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INTRODUCTION

The objective of this project is to identify genetic differences existing between atypical ductal hyperplasia (ADH), which are premalignant cancer precursor lesions, and ductal carcinoma in situ (DCIS), the earliest recognized human breast malignancy. We hypothesize that investigation of these differences should identify genes whose dysregulation is important to the transition to malignancy. To achieve this objective, the project's first aim is to optimize techniques to preserve mammary glands, cut and stain sections, identify and microdissect areas of interest (i.e, hyperplasia and cancer), extract RNA and assess its quantity and quality.

BODY

Task 1: Microdissect and extract mRNA from murine specimens. Months 1-18.

a. *Obtain Optimal fixed blocks from Drs Seldin and Cardiff's archive (starting with ~ 10 blocks containing mammary glands from 3-6 transgenic mice). Archive OCT-embedded and frozen mammary glands from mice currently being sacrificed for other experiments.*
b. *Section and stain fixed tissue with eosin, identify dysplastic vs malignant areas, laser capture microdissection (LCM) areas of interest.*
c. *mRNA extraction from microdissected areas, using techniques designed for small quantities. Determine quality and approximate quantity of mRNA.*
d. *If suboptimal, adjust staining conditions, mRNA extraction technique; if still not adequate, use OCT-embedded frozen tissues for sectioning, staining, microdissection and extraction and mRNA .*

As reported last year, we have completed Task 1. Neither Optimal-fixed, nor ethanol-fixed tissues generated adequate RNA. However, we found that frozen, OCT-embedded mammary tissue, stained with dilute H&E, permits histologic diagnosis and generates good quality RNA after LCM. We concluded that prospective collection and analysis of mammary tissues using this technique was needed to accomplish the project's second aim.

Task 2: Human tissue acquisition, preservation, LCM and mRNA extraction. Months 4-24

a. *Identify cases, acquire and preserve human breast tissues that would otherwise be discarded. If results from Task 1 indicate that Optimal fix preserves mRNA, then human tissues will be fixed with that preservative, in addition sections will*

be be OCT-embedded and frozen. If Optimal fix does not yield adequate RNA, tissues will be frozen only.

b. *Cut blocks, stain sections and identify areas of interest, perform LCM and mRNA extractions.*

We have been collecting human breast tissues, according to annually updated IRB approved procedures. Tissue acquisition continues to require considerable effort, since it demands communication and cooperation between surgeons, pathologists, nurses and ourselves. Despite these obstacles, we have been able to acquire breast tissue from 38 cases thus far (28 within the last year). These are all frozen in liquid nitrogen and placed in OCT.

However, we do not know until each case's tissues have been sectioned, stained and reviewed whether they contain the lesions of interest. As anticipated only a small fraction of the collected cases are useable since useable cases need to contain samples of both atypical ductal hyperplasia and carcinoma in situ. We have found that atypical ductal hyperplasia lesions are far less frequent than carcinoma in situ, and thus far have identified 5 cases with both ADH and DCIS, although not all 38 cases have yet been screened. However, from all 5 cases, we have been able to cut blocks, lightly stain sections, perform LCM and mRNA extractions and obtain good quality RNA. We have been fortunate to have access to a nanospec (Nanodrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc) which permits us to rapidly quantify small-sample RNAs.

In sum, work on Task 2 is progressing as scheduled. Although originally we were going to discontinue tissue collection after Month 24, we plan to continue collecting since we don't know for sure how many useable specimens we actually have.

Task 3: Suppression Subtractive Hybridization (SSH). Months 12-30.

a. *Perform SSH, clone amplification products into T/A cloning vector, isolate plasmid DNA, have clones (estimate: 10 – 100) sequenced.*
b. *Identify and analyze sequences using Blast or Vector NT programs.*

The goal of this task is to identify genes differentially expressed in premalignant lesions (ADH) vs malignant cancers (DCIS). In the last year we have made substantial progress on this aim. The technique we originally planned to use was suppression subtractive hybridization (SSH), but rapid technological advancements in the past 3 years, particularly the development and increasing availability of microarray facilities have made SSH obsolete. Our goal is particularly suited to microarray technology: one problem with microarray experiments is that they tend to generate too many differentially expressed sequences and it is difficult to distinguish meaningful from extraneous

sequences. However, in our experiments, the 2 RNA pools being compared (from ADH and DCIS) are taken from the same specimen. Therefore, many extraneous sequences not related to cancer progression will be present (or absent) in both samples. Consequently, our microarray results should yield fewer "false positive" differentially expressed sequences, and should be enriched for meaningful sequences.

