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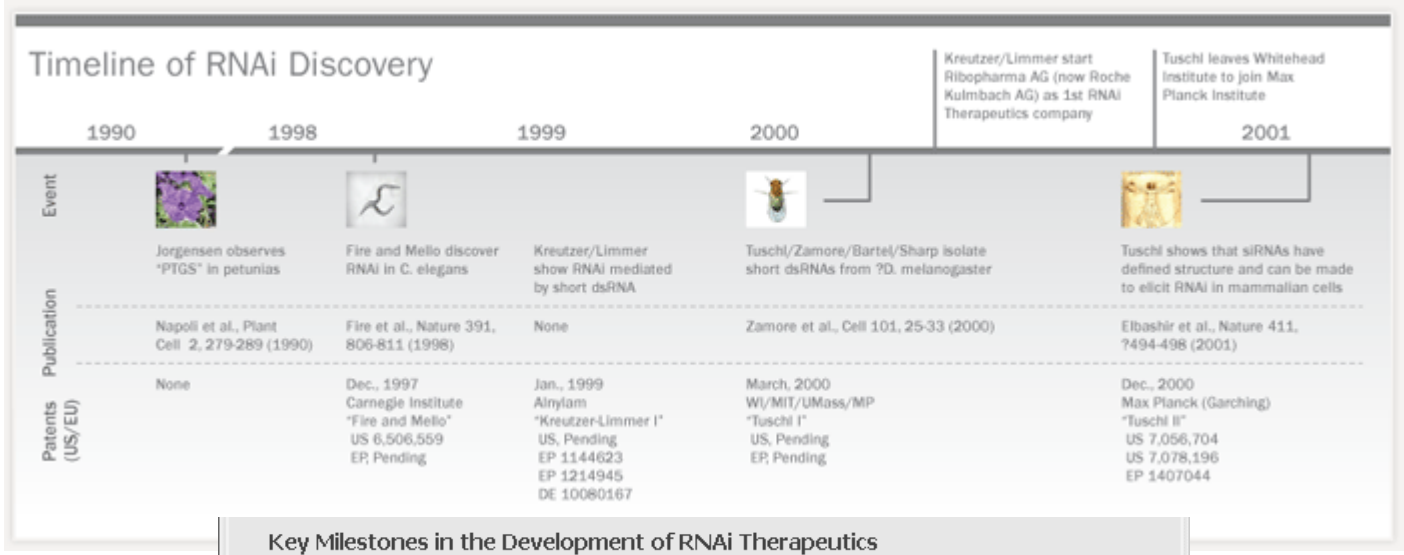
Application of RNA Interference to Anti-Doping

Matthew Fedoruk, Ph.D.

Gene & Cell Doping Symposium
2013, Beijing, China

From Plants to Worms to Humans: Discovery and Mechanism of RNAi



Key Milestones in the Development of RNAi Therapeutics

1990
First scientific observation of the "co-suppression" phenomenon in plants, today known to be caused by RNAi.

1998
Double-stranded RNAs shown to trigger gene silencing in the nematode worm *C. elegans*.

1999
RNAi is mediated by small dsRNA fragments including in mammalian cells leading to the formation of Ribopharma AG.

2001
siRNAs of 21-25 base pair length with 3' overhangs shown to induce efficient RNAi in mammals.*

2002
RNAi shown to inhibit viral replication, including HIV and HCV.*

2004
RNAi following systemic administration of siRNAs in adult mammals.*

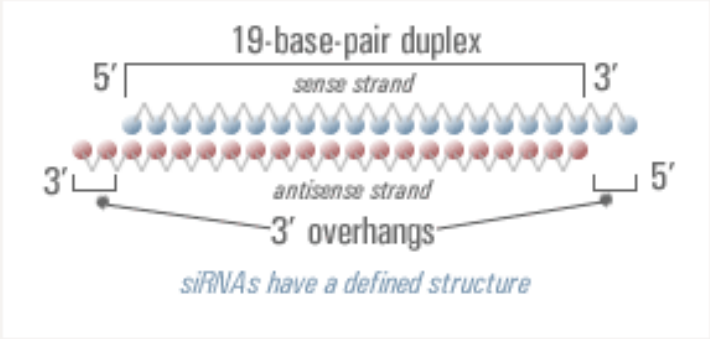
2006
RNAi following systemic administration of siRNAs in non-human primates.*

2008
An RNAi therapeutic demonstrated clinical efficacy in the double-blind, placebo-controlled, randomized GEMENI study.*

*Contributions made by scientists affiliated with Alnylam Pharmaceuticals, Inc.

