Presenters: Maggie Olson and Nick Struntz

The Discovery of Protein-Targeting Aptamers by SELEX

*Nat. Reviews Drug Discov.* 2010, 9, 537
What is an Aptamer?

- **Precedence**: Protein – DNA interactions in nature i.e. *Transcription Factors*

- **Characteristics of Aptamers**:
  - Double or single-strands of oligonucleotides which assume random 3D structures through base complimentarity
  - Able to bind specific molecular targets with low nanomolar – high picomolar binding affinities
SELEX Founders

“How many fundamentally different classes of structures can carry out the same catalytic activity?”

“Did the first biological catalysts arise from random sequence polymers?”

“How many random sequence polymers fold into stable, 3D structures with catalytic activity?”

Dr. Andrew Ellington
University of Texas at Austin

Dr. Jack Szostak
Massachusetts General Hospital

**In Vitro Selection**

Solid-Phase Phosphoramidite

Chemical Synthesis

DNA Pool

Selection

PCR

20-Fold Amplification

Oligo

100 random base sequence
Flanked by defined 5' and 3' ends
Approx. $10^{15}$ individual sequences

Ellington/Szostak Methodology

**Aim:**
Elucidate “site recognition” mechanisms of nucleic acid binding proteins

**Previous Work:**
Binding site comparison to determine consensus sequence

**Target:**
Interaction of bacteriophage T4 DNA polymerase (gp43) and mRNA ligand

Selective Evolution of Ligands by Exponential Enrichment

EVOLUTION

“Variation, Selection, Replication”

Often immobilized on nitrocellulose

PCR
Gold Methodology

Template Construction

1. T7 PRO
2. ligation
3. nnnnnnnn
4. ligation
5. 3

65,536 possible sequences

Tuerk, C. et al. Science 1990, 249, 505
Gold Methodology

65,536 possible sequences

Tuerk, C. et al. Science 1990, 249, 505
Gold Methodology

65,536 possible sequences
Selection of Consensus Sequence

Filter Binding Assay
Sequence gp43 bound RNA oligomers

Tuerk, C. et al. Science 1990, 249, 505
Recent Technological Advances: Combined Atomic Force/Fluorescence Microscopy

1. Library containing Aptamers
   Target Molecules on Substrate
   Bind (\& Wash)

2a. Oligo Construct:
   - Primer
   - Random Primer
   - Fluorophor

2b. Atomic force microscopy

3a. Specific binding

3b. AFM image

4. Align and overlay AFM \& fluorescence

5. Extract

6. Isolate
   Amplify \& Characterize

Peng et al. Microscopy Research and Technique 2007, 70, 372
Recent Technological Advances: Combined Atomic Force/Fluorescence Microscopy

Peng et al. *Microscopy Research and Technique* 2007, 70, 372
Recent Technological Advances: Combined Atomic Force/Fluorescence Microscopy

Peng et al. *Microscopy Research and Technique* 2007, 70, 372
Recent Technological Advances: Cell SELEX

Recent Technological Advances: Other Models

- 1. Capillary Electrophoresis SELEX
   Benefit: Eliminates stationary support and linker bias; collect as fractions

- 2. PhotoSELEX
   Benefit: Form photo-induced covalent bonding between aptamer and target protein allows for vigorous washing
Recent Technological Advances:
Other Models

1. Capillary Electrophoresis SELEX
   Benefit: Eliminates stationary support and linker bias; collect as fractions


2. PhotoSELEX
   Benefit: Form photo-induced covalent bonding between aptamer and target protein allows for vigorous washing
   Limitation: Requires 5-bromouracil

5-bromouracil

- Activated by absorption of light
- Absorbs UV in the 310 nm range
- Activated BrdU cross-links aromatic and sulfur-bearing amino acids
**Hypothesis:** Aptamer libraries (of random sequence) contain enough shape diversity to deconvolute a complex mix of molecular targets.

<table>
<thead>
<tr>
<th>Molecular Mass (kDa)</th>
<th>Identity</th>
</tr>
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<tbody>
<tr>
<td>72</td>
<td>Prothombin</td>
</tr>
<tr>
<td>180</td>
<td>C3 Complement</td>
</tr>
<tr>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td></td>
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## Validated Aptamer Targets

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<tr>
<th>Target</th>
<th>Nucleic Acid</th>
<th>$K_d$ (nm)</th>
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<td>VEGF</td>
<td>RNA</td>
<td>0.05 – 0.150</td>
</tr>
<tr>
<td>α-Thrombin</td>
<td>DNA</td>
<td>25 – 200</td>
</tr>
<tr>
<td></td>
<td>RNA</td>
<td>2.8</td>
</tr>
<tr>
<td>IgE</td>
<td>DNA</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>RNA</td>
<td>30</td>
</tr>
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<td>RNA</td>
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Advantages of Aptamers