Therefore, we have utilized the Boston University Microarray Resource (opened in 2002) to perform a series of experiments optimizing the techniques required to identify differentially expressed genes between RNAs microdissected from frozen breast tissue. Because the amount of RNA obtained from microdissected tissues is in the nanogram range, but the amount required for SSH or microarray is in the microgram range, the microdissected RNA needs to be amplified. Techniques for doing this have been published and are commercially available [1-6]. Our first experiment has been to demonstrate the reliability, reproducibility and correlation between isolations, amplifications and hybridizations of replicate amplified and unamplified samples. **Figure 1** diagrams the sample preparation and methods.

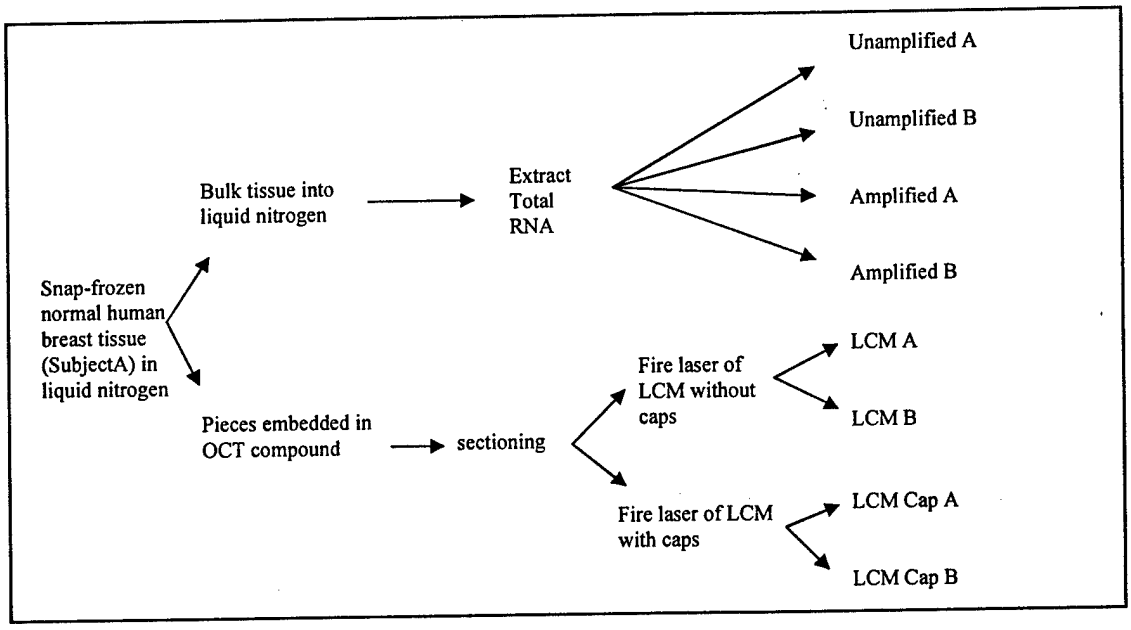

Figure 1. Schema of tissue acquisition and sample preparation. Normal human breast tissue was collected from specimen A after mastectomy and snap-frozen in liquid nitrogen. In one set of experiments, RNA is directly extracted from pieces of this tissue. Two 5 μg aliquots of RNA (unamplified A, unamplified B) are labeled and hybridized. Two 100 ng aliquots of RNA (amplified A, amplified B) are amplified, and then labeled and hybridized. In the other set of experiments, pieces of snap-frozen breast tissue are embedded in OCT compound and then sectioned by cryostat. In one subgroup (LCM A, LCM B), tissue is lasered directly (no cap is used) and scraped from the slides for RNA extraction. In the other subgroup (CAP A, CAP B), tissue is lasered and collected using caps. In both subgroups, RNA was processed in independent amplification, labeling, and hybridization steps.

As shown in the scatter plots of **Figure 2** (pg 8), we find strong correlation between all replicates (as shown in the column labeled replicates A vs replicates B), with all correlation coefficients > 0.93. In addition, we find strong correlation between all amplified samples (as shown in the rows labeled amplified, LCM and LCM Cap, which compare each of the 6 amplified samples vs the mean of the 5 other amplified samples) with correlation coefficients > 0.89.