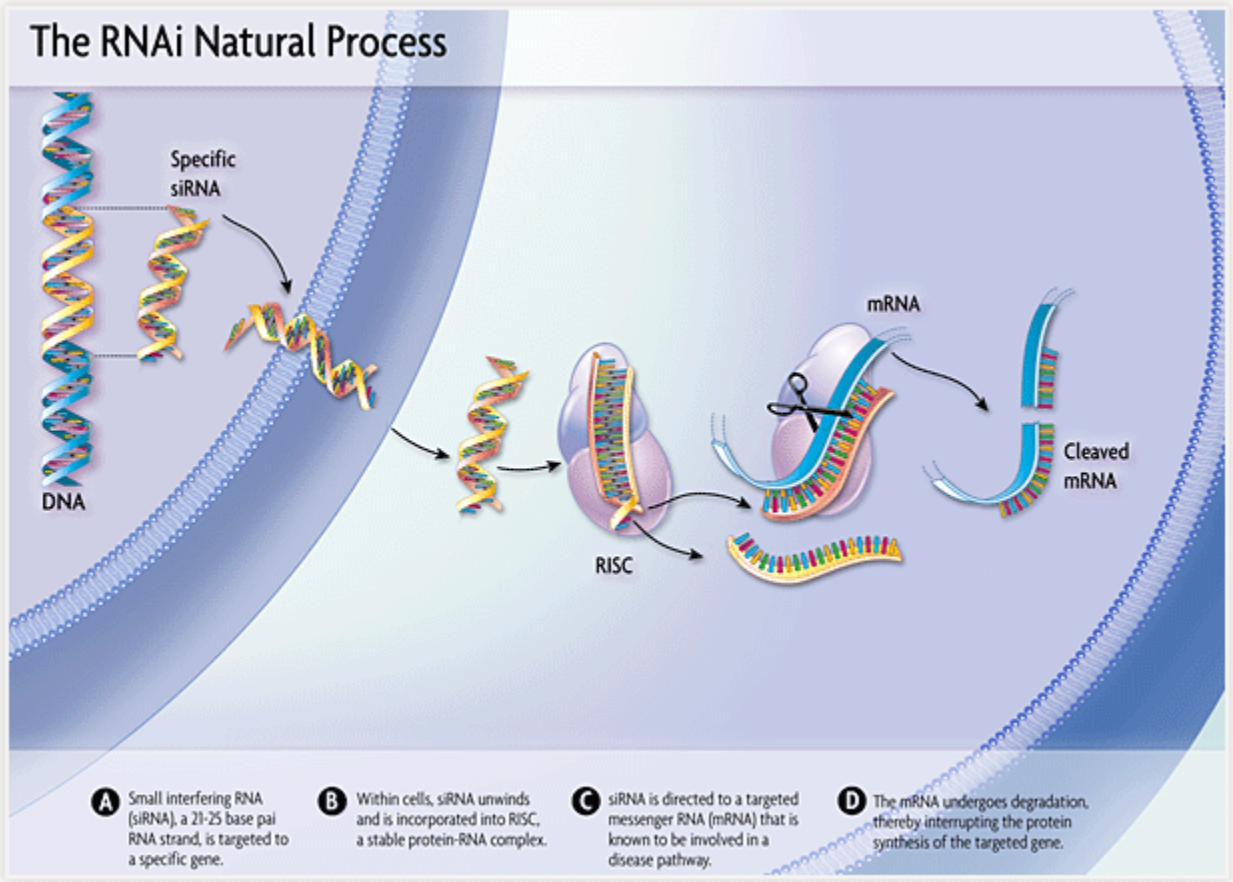
Short double-stranded RNAs (dsRNAs) mediate RNAi in human cells

siRNA Structure



Nature (2001) 411:494-8.

