buffers for purification of RNA (RNeasy, Qiagen, Valencia, CA).
5. β -mercaptoethanol (Sigma). Add 10 μ l β -mercaptoethanol per 1000 μ l to guanidine isothiocyanate-containing lysis/binding RLT buffer before use.
6. 100% molecular grade ethanol (Sigma).
7. RNase-free DNase (Qiagen): Mix 10 μ l DNase stock solution with 70 μ l of DNase dilution buffer (RDD) and add the diluted DNase directly to the gel membrane of the column.

2.4 Analysis and Quantitation of RNA

1. Agilent Bioanalyzer 2100.
2. RNA 6000 Nano Chip (Agilent, Santa Clara, CA). The RNA 6000 Nano Chip kit contains the gel matrix, spin filter columns, dye concentrate, marker mixture diluent, the RNA ladder (molecular weight markers), and the RNA chip. The priming station for pressurizing the chip is purchased separately.

2.5 Synthesis of First and Second Strand cDNA and cRNA

1. The MessageAmp II-Biotin Enhanced kit (Ambion #AM1791, Austin, TX). This kit contains all reagents for the synthesis of first and second strand cDNA and for the in vitro transcription synthesis of cRNA. This includes T7 Oligo (dT) primer, 10x first strand buffer, dNTP mix, RNase inhibitor, reverse transcriptase, 10x second strand buffer, DNA polymerase, RNase H, biotin-NTP mix, T7 10x reaction buffer, T7 enzyme mix, and filter cartridges and buffers for purification of cDNA and cRNA.
2. Non-DEPC-treated nuclease-free water (Ambion).
3. Bacterial RNA spikes were obtained from Applied Microarrays (Tempe, AZ).
4. MessageAmp II aRNA Amplification Kit (Ambion) if two rounds of cRNA synthesis are needed.
5. In vitro transcription (IVT) master mix: 12 μ l biotin-NTP mix, 4 μ l T7 10x reaction buffer, and 4 μ l T7 enzyme mix per sample plus 5% volume overage.

2.6 Hybridization

1. 5x Fragmentation buffer: 200 mM Tris acetate, pH 8.2, 500 mM potassium acetate, 150 mM magnesium acetate.
2. Hybridization buffer components A and B (Applied Microarrays, Tempe, AZ).
3. 0.75x TNT wash buffer: 75 ml of 1 M Tris-HCl, pH 7.6, 22.5 ml of 5 M NaCl, 0.375 ml of Tween 20, 902 ml of DEPC-treated water. Filter through 0.2 μ m filter.
4. 1x TNT buffer: 100 ml of 1 M Tris-HCl, pH 7.6, 30 ml of 5 M NaCl, 0.5 ml of Tween 20, 870 ml of DEPC-treated water. Filter through 0.2 μ m filter.
5. 0.1x SSC/0.05% Tween 20 wash buffer: 5 ml of 20X SSC buffer (Sigma), 0.5 ml of Tween 20 (Sigma), 994.5 ml of DEPC-treated water. Filter through 0.2 μ m filter.
6. TNB buffer: Dissolve 1 g NEN blocking reagent in 200 ml 1x TNT. Heat to 60°C then stir overnight, reheat to 60°C then filter through 0.88 μ m filter. Freeze at -20°C in 50 ml aliquots.
7. Streptavidin-Alexa 647 (Molecular Probes): Stock solution: Add 1 ml of 1x PBS to 1 mg lyophilized Streptavidin-Alexa 647. Freeze at -70°C in 100 μ l aliquots. Working dilution of Streptavidin-Alexa 647: Mix 6.8 μ l Streptavidin-Alexa 647 stock solution with 3.39 ml TNB buffer for each microarray plus 10% volume overage.

