Expanding the Scope of RNA-Seq
From Stem Cells to FFPE

September 10, 2014

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- Shows speaker bios
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- If you need help
Expanding the Scope of RNA-Seq
From Stem Cells to FFPE

September 10, 2014

Participating Experts

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Milwaukee, WI

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Transcriptome Sequencing: The Answer to Many Questions?

Audrey Papp
Technical Director
The Ohio State University College of Medicine
Center for Pharmacogenomics and Functional Genomics
Pharmacogenomics Core Laboratory

AAAS
September 10, 2014
The proportion of susceptibility to schizophrenia due to common SNPs (Lee et al., 2012)

Heritability due to SNPs significant in GWAS: <5%
Global heritability estimate of common SNPs: ~30%

“Estimating the proportion of variation in susceptibility to schizophrenia captured by common SNPs.”
PMID: 22344220

Comparison of heritability to schizophrenia explained by SNPs identified through GWAS and the global contribution of common variants to schizophrenia. Much of the heritability is hidden in the additive effect of common variants, but the individual variants are difficult, if not impossible to identify.  

Posted on July 30, 2012 by Ingo Helbig
In the human population, common DNA variants are likely to interact to affect wellness, and influence susceptibility to common health issues.
How do you find the functional variants that predict response?

- Assorted phenotypes
- Variable penetrance
- Multiple common variations
- Non-linear interactions
- Epigenetic influences
Why and how RNA analysis can provide insight into causative DNA variants

- Why RNA?
- Different Techniques for RNA analysis
Allelic Expression Analysis Reveals Functional DNA Variants

Allelic RNA expression indicates:

- Presence of regulatory SNPs
- Extent of effect on RNA
- Number of variants
- Linkage to indicator SNP
- Frequency of functional SNP
Pharmacogenes

**Transcription**
- ACE, VKORC1, CHRNA5, CYP2D6
  - Polymorphisms in coding region (cSNPs)
  - Altered protein sequence and function

**Splicing**
- TPH2, DRD2, CETP, SPA2
  - Altered splicing

**RNA Processing**
- CYP3A4, ABCB1
  - Structural RNA polymorphisms (srSNPs)
  - Altered mRNA processing and translation

**5', 3' UTR**
- HTR2A, NAT1

**Translation**
- DAT, OPRM1

Genes with specific functional variants identified by the OSU Pharmacogenomics Lab

Analysis of RNA transcripts in cellular compartments provides a more complete picture of RNA transcription and translation.
Pervasive Interactions Between all Classes of RNAs

- Long noncoding RNAs
  - Epigenetic regulation, structural, microRNA precursors, antisense and pseudo-genes

- Protein coding RNAs
  - Multiple transcripts per gene locus, mRNA processing, RNA editing

- Small noncoding RNAs
  - Sequestration, degradation, regulation

- Translated in polysomes, migrating to synapses, multiple polyA and TS sites, role of 3’- and 5’-UTRs

Challenge to our bioinformatics team
Distribution of RNA types in 10 brain regions

SOLiD Sequencing at OSU

Region specificity:
FPKM>2 in 2+ samples

protein coding  IncRNA  pseudogene  processed transcript

Whole transcriptome, 10 Individuals, 10 Brain Regions
Pathway Analysis of “Stable” Brain Genes
Differential expression of miRNA in human brain (Pairwise analysis)

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- t1  BA10
- t2  BA22
- t3  BA24
- t4  Insula
- t5  Amygdala
- t6  Hippocampus
- t7  Post. Putamen
- t8  Raphe Nuclei
What is the question?
Developing processes that test the hypothesis

Starting Material

Long transcripts

RNA Isolation

Quantitate Expression
What aspects?

Coding and Non-Coding?

Polyadenylated?

Stranded?

Splice Isoforms?

Short transcripts (micro RNA)

Targeted?