RNAi is a naturally occurring RNA gene silencing pathway in humans



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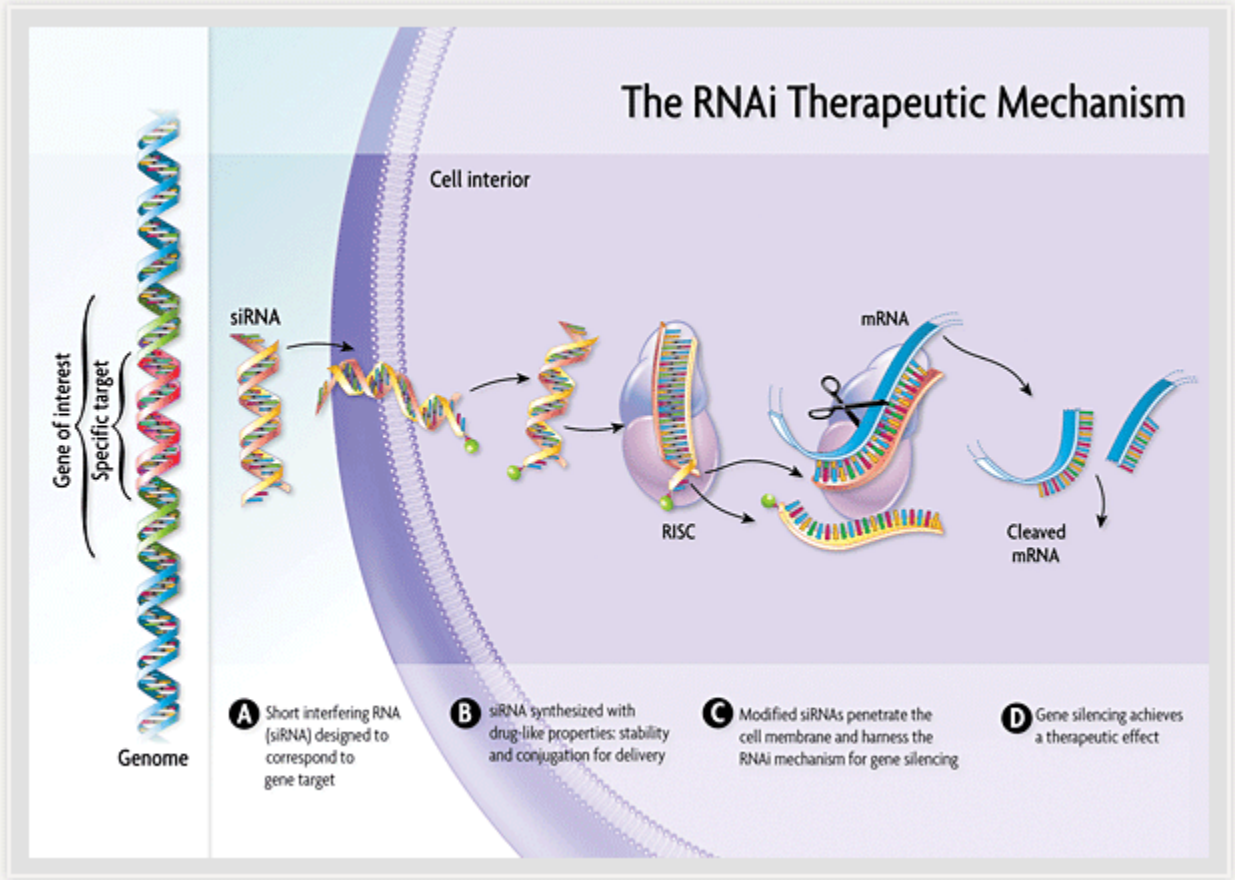
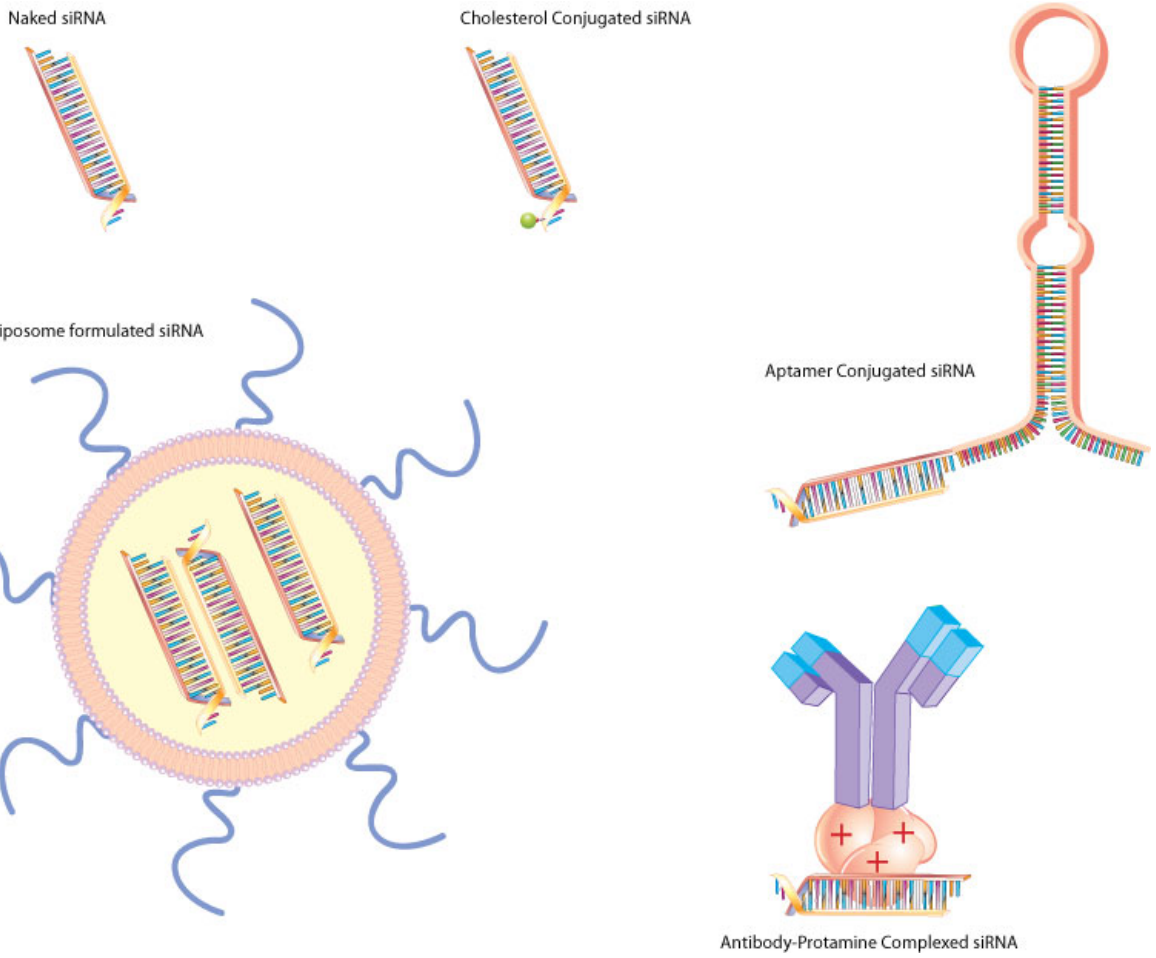
RNAi therapeutics target gene expression upstream of protein production