3. Methods

3.1 Preparation of Synchronized Mouse Ovaries for RNA Extraction

1. Maintain adult (90, 120, 150, or 180-day old) female mice on a 14:10 light/dark cycle with lights on at 0600.

2. To exclude gonadotropin-mediated effects, suppress late estrus/metestrus mice with a GnRH antagonist (Antide©, Bachem, Torrance, CA). Antide treatment may be initiated at any time during metestrus or diestrus. Inject (i.p.) mice with 10 µg/g body weight (BW) Antide on the morning of Day 1 of treatment and again on the morning of Day 4.
3. On the evening of Day 5 (~36 hours after the last Antide injection), inject mice with 1 IU eCG (Sigma) per 5 gram BW (i.e. 5 IU for a ~25g mouse) to stimulate coordinated follicle development. Euthanize the mice by cervical dislocation 36 hours after eCG injection (*see* Note 1).
4. Quickly dissect the ovaries free from surrounding tissue and immediately place them in a sterile glass petri dish in ice-cold PBS + 0.1 % BSA (RNAlater can be substituted for PBS/BSA in this dissection). Rapidly trim all surrounding fat and connective tissue from the ovaries under a dissecting microscope. One ovary is placed in a 1.8 mL cryovial containing 1.0 mL RNAlater and placed on ice for 5–10 minutes. After 5–10 minutes, cryovials are placed in a –70°C freezer or in liquid nitrogen until RNA extraction. The contralateral ovary may be placed in cell lysis buffer (Sigma), homogenized, centrifuged, and extracts stored at –70°C for later protein analysis by immunoblot, or it may be fixed and embedded for immunohistochemistry.

3.2 RNA Hygiene

1. Ribonucleases (RNases) are very sturdy enzymes and are ubiquitous in the environment. The surface of the skin is an especially rich source of RNases. In order to preserve the integrity of RNA during the extraction and processing of samples it is important to practice careful RNA hygiene.
2. Purified water (resistivity of 18.2 M) should be treated with diethylpyrocarbonate (*see* Note 2) to destroy RNases. For enzyme reactions, use commercial nuclease-free water rather than DEPC-treated water as DEPC may interfere with some enzymes. DEPC will also interfere with UV spectrophotometry so use commercial nuclease-free water to dilute nucleic acids for spectrophotometry.
3. Treat instruments with an RNase reducing agent such as RNase ZAP and rinse with DEPC-treated water. Autoclaving alone does not destroy RNases. Pipet tips, centrifuge tubes, and other disposables should be certified nuclease-free. Gloves should be worn at all times when working with RNA.

3.3 RNA Extraction

1. To extract RNA, blot the RNAlater from the ovaries, weigh the tissues, and mince the ovaries into 600 µl TRI reagent. A single mouse ovary weighs 2.4–4.0 mg. If

¹The synchronization with Antide should yield ovaries comparable to a natural cycle on the morning of proestrus. The timing may be adjusted to obtain earlier or later stage ovaries. To obtain luteinized ovaries, hCG (1 IU/5g BW) may be given 48 hours after eCG with euthanasia 12–24 hr later.

²Add 1 ml of DEPC to 999 ml water and stir on a stir plate overnight. Autoclave the water to destroy the DEPC.

ovaries are being pooled, 30–50 mg of tissue can be processed in the volumes specified.

2. Homogenize the tissues with a Polytron homogenizer using a 7 mm probe. (The 7 mm probe fits well in a 12 × 75 mm test tube or a 1.5 ml Eppendorf centrifuge tube.) The probe must be thoroughly cleaned before use (*see* Note 3). Place the tube containing the sample in a beaker of ice. Homogenize the sample for two pulses of 10 sec each, separated by 30 sec rest on ice to prevent overheating of the sample. Run the Polytron probe in 400 µl fresh TRI reagent to rinse any residual tissue fragments from the probe.
3. Combine the two aliquots of TRI reagent into one (*see* Note 4). Add 200 µl bromochloropropane and 60 µl 3 M sodium acetate to the homogenate in TRI reagent, mix well, then incubate on ice for 15 min.
4. Transfer the sample to a 1.5 ml centrifuge tube and centrifuge for 5 min at 8,000× g. The clear aqueous (top) layer contains the total RNA.
5. Remove the aqueous layer, carefully avoiding the interface between layers (*see* Note 5). Add 1 ml RLT buffer to the aqueous phase (approximately 450 µl) and mix. Add 1.2 ml of 100% ethanol and mix.
6. For each sample, place a silica gel membrane spin column in a collection tube. Add 700 µl of the sample mixture to the membrane and centrifuge for 30 sec at 10,000 × g.
7. Discard the flow through and repeat the addition of sample mixture in 700 µl aliquots until all of the sample has been centrifuged through the membrane.
8. Wash the membrane with 350 µl of wash buffer RW1 and discard the flow through.
9. Add 80 µl of diluted DNase directly to the silica gel column and incubate at room temperature for 15 min. This step is necessary to ensure removal of residual DNA. DNA does not bind well to this silica gel membrane, but since the RNA will be used for microarray it is imperative that all traces of genomic DNA be removed from the sample.
10. After the incubation, add 350 µl of wash buffer RW1 to the membrane and centrifuge for 30 sec at 10,000× g and discard the flow through.