- High Affinity/Selectivity
- Nontoxic/Nonimmunogenic
- Predictable Pharmacokinetics
- Easily prepared in bulk ($200 - $2000/g)
- Long Shelf Life
- Simple Antidote Generation
- Will refold after exposed to denaturing conditions
Disadvantages

- Short *in vivo* half life, except in ocular compartment
- Oligonucleotides highly charged and thus low bioavailability
- Targets should be in the bloodstream or on cell surfaces
Therapeutic Relevance
Case Study: Macugen® Pegaptanib

• Age-related macular degeneration (AMD) is the leading cause of blindness in people over 50
• No effective treatments

Vascular Endothelial Growth Factors

- Tyrosine kinase receptors
- Stimulate vasculogenesis and angiogenesis
- Validated as a major regulator of aberrant and excessive blood vessel growth in the eye

![Diagram of VEGF receptors](image)

**VEGF-B**
**VEGF-A**
**VEGF-C**

**VEGF-B**
**VEGF-E**
**VEGF-D**

**VEGFR-1** (Flt-1)
**VEGFR-2** (Flk-1/KDR)
**VEGFR-3** (Flt-4)

*Nat. Rev. Drug Discov.* 2006 5, 123
• The earliest work in 1994 isolated aptamers that inhibit VEGF *in vitro*
• NeXstar Pharmaceuticals carried out three separate iterations of SELEX

<table>
<thead>
<tr>
<th>Aptamer</th>
<th>Modification</th>
<th>Half-life in urine (hours)</th>
<th>Dissociation half-life</th>
<th>Binding affinity for VEGF ($K_d$, nM)</th>
<th>Binding affinity for PDGF ($K_d$, nM)</th>
<th>Ratio $K_d$ PDGF/VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX-107</td>
<td>None (minimal ligand)</td>
<td>1.4</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>NX-178</td>
<td>3’ and 5’ caps</td>
<td>17</td>
<td>12 seconds</td>
<td>2.4</td>
<td>75</td>
<td>31</td>
</tr>
<tr>
<td>NX-213</td>
<td>3’ and 5’ caps + 2’-OMe purine substitution</td>
<td>131</td>
<td>8 minutes</td>
<td>0.14</td>
<td>91</td>
<td>650</td>
</tr>
</tbody>
</table>
Aptamers containing both 2’-F and 2’-OMe modifications were highly stable *in vivo*

<table>
<thead>
<tr>
<th>Aptamer</th>
<th>Length (nucleotides)</th>
<th>Binding affinity for VEGF ($K_a$, pM)</th>
<th>Dissociation half-life (seconds)</th>
<th>$T_m$ ($^\circ$C)</th>
<th>Binding dependent on divalent cations</th>
<th>VEGF$<em>{165}$ IC$</em>{50}$ for VEGFR2 (M)</th>
<th>Miles assay (inhibition at 0.1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t22-OMe</td>
<td>23</td>
<td>72</td>
<td>60</td>
<td>49</td>
<td>No</td>
<td>2-3 x 10$^{-12}$</td>
<td>13%</td>
</tr>
<tr>
<td>t2-OMe</td>
<td>29</td>
<td>130</td>
<td>170</td>
<td>66</td>
<td>No</td>
<td>6 x 10$^{-11}$</td>
<td>None</td>
</tr>
<tr>
<td>t44-OMe (pegaptanib)*</td>
<td>27</td>
<td>49</td>
<td>90</td>
<td>62</td>
<td>Yes</td>
<td>2-3 x 10$^{-12}$</td>
<td>48%</td>
</tr>
<tr>
<td>Scr-t44-OMe†</td>
<td>27</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>5 x 10$^{-8}$</td>
<td>None</td>
</tr>
</tbody>
</table>

*Inhibition of vascular leakage

Attachment of a 5’-linked 40-kDa polyethylene glycol improved inhibition to 83%
Therapeutic Relevance
Case Study: Pegaptanib

(a) A A
    G   U
    U-G
    G-C
    A-U
    C
    U
    A
    A
    U
    A
    C
    G

40-kDa PEG-5'  3’-3’-dT-5’

(b) Photo-crosslink

C137

C
U14

2’-Fluoro

2’-Methoxy

Unmodified
Therapeutic Relevance Case Study: Pegaptanib

- Transient adverse effects
- FDA approved in 2004 with an IC$_{50}$ of 49pM
Therapeutic Relevance
Case Study: AS1411

• The Bates’ group at the University of Louisville made a Purine Motif TFO that was specific for the promoter of the *uPA* gene
• Gave moderate inhibition of DU145 growth

• Negative control was all G and T with no complementarity to gene
• Gave a much larger inhibition

*Experimental and Molecular Pathology* 2009, 86, 151
Therapeutic Relevance  
Case Study: AS1411  

5′-GGTGGTGGTGGTGGTTGTGGTGGTGGTGGG  

• Aptamers made by SELEX frequently for G-quadruplexes  
• Certain cell types (including cancer and some immune cells) preferentially internalize G-quadruplex-forming oligos
**Therapeutic Relevance**

