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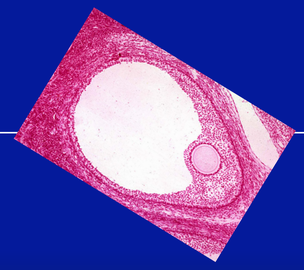

Yield of Ovarian Granulosa Cell RNA from single follicles during IVF

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Abstract

Background: There is considerable evidence of bidirectional communication between the oocyte and the surrounding granulosa cells (GCs) within the maturing human ovarian follicle. Study of GC gene expression during *in vitro* fertilization (IVF) could provide insight into predicting oocyte quality and embryo outcomes. This study aims to establishing a model for future research with primary human GCs.

Methods: Two follicles and their associated oocytes were harvested from 180 IVF patients using Ultrasound-guided transvaginal retrieval. Oocytes were stripped and the GCs were isolated and frozen. The oocyte and GC samples were tracked and matched to the ultimate embryo fragmentation score as the primary IVF outcome. Total GC RNA was extracted and RNA extracts were quantified and qualified, converted into cDNA and analyzed by single gene qPCR to establish their utility in future gene expression studies using Focused RT-PCR Arrays.

Results: Both embryos and GC RNA were successfully obtained from 130 oocytes out of the total 355 follicles sampled. Of the 130 samples, which had both GC samples and embryo outcomes, only 31 GC samples yielded sufficient quantity and quality of total RNA (A_{260}/A_{280} ratios between 1.8-2.0 and a minimum 3ug) for focused gene expression PCR array studies. Initial qPCR , run using a GAPDH housekeeping gene and CYP11A1 target gene results have shown presence of good quality cDNA in these samples.

Conclusion: Investigation of the associations between GC gene expression and oocyte/embryo outcomes is dependent on obtaining both an embryo and sufficient amounts of high quality GC-RNA. We have shown here that, during follicle sampling of standard clinical IVF cycles, the expected efficiency of obtaining both embryos and sufficient extracted RNA is very low; <10% of sampled follicles.

Introduction

Current success rates are less than 35 percent for all *in vitro* fertilization (IVF) cycles. Currently, there are no biochemical markers of oocyte quality which could increase the chance of a successful pregnancy with IVF by optimizing oocyte and embryo selection, allowing fewer embryos to be transferred and reducing multiple pregnancy rates.

Granulosa Cells (GCs) and follicular fluid (FF) surround the developing oocyte in the ovarian follicle and may provide a biochemical window into the metabolic processes important to oocyte and embryo quality. [1] Although limited in scope, several small studies have shown promise for identifying potential biomarkers by targeting GC gene expression using state-of-the-art RT-PCR arrays. [2,3,4]
Conducting these studies requires a one follicle→one embryo design allowing the GC gene expression to be tracked to a single IVF outcome.[5] Furthermore, these studies can only be ethically conducted under the standard of care. Consequently, as oocyte retrieval is the primary clinical goal, obtaining suitable GCs is a highly variable process. Accurate estimates of the amounts and quality of GC mRNA that can be obtained from a single ovarian follicle, within the clinical standard of care setting have not been reported.

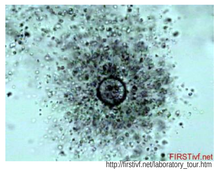
Here, we report on the quantity and quality of GC mRNA obtained from single ovarian follicles collected during a prospective study of the associations between the biochemical components of ovarian follicular fluid and embryo outcomes in women undergoing IVF. We further report the number of follicles which generated embryos suitable for morphological analysis in the context of sufficient GC mRNA for focused RT-PCR array analysis.

These estimates are critical for determining the size of participant and follicle cohorts for future studies examining GC gene expression and embryo outcomes.

Materials and Methods

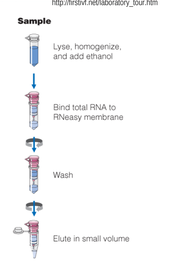
Participants: This study was approved by the UCSF Committee on Human Research and the University at Buffalo Health Sciences Institutional Review Board. One hundred eighty (180) patients undergoing IVF treatment at the UCSF Center for Reproductive Health were enrolled prospectively, prior to completion of their IVF cycle, with full informed consent.

Granulosa Cells (GCs): Following gonadotropin-induced ovarian stimulation per clinic protocols, oocytes, follicular fluid and GCs from an individual mature 18-20 mm follicle were aspirated using a single lumen 17-gauge 35-cm aspiration needle guided by transvaginal ultrasonography.



GCs were stripped from the oocyte by treatment with hyaluronidase, layered over a ficol-hypaque gradient , centrifuged aspirated, pelleted, snap frozen and stored at -80ºC.

Total RNA Extraction:
RNeasy Mini Kit from Qiagen was used to purify RNA from GCs. All procedures were performed at 4 ºC. Contrary to regular protocol cell lysis buffer (RLT) was added prior to thawing of the cells to prevented the release of Rnases during thawing.