³To clean the 7 mm Polytron probe, fill a 50 ml graduated cylinder with DEPC-treated water and laboratory detergent. Run the Polytron probe in the soapy water. Run the Polytron probe in clean DEPC-treated water several times to rinse. Spray the outside and inside of the probe with RNase ZAP and rinse thoroughly with DEPC-treated water. When homogenizing multiple tissues, run the probe in a graduated cylinder of DEPC-treated water between each sample to prevent cross contamination of samples. It is also important to clean the homogenizer probe immediately after use. TRI reagent is corrosive and will ruin the probe if it is not removed. Clean with laboratory detergent in DEPC-treated water as at the beginning of the homogenizations.

⁴When working with small quantities of tissue this technique rescues the residual sample that is retained in the Polytron probe and would otherwise be lost. An alternate method when working with very small samples is to use a reagent kit such as the Ambion RNAqueous micro kit. This kit is designed for extraction of RNA or DNA from paraformaldehyde fixed tissue sections, but works very well for small unfixed tissues as well. The kit uses a protease mix to digest tissue and release the RNA and does not utilize a homogenization step.

⁵After centrifugation, the aqueous (clear, top) layer contains the RNA, the organic (pink, bottom) layer contains proteins, and the DNA is at the interface. It is important to avoid the interface when withdrawing the RNA layer in order to reduce DNA contamination of the RNA sample. Proteins can be extracted from the organic layer as described elsewhere (11).

11. Wash the membrane twice with 500 μ l RPE wash buffer, passing each wash through the membrane by centrifugation for 30 sec at 10,000 $\times$ g and discarding the flow through.
12. Centrifuge the membrane without adding buffer for 2 min at 14,000 $\times$ g to dry the membrane. Move the membrane to a new, well-labeled 1.5 ml collection tube.
13. To elute the RNA, add 50 μ l nuclease-free water to the center of the membrane. Incubate for 10 min at room temperature.
14. Centrifuge the membrane for 1 min at 10,000 $\times$ g. Repeat the elution step with another 50 μ l of nuclease-free water, incubate at room temperature for 10 min, and centrifuge into the same collection tube.
15. Discard the membrane. The purified RNA is now located in 100 μ l of nuclease-free water (*see* Note 6). Store the purified RNA at -70° C.

3.4 Analysis and Quantitation of RNA

1. Assess the quality and quantity of the purified total RNA using the Agilent RNA 6000 Nano LabChip in an Agilent Bioanalyzer. This microfluidics chip replaces both quantitation by UV spectrophotometry and analysis of quality by agarose gel electrophoresis and uses only 1 μ l of RNA sample.
2. Place 550 μ l of the gel matrix on a spin filter cartridge included with the reagent kit and centrifuge for 10 min at 1500 $\times$ g. Store at 4° C in 65 μ l aliquots.
3. Before use, bring reagents to room temperature including one 65 μ l aliquot of gel matrix, the dye concentrate, and the marker mixture diluent. Vortex the dye concentrate and centrifuge 5–10 sec at 10,000 $\times$ g.
4. Add 1 μ l of dye concentrate to the aliquot of gel matrix. Vortex well to mix and centrifuge at 13,000 $\times$ g for 10 min in a 1.5 ml centrifuge tube.
5. Pipet 9 μ l of the gel matrix into the 3 specified wells on the chip and pressurize one specified well with the accompanying priming station.
6. Pipet 5 μ l marker mixture diluent into the 12 sample wells as well as the RNA ladder well. Denature the RNA samples and ladder at 70° C for 2 min and chill on ice. Add 1 μ l of ladder to the appropriate well and 1 μ l of sample to each sample well.
7. Vortex the chip for 1 min at 2400 rpm using the vortex adaptor.
8. Place the chip in the Agilent Bioanalyzer for analysis. The readout from the Agilent Bioanalyzer is illustrated in Fig 1.

⁶The RNA can be eluted into a smaller volume, but our experience is that recovery is better if we use two elution steps with 50 μ l volume each. If the sample is too dilute for downstream purposes the RNA can be concentrated with a SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA). In our hands, the silica gel membranes yield a more consistent RNA sample than the previous method using phenol/ chloroform/ isoamyl alcohol (11). The spin column purification technologies for RNA continue to evolve and a variety of these matrices are now available. The RNeasy silica gel membranes exclude RNA species smaller than 200 nucleotides so this method cannot be used if microRNAs are desired; however, the RNAqueous micro Kit (Ambion) uses a similar spin column technology and will recover microRNAs.