**Case Study: AS1411**

- Tested at NCI in 60 different cancer cell lines
- Displayed antiproliferative activity in almost all at low μM concentrations
- Non-malignant cells unaffected at 10 μM
- Cytostasis occurs as a result of cell division being inhibited

<table>
<thead>
<tr>
<th>A549 non-small cell lung cancer</th>
<th>Hs27 non-malignant skin fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="untreated.png" alt="Untreated A549" /></td>
<td><img src="untreated.png" alt="Untreated Hs27" /></td>
</tr>
<tr>
<td>![3 μM AS1411 A549](3uM AS1411.png)</td>
<td>![10 μM AS1411 Hs27](10uM AS1411.png)</td>
</tr>
</tbody>
</table>

**Images:**
- Untreated vs. treated with AS1411 at different concentrations for A549 and Hs27 cell lines.
Therapeutic Relevance
Case Study: AS1411

• Southwestern blot gave a protein ~110kDa
• Hypothesized that it was Nucleolin, a telomere binding protein

Proposed Model for AS1411 Mechanism of Action

- AS1411 interferes with molecular interactions and functions of nucleolin
  o Shuttling of PRMT5-nucleolin complex
  o Sequestration of NEMO-nucleolin complex
  o Binding of nucleolin to bcl-2 mRNA
  o Anticipate other effects, not yet known

- Uptake of AS1411 depends on cell surface nucleolin

- Cancer Selectivity

- Antiproliferative Effects
  o Cell cycle arrest
  o DNA replication block
  o Inactivation of NF-κB
  o Induction of tumor suppressors
  o Induction of cell death
  o Reduced Bcl-2
  o Other unknown effects

- Normal Cell
  - No surface nucleolin, no uptake

- Cancer Cell

Key:
- AS1411
- Nucleolin

• Entered in Phase II clinical trials for treatment of acute myeloid leukemia in late 2007
Therapeutic Relevance
Case Study: REG1

• Anticoagulation drugs are used extensively to treat acute venous and arterial thrombosis
• Major hemorrhage occurs in 4% of patients with acute coronary syndrome
• 5% of patients undergoing artery bypass require a second operation to control bleeding
• There is a need for a controlled anticoagulant

*Circulation* 2008, 2865
Qiagen
Therapeutic Relevance
Case Study: REG1

TF-Bearing cell

INITIATION

TF
VIIa
X
II

RB006

RB007

RB006-RB007 complex

VIIa

IX

IXa

Activated Platelet

AMPLIFICATION

VIIIa

VIII/vWF

AMPLIFICATION

X

II

Xa

Va

Platelet

V

Va

XI

Xla

• The Rusconi Group 2.8nM affinity for IXa
Therapeutic Relevance
Case Study: REG1

- aPPT – activated partial thromboplastin time
- A performance indicator of the pathway

• Currently in Phase II Clinical Trials
• Diabetic nephropathy is a progressive kidney disease caused by a weakening of the kidney capillaries from dysregulated blood sugar.

• Cytokine antagonism is a powerful strategy to prevent tissue damage in chronic inflammation.

Noxxon Pharmaceuticals
*The Journal of Pharmacology and Experimental Therapeutics* 2009, 328, 371
Therapeutic Relevance
Case Study: NOX-E36

• High-affinity aptamers to D-mCCL2 were identified after 11 rounds of *in vitro selection*

- Biologically stable
- Non-immunogenic
- Safe & well tolerated in animals & man

D-RNA

- Biologically unstable
- Frequently immunogenic
- Approved product

L-RNA (Spiegelmer)

Noxxon Pharmaceuticals
Therapeutic Relevance
Case Study: NOX-E36

- Currently in Phase I Clinical Trials
Therapeutic Relevance
Drug Delivery

a) aptamer + drug \[\rightarrow\] aptamer – drug physical conjugate

b) PSMA aptamer

doxorubicin

Therapeutic Relevance
Drug Delivery

- Titration of aptamer with constant concentration of doxorubicin

Therapeutic Relevance
Drug Delivery

Possess PSMA
Does not possess PSMA

Conclusions

- Aptamers are oligonucleotide strands which bind molecular targets with good binding affinity.
- SELEX provides the ability to select oligonucleotides with high binding affinities for molecular targets from random oligo pools.
- Though SELEX introduced a highly efficient method of selecting and amplifying high binding affinity oligo- strands, continued advances in SELEX may produce aptamers with increased therapeutic potential.
Conclusions

- An FDA approved drug and several clinical trial drugs validate aptamers as therapeutics
- Aptamers can also be used in drug delivery and other applications

2. [http://www.instantcast.com/AllStars/CREB](http://www.instantcast.com/AllStars/CREB)


5. [http://mcdb.colorado.edu:8081/mcdb/faculty/researcherobjects/Larry-Gold](http://mcdb.colorado.edu:8081/mcdb/faculty/researcherobjects/Larry-Gold)