In addition, we find acceptable correlation between amplified and unamplified samples, with r-values > 0.70. When we use a histogram to compare the frequency of > 2-fold changes in expression by signal intensity, we find that, as expected, amplified samples demonstrate some biasing of results away from low-abundant transcripts. These results are shown in **Figure 3** (pg 9), top panel. Furthermore, in amplified samples there are more repressed genes than induced genes (**Figure 3**, middle panel). This seems to be a function of the amplification process, since there is no biasing between replicates, (**Figure 3**, bottom panel).

This is the first direct comparison, we believe, of hybridizations utilizing primary tissue and comparing hybridization results from unamplified, amplified and amplified from microdissected without, and with, a cap. These results indicate that independent isolations, amplifications and hybndizations, regardless of method of isolation, yield consistent results. We are prepared to move forward utilizing the ADH and DCIS samples we have been collecting.

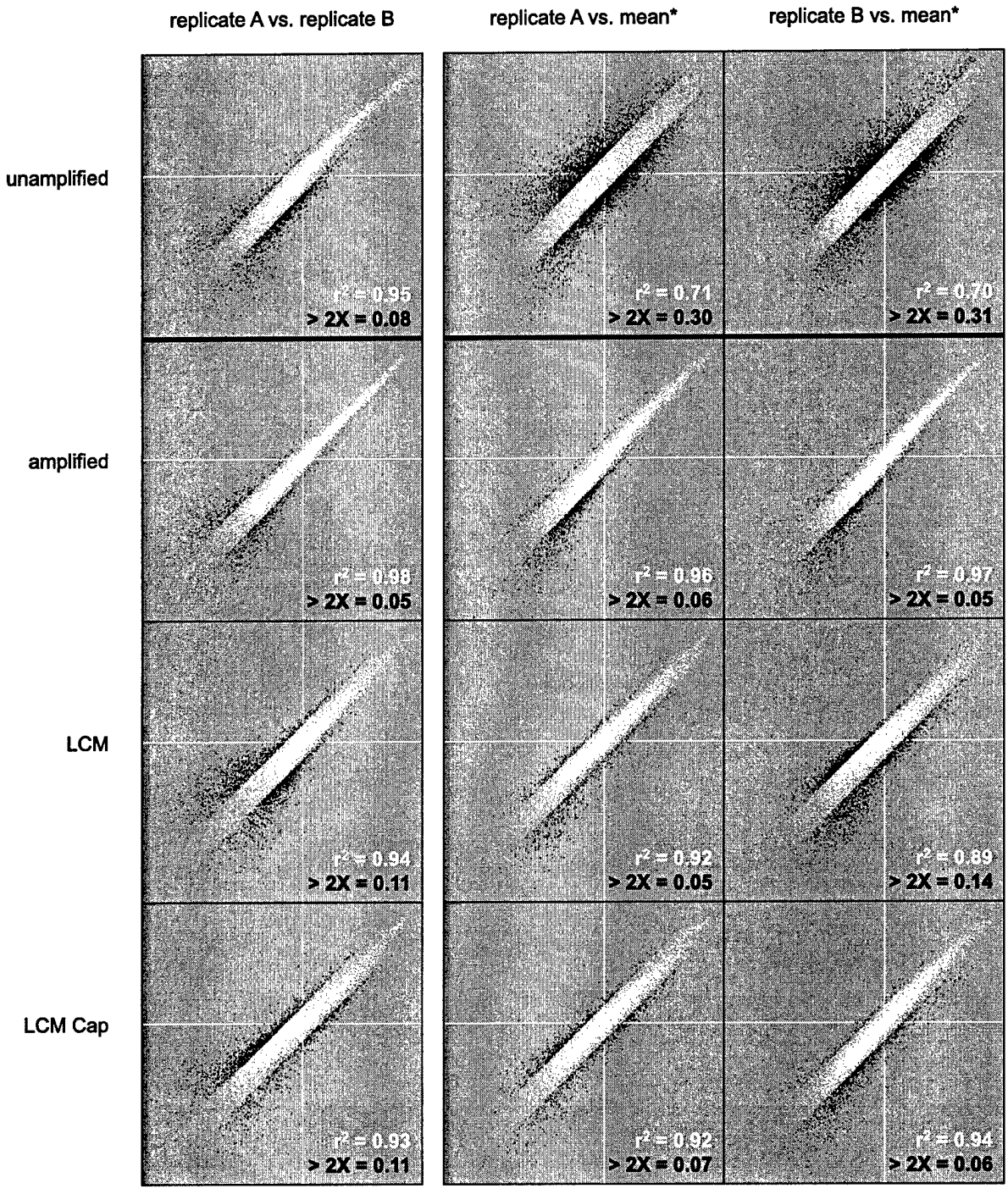
replicate A vs. replicate B
replicate A vs. mean*
replicate B vs. mean*
unamplified
amplified
LCM
LCM Cap
r² = 0.95
> 2X = 0.08
r² = 0.71
> 2X = 0.30
r² = 0.70
> 2X = 0.31
r² = 0.98
> 2X = 0.05
r² = 0.96
> 2X = 0.06
r² = 0.97
> 2X = 0.05
r² = 0.94
> 2X = 0.11
r² = 0.92
> 2X = 0.05
r² = 0.89
> 2X = 0.14
r² = 0.93
> 2X = 0.11
r² = 0.92
> 2X = 0.07
r² = 0.94
> 2X = 0.06