3.5 Synthesis of First and Second Strand cDNA and cRNA

1. This protocol is designed to synthesize enough labeled cRNA for one microarray using 0.2–2.0 µg total RNA as the starting material (*see* Note 7). The volume of total RNA must be 10 µl or less. If the volume is greater than 10 µl, use vacuum concentration (i.e., SpeedVac) to reduce the volume. If the volume is less than 10 µl, add nuclease-free water.
2. Mix the 10 µl of total RNA with 1 µl of diluted bacterial control spike RNA and 1 µl of T7 Oligo (dT) primer. Incubate the mixture for 10 min at 70°C then chill the sample on ice. The bacterial control RNA is carried through the entire reaction series and serves as a control for the reaction series and also for the hybridization as bacterial samples are spotted on the microarrays as well as genes of the species of interest. The T7 Oligo (dT) primer is composed of a tract of oligo (dT) sequence and the sequence of the T7 promoter. The oligo (dT) section of the primer hybridizes with the poly-A tail of messenger RNAs in the sample. During the reverse transcription reaction in the next step, the sequence of the T7 promoter is added to each mRNA that is reverse transcribed.
3. Prepare a reverse transcription (RT) master mix during the priming reaction. The RT master mix contains 2 µl of 10x first strand buffer, 4 µl dNTP mix, 1 µl RNase inhibitor, and 1 µl ArrayScript reverse transcriptase for each sample of the reaction plus an additional 5% volume overage.
4. Pipet 8 µl of RT master mix into each RNA sample, mix by pipetting, and centrifuge briefly to collect all volume at the bottom of the tube. Incubate at 42°C for 2 hr then chill on ice.
5. Prepare second strand master mix during the first strand synthesis incubation. For each sample, include 63 µl nuclease-free water, 10 µl second strand buffer, 4 µl dNTP mix, 2 µl DNA polymerase, and 1 µl RNase H plus 5% volume overage.
6. Add 80 µl second strand master mix to each sample, mix by pipetting, and centrifuge briefly. Incubate for 2 hr at 16°C. This reaction is especially sensitive to temperature, to the extent that if using a thermal cycler with a heated lid the heat should be turned off or the lid should be left open because the heated lid will prevent the reaction from occurring at the appropriate temperature.
7. During the second strand synthesis reaction nuclease-free water should be heated to 50–55°C for the cDNA purification step.
8. When the second strand cDNA incubation is completed, add 250 µl of cDNA binding buffer to each sample and mix by pipetting up and down.

⁷If less than 0.2 µg total RNA is available, two successive rounds of cRNA synthesis can be employed—see section 3.6.3. Be aware of the time demands of this protocol. From initiation of the synthesis of first strand cDNA to the final scanning of microarray slides requires 3 days. There are several points (noted in the methods) at which the samples can be frozen and the experiment resumed at a later time.

9. Transfer the mixture to a cDNA filter cartridge that is seated in a 1.5 ml collection tube for removal of salts and enzymes. Centrifuge the filter cartridge for 1 min at 10,000× g and discard the flow through.
10. Wash the filter cartridge once with 500 µl wash buffer, centrifuge for 1 min at 10,000× g, and discard the flow through. Centrifuge the filter cartridge for 1 min at 10,000× g without the addition of buffer to dry the cartridge, then move the filter cartridge to a new, well-labeled 1.5 ml collection tube.
11. Pipet 12 µl of preheated (50–55°C) nuclease free water onto the center of each cartridge. Incubate at room temperature for 2 min then centrifuge for 1.5 min at 10,000× g to elute the cDNA.
12. Add a second 12 µl aliquot of preheated nuclease-free water to each cartridge, incubate for 2 min at room temperature, and centrifuge at 10,000× g for 1.5 min to elute into the same collection tube.
13. The purified double-stranded cDNA is now located in the approximately 20 µl volume that was eluted in two steps from the filter cartridge. Discard the cartridge. Store the purified cDNA at –20°C or proceed with the in vitro transcription (IVT) reaction for the synthesis of biotinylated cRNA.
14. The IVT master mix can be prepared during the second strand cDNA incubation and held on ice during the cDNA purification step.
15. Add 20 µl of IVT master mix to each sample of purified double-stranded cDNA (20 µl) and mix by pipetting.
16. The IVT reaction is incubated at 37°C for 14 hr. The T7 enzyme is an RNA polymerase that will transcribe any sequence containing the double-stranded T7 promoter at its 5′ end. Because the T7 oligo (dT) primer used in the reverse transcription reaction (step 3.5.2 above) contained the sequence of the T7 promoter, each double-stranded cDNA transcript synthesized in step 3.5.6 will contain a double-stranded promoter for the T7 RNA polymerase enzyme at its 5′ end and will thus serve as a template for the T7 RNA polymerase. The biotin-NTP mix contains ATP, GTP, CTP, and biotin-11-UTP, consequently the cRNA synthesized in the IVT reaction is labeled with biotin.
17. Add room temperature nuclease-free water (60 µl) and RNA binding buffer (350 µl) to the IVT reaction product.
18. Add ethanol (250 µl) to each sample, pipet 3 times to mix, and transfer immediately to an RNA filter cartridge seated in a collection tube (*see* Note 8). This step is performed to remove enzymes, salts, and excess nucleotides from the cRNA.
19. Centrifuge the filter cartridges for 1 min at 10,000× g and discard the flow through.