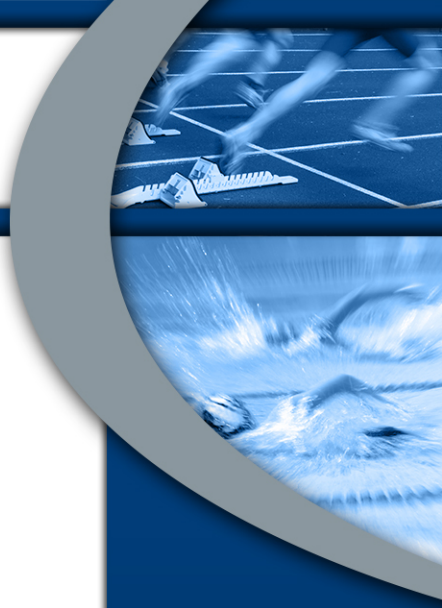


Delivery Approaches



RNA Interference Delivery Systems

- Plasmid vectors
- Polyethyleneimine carriers
- Hydrodynamic injection
- Local injection
- Modified viral vectors



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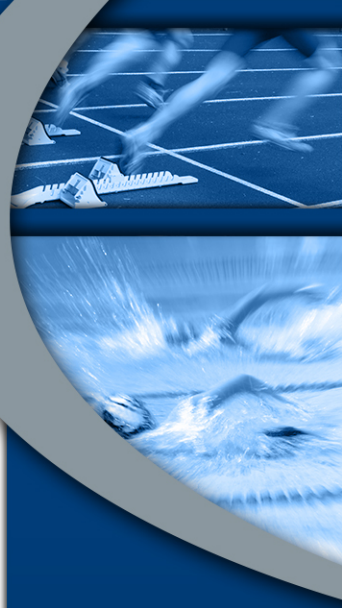
Challenges of Therapeutic siRNA



- Stability and targeting
  - An RNAi therapeutic has to cross the cellular membrane barrier
  - Resist degradation – naked siRNA subject to Rnases in minutes
- Off-target silencing
- Bio-distribution
  - Delivery routes: local (eye & lung) vs. systemic (liver, kidney & spleen)
- Activation of immunogenic and inflammatory response (modifications necessary)
- Barriers to delivery