Figure 2

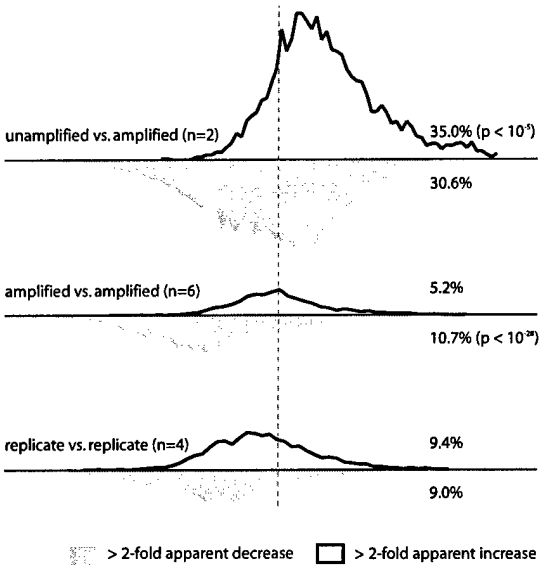
unamplified vs. amplified (n=2)
35.0% (p < 10⁻⁵)
30.6%
amplified vs. amplified (n=6)
5.2%
10.7% (p < 10⁻²⁶)
replicate vs. replicate (n=4)
9.4%
9.0%
> 2-fold apparent decrease
> 2-fold apparent increase

Figure 3

Figure Legends:

Figure 2: Scatter plots graphing hybridization intensity of one sample against another. Each x-axis is the log-average intensity of all amplified samples except for the sample on the y-axis (if it is an amplified sample). Each y-axis is the log-intensity of the single sample indicated. White cross lines are the location of the mean intensity of scaled hybridization signal. Black dots are probe-sets (i.e., genes) that are > 2-fold different between the two samples being compared. Almost all of these black dots fall below the mean intensity of both samples' probe-sets. The percent of probe-sets that are > 2-fold different between the 2 samples is provided in the bottom right corner of each graph, as is the correlation coefficient (r^2) of the 2 samples as a whole.

Figure 3: Histograms showing the frequency (y-axis) of > 2-fold apparent decrease (shaded area) and > 2-fold apparent increase (outlined white area) in probe-set expression per array as a function of signal intensity (x-axis). White cross lines are the location of the mean intensity of scaled hybridization signal.

KEY RESEARCH ACCOMPLISHMENTS

- Acquisition of human mammary tissue samples is proceeding, and samples with both ADH and DCIS are being identified, sectioned, stained, LCM'd and RNA extracted and quantitated.
- Demonstration that Affymetriz GeneChip data from independently isolated (with or without LCM), amplified, and hybridized small-sample specimens are comparable.

REPORTABLE OUTCOMES

- Manuscript in preparation demonstrating that independently isolated (no LCM, LCM with and without cap), amplified and hybridized small-sample specimens are comparable.

CONCLUSIONS

- Laser capture microdissection, amplification and hybridization of RNA from ADH and DCIS from a series of independent primary human breast tumors should yield information important to the understanding of early steps in cancer progression. These experiments are in progress, according to the time-table set out in the Statement of Work.

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APPENDICES None.