⁸Add the water and RNA binding buffer to all of the samples. Add ethanol to one sample and transfer that sample to its filter cartridge before adding ethanol to the next sample. The cRNA is in a semi-precipitated state after addition of the ethanol so it is important to transfer it to the cartridge rapidly.

20. Wash each cartridge with 650 μ l wash buffer and centrifuge for 1 min at 10,000 $\times$ g. Centrifuge the filter cartridge for 1 min at 10,000 $\times$ g without the addition of buffer to dry the cartridge, then move the filter cartridge to a new, well-labeled tube.
21. Pipet 100 μ l of preheated (50–60°C) nuclease-free water onto the filter cartridge and incubate at room temperature for 2 min. Nuclease-free water must be heated to 50–60°C before beginning cRNA purification.
22. Centrifuge the cartridge for 1.5 min at 10,000 $\times$ g to elute the cRNA from the cartridge. Discard the cartridge.
23. Quantitate cRNA: Dilute 2 μ l of cRNA in 78 μ l nuclease-free water (dilution factor of 40) and read the UV absorbance at 260 nm and 280 nm using an 80 μ l quartz cuvette.
24. Calculate the concentration of cRNA by the equation:
$$\text{Absorbance}_{260} \times \text{dilution factor} \times 40 \mu\text{g/ml} \times 0.001 \text{ ml}/\mu\text{l} = \text{concentration in } \mu\text{g}/\mu\text{l}.$$
25. If the absorbance of cRNA at 260 nm is 0.54 then the concentration of cRNA is 0.864 $\mu\text{g}/\mu\text{l}$ ($0.54 \times 40 \times 40 \times 0.001$). Store the purified cRNA at –70°C or proceed to the next step.

3.6 Hybridization

1. cRNA must be fragmented prior to hybridization. 10 μg of purified biotinylated cRNA is needed for each microarray in a volume of 20 μl or less. If the volume containing 10 μg is greater than 20 μl , the sample should be concentrated using a vacuum centrifuge (i.e., SpeedVac).
2. Using the sample in the example in step 3.5.25 above, 11.57 μl of sample will contain 10 μg cRNA ($10 \mu\text{g}/0.864 \mu\text{g}/\mu\text{l} = 11.57 \mu\text{l}$). Add 8.43 μl of nuclease-free water to 10 μg cRNA to bring the volume to 20 μl .
3. If the synthesis reaction did not yield 10 μg of cRNA (this typically occurs if the starting material is less than 0.2 μg of total RNA), two rounds of cRNA synthesis may be employed in order to provide enough cRNA for the microarray. In this case, the first round of cRNA synthesis is the same as a single round synthesis protocol except that none of the NTP's in the IVT reaction are modified; biotinylated cRNA cannot be used as the source material for cDNA synthesis.
 1. The purified, unmodified single-stranded cRNA (up to 2 μg in a maximum of 10 μl) from the first strand reaction is primed for the second round of cDNA synthesis using 2 μl random primers. Incubate at 70°C for 10 min then chill on ice.
 2. Mix 2 μl 10x first strand buffer, 4 μl dNTP mix, 1 μl RNase inhibitor, and 1 μl reverse transcriptase plus 5% volume overage for each sample to prepare a first strand master mix.