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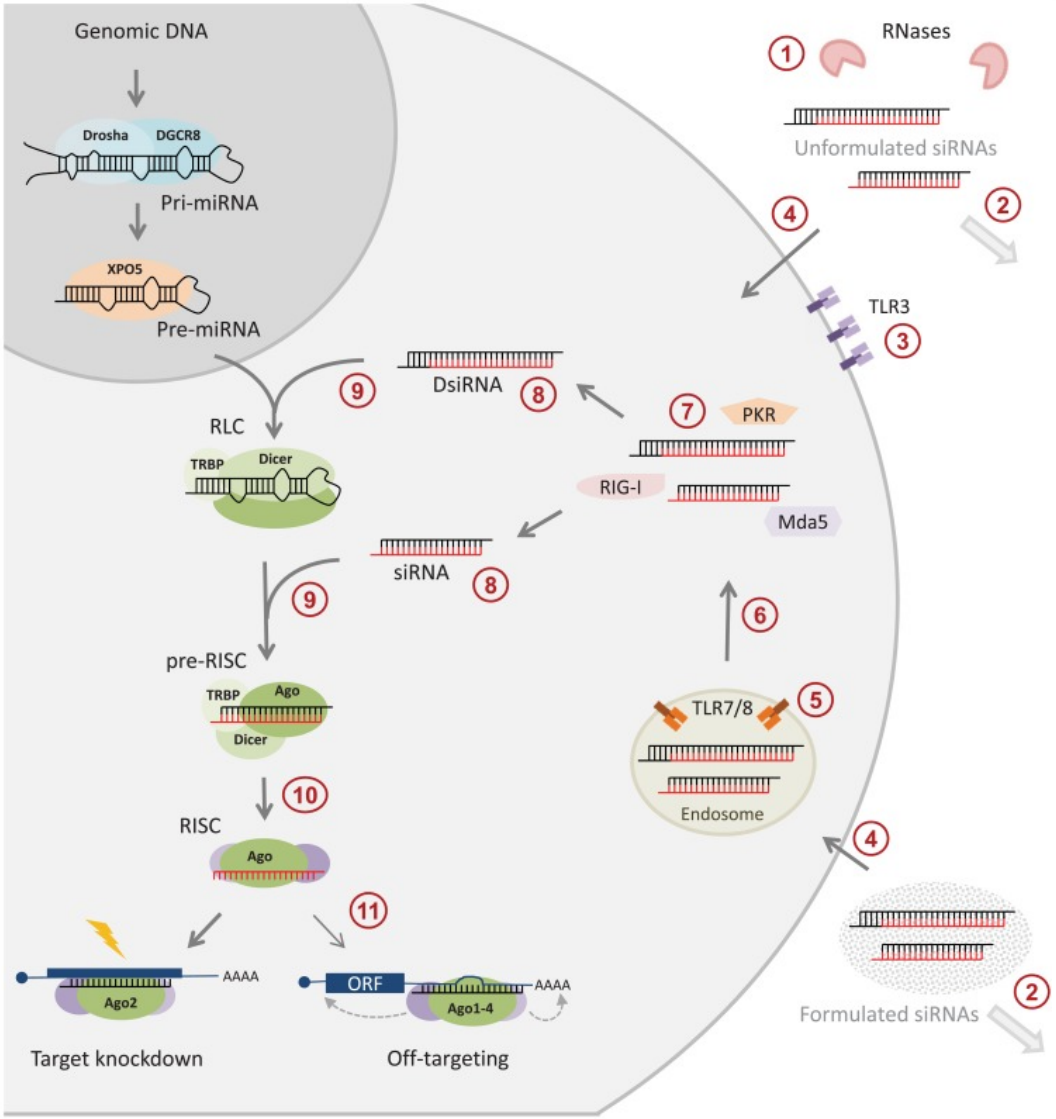
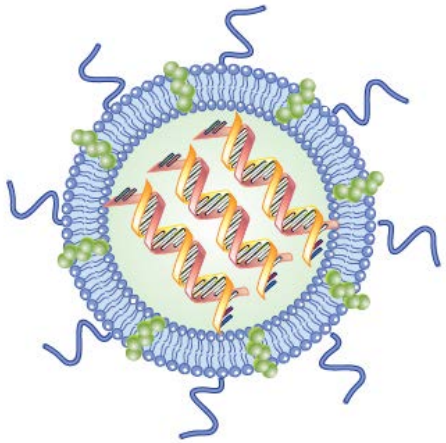


1 Degradation by nucleases
2 siRNA body clearance
3 TLR3-mediated immunogenicity
4 Poor intracellular delivery
5 TLR7/8-mediated immnunogenicity
6 Poor endosomal escape
7 Immune activation by cytoplasmic PRRs
8 Intracellular availability/stability
9 siRNA/miRNA competition
10 siRNA clotting effects
11 Off-target effects

Front Genet. 2012; 3: 154. .