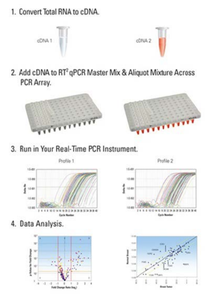


http://www.sabiosciences.com/pathwaymagazine/pcrhandbook/qiagen-measy-kits.php

RNA Yield, Purity and quality:
RNA yield and purity were measured using NanoDrop2000 UV-Vis microvolume Spectrophotometer. A_{260}/A_{280} and A_{260}/A_{230} ratios were observed for checking protein and phenol contamination. RNA concentration was measured as:

RNA (µg/ml) =(A_{260}) x (dilution factor) x (40µg RNA/ml)/(1 A_{260} unit)

RT² First strand synthesis and single gene qPCR for QC:
RT² First strand kit with built in genomic DNA elimination and an external RNA control was used for cDNA synthesis. Samples with A_{260}/A_{280} between 1.8-2.0 and a minimum RNA yield of 3.0ug were subjected to a quality control check by running single gene qPCR. GAPDH and CYP11A1 were examined as a housekeeping Gene and a gene know to be expressed in luteinized GCs.



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Results

Table 1. The available sample and data set of Embryos with GC available for RNA isolation. Percent loss of efficiency of the samples is also included.

Sample	Number	% Yield
Patients	180	100
Total follicles	355	98.6
Follicles with oocytes	209	58.9
Oocytes fertilized	197	55.5
Embryos generated	153	43.1
Embryos with GCs	130	36.7

Results

Table 2: EFS and total RNA yield from 130 available GC samples.

EFS	Total GCs available	Total GC extracted	Usable* Samples	% usable samples
1	28	28	12	42.9
2	68	38	9	23.7
3	25	25	6	24.0
4	8	8	4	50.0
5	1	1	0	0
Total	130	100	31	31.0

EFS- *Embryo Fragmentation score;* GC- *Granulosa Cells*
**Usable defined as >3 µg of RNA with* A_{260}/A_{28} =1.8-2.0

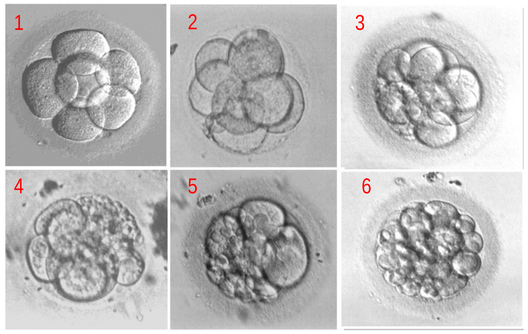

Figure 1: Embryo Fragmentation scores (1-Best, 6-Worst)

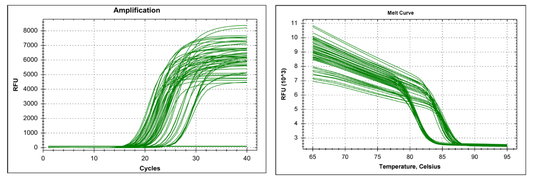

Figure 2: Single gene qPCR quality control run for first 14 samples using GAPDH and CYP11A1. Figure shows both amplification and melt curves.

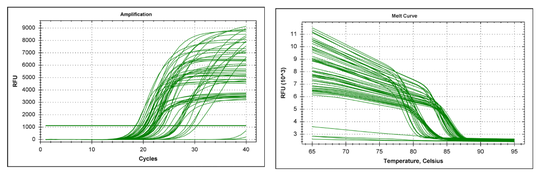

Figure 3: Single gene qPCR quality control run for second14 samples using GAPDH and CYP11A1. Figure shows both amplification and melt curves. This run also includes a Negative control.

Results

- Of 355 sampled follicles only 130 (36.7%) yielded both a successfully fertilized embryo and a sample of GCs for RNA extraction
- Of theses 130 GC samples only 31 (31%) yielded >3 µg of sufficiently pure total RNA
- Less than 10% of all sampled follicles ultimately yielded the samples necessary to examine the association between embryo outcomes (EFS) and GC gene expression
- The frequency of obtaining sufficient amounts of high quality GC RNA was highest with the best quality embryos with the lowest EFS.
- Maximum difference in gene expression studies is expected between high score and low score EFS GC.
- Single gene qPCR of GAPDH and CYP11A1 have a Ct value below 30, indicate presence of intact, suitable quality and quantity cDNA.
- Melt curve analysis on all qPCR samples indicates the presence of single amplicons for each gene.
- Non-amplification of the negative control in the second run indicates lack of cross contamination.
- Cell counting was not performed on GC after stripping

Conclusion and Future Directions

- The study design with a 180 patients with 355 individual follicles resulted into successful RNA isolation and cDNA conversion of only 31 samples.
- The efficiency of these procedures can be observed at approximately 10% from the oocyte retrieval phase.
- Once GC are extracted 25-50% yield high quality and quantity mRNA to be used for focused PCR array studies
- Future studies will need to account for low yield of matched GC samples with successful embryo generation to provide sufficient statistical power to assess the role of GC gene expression on embryo outcomes.
- Cell counting of Granulosa Cells before freezing could help shed some more perspective on the reasons of low yield of RNA.

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