3. Add 8 μ l of this master mix to each sample, mix, and incubate at 42°C for 2 hr.
4. Add 1 μ l RNase H to each sample and incubate for 30 min at 37°C. The RNase removes the cRNA template so that only the newly synthesized first strand cDNA remains to serve as the template for second strand cDNA synthesis.
5. Add 5 μ l T7 Oligo (dT) primer to each sample and incubate at 70°C for 10 min then chill on ice. This is the same primer used for the reverse transcription reaction in the first round of amplification. It will prime the second strand synthesis of cDNA and ensure that each transcript bears the T7 promoter sequence required for the IVT reaction.
6. Prepare second strand master mix while the priming reaction incubates. Include 58 μ l nuclease-free water, 10 μ l 10x second strand buffer, 4 μ l dNTP mix, and 2 μ l DNA polymerase per sample plus 5% volume overage.
7. Add 74 μ l second strand master mix to each sample and incubate at 16°C for 2 hr. Complete the purification of double-stranded cDNA. Synthesize, purify, and quantitate biotinylated cRNA as described above.
4. Add 5 μ l of Fragmentation buffer to 10 μ g cRNA and incubate the sample at 94°C for 20 min, then chill on ice for a minimum of 5 min (*see* Note 9).
5. Prepare a hybridization solution master mix containing 78 μ l hybridization buffer component A, 130 μ l component B, and 27 μ l nuclease-free water for each sample plus 5% volume overage.
6. Add 235 μ l of the hybridization solution master mix to each fragmented sample of cRNA. Mix the samples by vortexing and denature the cRNA by incubating at 90°C for 5 min. Chill samples on ice for 5 min before loading the microarrays.
7. Use CodeLink (6–8) Whole Mouse Genome microarrays. These microarrays contain approximately 30,000 single-stranded 30-mer oligonucleotide probes for mouse genes/transcribed sequences. CodeLink microarrays are treated glass microscope slides designed with a removable flexible coverslip with a tab. The coverslip is glued around the edges with a port at each of the four corners.
8. Using a wide-bore 1 ml pipet tip, 250 μ l of hybridization solution containing fragmented biotinylated cRNA is slowly injected through one of the ports.
9. The ports are sealed with sealing strips and the microarrays are incubated at 37°C for 18 hr in a shaking incubator set at 300 rpm.
10. In preparation for post-hybridization wash steps, 240 ml of 0.75X TNT buffer should be brought to 46°C in a waterbath overnight.

⁹The cRNA is fragmented by metal-induced hydrolysis. The purpose of fragmentation of the cRNA is to reduce secondary and tertiary RNA structure that interferes with RNA hybridization to oligonucleotide spotted on the microarray slide, thereby improving hybridization kinetics (12).

3.7 Post-hybridization processing

1. Remove the microarrays from the hybridization incubator and carefully peel off the flexible coverslips by lifting the tab. Place the microarrays in room temperature 0.75x TNT until all coverslips have been removed.
2. Place a rack holding all of the microarrays into 0.75x TNT buffer at 46°C, and incubate the arrays for exactly one hour to remove non-hybridized and nonspecifically hybridized cRNA.
3. Move the rack of microarrays into a tray containing Streptavidin-Alexa 647 (*see* Note 10) and incubate covered at room temperature for 30 min.
4. Fill four 250 ml reservoirs with 1x TNT buffer at room temperature. Move the rack of microarrays from the Streptavidin-Alexa fluor 647 into the first reservoir and incubate for 5 min.
5. Wash 3 more times in fresh 1x TNT, 5 min each wash. Wash the slides a final time in 0.1x SSC/0.05% Tween 20 for 30 sec with continuous movement up and down of the slides in the buffer.
6. Dry the slides by centrifugation using a 96-well plate rotor to carry the rack holding the microarrays. Place the slides in an opaque slide box until they can be scanned.
7. Scan the microarrays with a GenePix Pro 4000B scanner (Molecular Devices, Sunnyvale, CA). The scanner should be turned on and the GenePix Pro software opened 15 min before use. Scan the slides, saving each with a unique name that includes the serial number of the microarray.
8. If regions of high background are identified in the scans, the final washing step in 0.1x SSC/0.05% Tween 20 for 30 sec should be repeated and the slide scanned again.