Allelic Expression?
RNA Analysis on the Ion Torrent™ platform

- Ion Total RNA-Seq Kit v2: Ribominus, Rnase III shearing, Stranded,
- Nugen Ovation RNA Seq v2: Low input, Ribosomal reduction combined with linear amplification, Covaris shearing, Non-stranded
Ion Proton™ Transcriptome Expectations

- Each Proton PI run should yield ≥ 70M total library reads
- For paired transcriptomes - each Barcode must contain at least 30M total library reads
- Mean read length of >90bp.
- The ERCC $R^2$ value for each library must be $\geq 0.85^*$ based on a min count of 10.
- $\geq 90\%$ of filtered reads mapped
- 17,000 genes detected at $\geq 10$ counts
- High Accuracy
Ion AmpliSeq™ Transcriptome Human Gene Expression Kit
Ion AmpliSeq™ Transcriptome
Experimental results on different tissue types

Differential expression analysis of stimulated and unstimulated IPSC derived brain organoids

<table>
<thead>
<tr>
<th>Barcode ID</th>
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<th>Mapped Reads</th>
<th>On Target</th>
<th>Targets Detected</th>
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<td>1.302</td>
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</tbody>
</table>
Method comparisons: Expression Correlations

Expression Correlation : Multiple Samples, Same sample type, Same method:
0.95-0.99 with all three methods (Ampliseq RNA, Ion Total RNA ,Nugen)

Expression Correlation of similar tissues
Human Brain Reference Pool compared to Prefrontal Cortex RNA:
0.893 Ampliseq RNA
0.895 Ion Total RNA-Seq Kit v2
0.905 Nugen Ovation RNA Seq v2

Expression Correlation between different library preparation methods
Human Brain Reference Pool:
0.652 Ampliseq RNA : Ion Total RNA-Seq Kit v2
0.646 Ampliseq RNA : Nugen
0.686 Ion Total RNA-Seq Kit v2: Nugen Ovation RNA Seq v2
Method Comparisons: Variant calling

RNA Variant Calling: 94% Concordance
Ion Total RNA-Seq Kit v2: Nugen Ovation RNA Seq v2

Allelic Expression: 59% Concordance
Ion Total RNA-Seq Kit v2: Nugen Ovation RNA Seq v2

Average Allelic Expression Imbalance
1.75 Ion RNA-Seq /RNaseIII
1.65 Nugen/Covaris
Summary:

RNA techniques for identification of functional biomarkers

- Quantitate RNA expression
- Accurately measure allelic expression
- Interrogate non-coding RNAs, microRNA’s
- Identify correlations between RNA transcript levels and variants
  - Consider isoforms (splicing, 5’UTR, 3’UTR, polyA, RNA editing)
- Focus on targeted genes
- Define functional DNA variants
- Epistatic analysis (gene:gene and gene network)
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Jose Cienfuegos
Scott Dewell

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Milwaukee, WI

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life technologies
A Thermo Fisher Scientific Brand
Using Patient Specific iPSC-Cardiomyocytes for Disease Modeling

Ulrich Broeckel, MD

Section of Genomic Pediatrics
Department of Pediatrics

Medical College of Wisconsin
Conflict of Interest

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Milwaukee WI, USA
CONGRATULATIONS!

Genetics and Genomic Medicine doesn’t need any advertising anymore

We can analyze more DNA, genes samples than ever before. And it will only get better...
Questions:

What does this gene do?

What can we do with this gene?

Oh – wait, there is no gene?

Let’s move from

*statistical association* of DNA using large populations to

*functional gene and genome analysis* in populations

Let’s study the human genome at work
Left Ventricular Hypertrophy

Definition: Increase in weight of the left heart - ventricle

Causes: Hypertension, Kidney disease
Diabetes
Age
Gender
Race

Metabolic Disease
Valvular Disease
Left Ventricular Hypertrophy

Increase of Left Ventricular Mass

Independent cardiovascular risk factor
Considerable inter-individual variability at equal blood pressure
Leads to Heart Failure;
‘Hypertensive and Diabetic Heart Disease’

Devereux et al. JAMA 2004
Next Steps Concept

Develop a disease model:

Human cells → Cardiomyocytes
Recapitulate disease → Induce disease state

Understand disease mechanisms, inter-individual variability, test for treatment
The Power of iPS cells

Cell unique as individual
Should recapitulate each individual's disease phenotype

Yamanaka, Blau. Nature 2010
HyperGEN – NHLBI Family Blood Pressure Program:

African-American and Caucasian Cohort
Phenotyping: Cardiovascular phenotypes and risk factors
Family-based ascertainment

Generate 250 iPSC lines and Cardiomyocytes
Human Cardiomyocyte Model of Hypertrophy

**Figure A:**
- Relative Cell Size by Stimulants:
  - Angiotensin II: 6.59E-05, 275.4
  - Endothelin-1: 0.0029, 226.5
  - Isoproterenol: 0.0043
  - Control: 170.1