3.8 Analysis of Microarray Data

1. Scanned microarray images are imported into CodeLink 5.0 software (Fig 2A). This software associates the image of each spot on the array with an expression value that reflects the pixel intensity of the fluorescence of the hybridized sample (Fig 2B). The software also associates the expression value of each spot with gene identifiers (i.e., gene name, GenBank accession number, Locus Link identifier). CodeLink software also provides several tools to analyze the quality of the microarrays. For example, the number of spots with background contamination can be visualized as well as the coefficient of variation of the microarray slide set.
2. A useful tool allows all of the spots on one microarray to be graphed against all of the spots on a second microarray. When two arrays measuring gene expression from the same tissue are graphed against each other, there is a strong correlation between the two data sets because the majority of genes are expressed identically (Fig 3A). The broadening of the line into an arrow shape reflects genes whose

¹⁰The Alexa 647 fluorescent signal does not photobleach as rapidly as some other fluorescent probes.

expression is near background. In contrast, when there is a problem with a sample, the graph yields a “cloud effect” (Fig 3B).

3. If the cloud effect is observed when two biological replicates are graphed, then the array that is the source of the problem must be discarded and the microarray for that sample run again.
4. Data can be exported from CodeLink software in a variety of formats that support software programs for further analysis of microarray data. We routinely export data in Excel format and in a tab-delimited format specified for upload to GeneSpring software (*see* Note 11).
 1. Data from the Excel output are uploaded to Acuity software (Axon). This program is the first point at which we can observe gene names associated with expression values.
 2. Data from the tab-delimited format are uploaded to GeneSpring 7.0 (Agilent) (Fig 2C). GeneSpring is used to normalize the data.
 3. The expression of each gene is normalized to the median gene expression, and each microarray is normalized to the 50th percentile of gene expression. Gene Spring also transforms the data to log base 10. Statistical analysis is performed using the normalized and log-transformed data.
 4. GeneSpring 7.0 performs t-tests for comparisons of two groups or analysis of variance for comparisons of multiple groups. The p value can be varied and should be set at 0.05 or lower.
 5. Since thousands of comparisons are performed across the microarray, a multiple testing correction such as the Benjamini and Hochberg False Discovery Rate should be applied. With this test, approximately 5% of the genes pass the test by chance. GeneSpring also provides tools for cluster analysis and for gene ontologies (*see* Note 12).
 6. Many genes, sometimes several thousand, are often identified as exhibiting significantly different expression (Fig 2D). The significance lists include genes whose expression is near the limits of detection (near background) as well as genes for which the difference between the comparison groups is minimal. To make the lists of genes more manageable, we eliminate genes for which the means of all comparison groups are near background (< 0.02) as well as genes for which the group means are not greater than 2-fold different from each other ($\pm 10\%$) (*see* Note 13).

¹¹More recent versions of GeneSpring appear to have changed their algorithms for statistical analysis as well as the user interface and are less user friendly than the 7.0 version.

¹²CodeLink microarrays use a single sample on each array, and therefore a single dye for each array. Data analysis therefore deals with fluorescent intensity rather than intensity ratios.

¹³The 2-fold cut-off is an arbitrary point widely used in gene expression studies. It is based on the assumption that if the means of two groups differ by less than 2-fold, the differences are not likely to be biologically significant even if they are mathematically significant. It is certain that some biologically important genes will be missed by using the 2-fold cut-off, but it is expected that the majority of genes will be identified in most tissues.

7. CodeLink microarrays contain many expressed sequence tags (ESTs) and gene sequences that are not named and whose functions are unknown. These genes are typically set aside but not discarded. With time, these genes will be associated with a name and function and it will be possible to fit them into known gene ontologies (Table 1).
8. Other software packages are available that are designed specifically to identify relationships among differentially expressed genes in a given experimental paradigm, such as Ingenuity Pathways Analysis (Redwood City, CA) and Ariadne Pathway Studio (Rockville, MD). These programs can be useful for data analysis. However, there is no substitute for analyzing your own data. The pathway analysis software programs recognize relationships that fit specific algorithms. If relationships in your data do not fit those algorithms, the relationships will be missed by the software.
9. It is important to carry out confirmatory studies with selected genes using complementary technologies. Real time RT-PCR (5,6) confirms differential gene expression. Similarly, immunoblot (6), enzyme linked immunosorbent assay, and immunohistochemistry (5) confirm differential expression of the protein products of selected genes.
10. Microarray data should be deposited in a public database such as the Gene Expression Omnibus (GEO) database that is maintained by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/geo) as recommended by Minimum Information About a Microarray (MIAME) standards (9). Many journals require public deposition of microarray data before publication.
11. The study of the effect of aging and obesity on gene expression in the mouse ovary (10) is a good example of the types of data and results that can be gained from DNA microarray experiments. Gene expression data were compared between young (90 day) versus old (180 day) mice with the lethal yellow gene mutation and 90 versus 180 day old lean black littermates. In addition, comparisons were made between black versus lethal yellow at 90 days of age and between black versus lethal yellow at 180 days of age. A number of genes related to sterol synthesis and metabolism were shown to be differentially expressed in the ovaries of aging (180 day old) lethal yellow mice compared to black mice but those genes were not differentially expressed at 90 days of age. The data suggest that differential expression of these genes is directly related to acquired obesity and not to the mutation that causes the lethal yellow phenotype.