**Figure B:**
- Images of Cardiomyocyte Treatments:
  - Ang-II
  - EL-1
  - ISO
  - Control

**Principal Component Analysis Diagram:**
- PC1 and PC2 scatter plot with various markers indicating different treatment groups:
  - Control LV biopsy
  - Hypertrophic LV biopsy normal EF
  - Hypertrophic LV biopsy low EF
  - Control iPSC cardiomyocyte
  - ISO iPSC cardiomyocyte
Substantial phenotypic variation in response to stimulation
miRNA and Cardiovascular Disease

- miRNA modulate the expression of groups of RNAs
- Play a key role in cardiovascular development
- Interest in miRNA as drug targets and as diagnostic markers
- Analysis: Array based approach: Analysis of known miRNA
  - Sequencing - novel miRNAs?

Small, Olson. Nature 2011
miRNA Sequencing

Resources:
- Databases with annotated miRNA sequences
- Sequencing allows for the identification of known & potential novel miRNAs

Using small RNA sequencing on Ion Torrent PGM 318 chip

Analysis:
- SHRiMP2, short read gapped aligner was used to map raw reads against hg19
- miRDeep2 package was used to predict novel (undescribed miRNAs)
- Quantified miRNA sequence data for known human and novel mature predicted miRNA using HTSeq
- DESeq determine differential expression
We identified significantly differentially expressed known and novel miRNAs.
Differential Expression

Known ‘hypertrophy’ miRNAs
qPCR confirms sequencing results

Predicted miRNAs
Knock down of a novel miRNA reduces BNP expression (hypertrophy) in response to ET-1 stimulation.
Knock Down: Expression Analysis

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<th>Pathway Name</th>
<th>#Gene</th>
<th>EntrezGene</th>
<th>Statistics</th>
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</thead>
<tbody>
<tr>
<td>Dilated cardiomyopathy</td>
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<td>Vascular smooth muscle contraction</td>
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</table>

TFT

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</table>
Variation Between Lines

iPSC-CMs

Significant variation in miRNA expression between different cell lines
Ion AmpliSeq™ Transcriptome Kit

Selective amplification of RNA targets
Quantitative expression analysis using sequencing platform

- Design
  - > 20,800 amplicons – one per coding, non-coding RNA. exon-exon conserved junctions
  - very high concordance with TaqMan and RNA-Seq for shared targets ($r^2$ values of 0.96 and 0.94 respectively for differential expression using UHR and HBR)
RNA Ampliseq

Pros

• Faster workflow
• Ability to pool multiple samples per sequencing run
• Reduced library prep and analysis time
• Cost
• Only target a few hundred base pairs per transcript
  • Thus potentially avoiding transcript length bias
  • Do not need 40-50 million sequencing reads per sample

Cons

• No differential isoform expression analysis
• No RNA-editing analysis
Sequencing Control Libraries

UHR – Universal Human Reference RNA

HBR – Human Brain Reference RNA

<table>
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<tr>
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<td>HBR6</td>
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Sequencing Control Libraries

- Broeckel lab sequencing run
- Life Technologies in-house sequencing run
RNA Sequencing iPSC-CM Lines

Table:

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<th>Sample</th>
<th>Mapped Reads</th>
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<th>Targets Detected</th>
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<td>93.32%</td>
<td>56.05%</td>
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<td>10,611,527</td>
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<td>1131 ET-1</td>
<td>11,992,673</td>
<td>94.60%</td>
<td>58.95%</td>
</tr>
</tbody>
</table>

at 10X or more
RNA Seq QC: ERCC Controls

We include ERCC controls in all RNA seq samples for QC

High correlation for using Ion Torrent P1 sequencing chip
1077 Unstimulated

1077 ET-1

1099 Unstimulated

1099 ET-1

1104 Unstimulated

1104 ET-1

1131 Unstimulated

1131 ET-1
Variation between iPSC-CM Lines

Differences between subject cell lines represents larger source of variation
Summary

• iPSC derived cardiomyocyte resemble human disease phenotypes

• Functional RNA analysis can lead to the identification of novel disease markers and mechanisms (miRNA...)

• Unprecedented platform for functional genome analysis

• Multiple RNA analysis platforms exist now which can be tailored to specific experimental needs

• RNA sequence analysis will continue to expand: whole transcriptome as well as targeted RNA analysis
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Expanding the Scope of RNA-Seq
From Stem Cells to FFPE

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