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Fig 1.

Readout from Agilent Bioanalyzer RNA 6000 Nano LabChip. The bioanalyzer scans each RNA sample and produces an electropherogram for each sample. The electropherograms are interpreted into an gel view that mimics the appearance of the traditional agarose gel (bottom left corner) showing the RNA molecular weight marker ladder and the 18s and 28s ribosomal RNA bands of the 12 samples. In the figure, sample 1 is highlighted. The individual electropherogram for sample 1 is shown in the center of the figure with the gel view for that sample to its right. The RNA concentration is shown below in ng/μl.

Fig 2.

Images of DNA microarray analysis. Panel A shows the appearance of raw data from the microarray scanner; the varying intensities of each spot on the array can be seen. Panel B illustrates data from CodeLink 5.0 software which assigns a numeric value to the pixel intensity of each spot on the array associates that information with the gene name. Data from GeneSpring 7.0 software comparison of gene expression in the ovaries of 90 versus 180 day old lethal yellow mice are shown in Panels C and D. Each line in these graphs represents one gene. All genes on the microarray are shown in the graph in Panel C, whereas Panel D shows only the genes that were found to be significantly different between 90 and 180 day old mouse ovaries.

Fig 3.

Log base 10 data from two microarrays are graphed against each other. Panel A: The majority of genes in a given tissue are expressed equally; therefore, graphing the log base 10 data from two microarrays shows a strong correlation with less than 2-fold difference in expression. (The faint lines on each side of the regression line illustrate 2-fold difference in expression.) The broadening of the data into an arrow shape, shown in lighter gray, reflects genes whose expression is near background. Panel B illustrates the “cloud effect” observed when a failed microarray is graphed against a valid microarray. The data from the microarray in panel B was discarded.

Gene expression data from DNA microarray analysis

Table 1

Table 1 Genes expressed in the ovaries of 90 day old (90 d) versus 180 day old (180 d) lethal yellow mice were compared. Shown here are the expression values at 90 and 180 days for 9 expressed sequence tags that represent unnamed genes cloned from ovary tissue or embryonic stem cells. In addition to the gene name or description of the expressed sequence tag and expression data, other types of data that can be obtained from DNA microarray analysis include the p value that denotes significant differences in gene expression and fold expression values (Fold Exp) (expression at 180 days divided by expression at 90 days). In addition, the GenBank accession number allows searching of NCBI databases for a variety of types of gene and protein information. Expressed sequence tags eventually are identified and associated with a cellular function. For example, an NCBI search found that AK054275, denoted with an asterisk in the table, has been identified as Lcor, a ligand dependent nuclear receptor corepressor, also known as transcription factor *Mir2* (13)

GenBank ACCN#	Expressed Sequence Tag Description	90 d	180 d	p value	Fold Exp
AK087684	2 days pregnant adult ovary cDNA, RIKEN clone:E3300004G06	0.22	1.70	0.048	7.7
AK054275*	2 days pregnant adult ovary cDNA, RIKEN clone:E3300009D23	0.63	1.99	0.030	3.2
AK054483	2 days pregnant adult ovary cDNA, RIKEN clone:E330031A18	0.61	1.41	0.026	2.3
AK054536	2 days pregnant adult ovary cDNA, RIKEN clone:E330038L21	1.82	4.95	0.017	2.7
AW553537	Mouse Newborn Ovary cDNA Library cDNA clone L0228E02 3'	1.34	0.52	0.018	0.4
AW555412	Mouse Newborn Ovary cDNA Library cDNA clone L0255E08 3'	0.21	0.44	0.038	2.2
BB556413	RIKEN 2 days pregnant adult ovary cDNA clone E330023M09 3'	0.63	1.33	0.013	2.1
BB559749	RIKEN 2 days pregnant adult ovary cDNA clone E330040F24 3'	1.53	4.67	0.013	3.1
AV093810	C57BL/6J ES cell cDNA clone 2400004K12	19.62	141.63	0.01	7.2