Proposed Detection Strategies

- Direct
  - Delivery vector
  - Commercial detection methodology (TaqMan® MicroRNA Assays)
  - Chromatographic & mass spectrometric
  - mRNA imaging
- Indirect
  - Immune reaction
  - Proteomic changes
  - Longitudinal biomarker analysis



Liposome formulated siRNA

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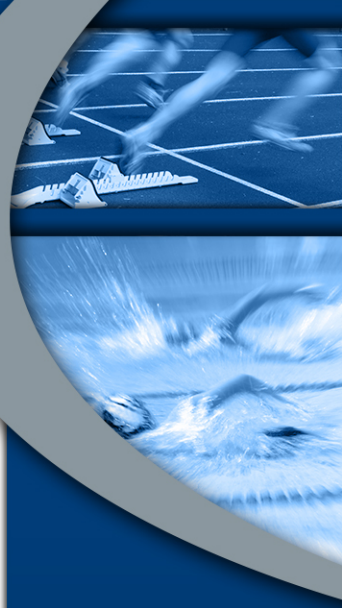
WADA grants targeted to siRNA



- *Detection of small interfering RNA (siRNA) as gene doping strategy using combined biochemical and mass spectrometric approaches*
- M. THEVIS et al.
- German Sport University, Germany

- *Development of a Highly-Sensitive Quantitative Assay to Detect siRNA- Mediated Gene Doping*
- J. RUPERT & M. FEDORUK
- University of British Columbia, Canada

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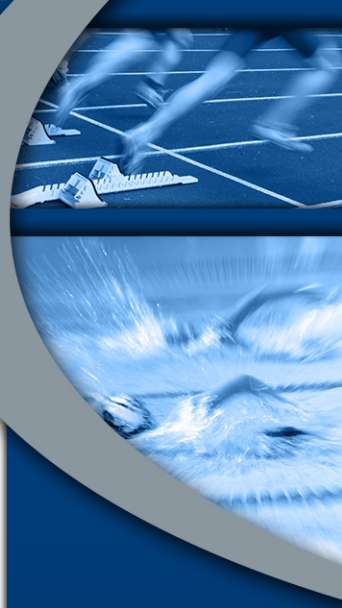
RNAi Targets for Performance-Enhancement



- Myostatin
- EglN family of 2-oxoglutarate-dependent dioxygenases - (Hypoxia-Inducible Factor-prolyl hydroxylase (HIF-PHD) pathway)
- Glucocorticosteroid-like effects (Inflammation)
- Energy metabolism & weight loss
- Pain killers
- Wound healing
- Respiratory targets
- Unknown!

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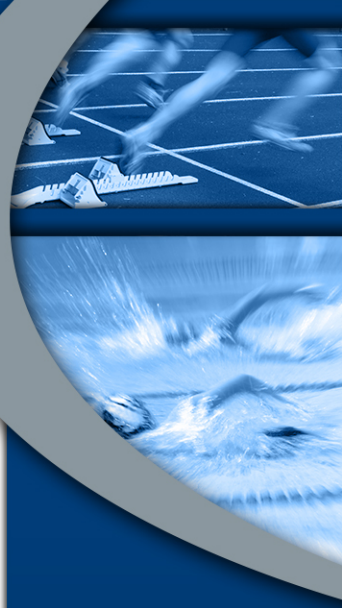
Myostatin



- Negative regulator of muscle growth
- Natural genetic mutations generate myostatin-deficient individuals
- Specifically mentioned on WADA Prohibited List
- Supplement industry – myostatin blockers
- Limited support for transient increases in performance
- Pre-clinical and clinical investigations – unproven
- Localized and efficacious delivery to muscle problematic
- Interest from athletic community

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siRNA targeted to EglN family

blood

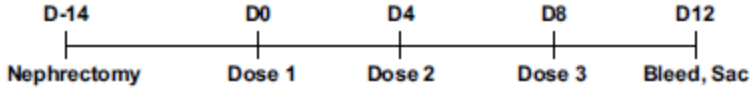
Prepublished online May 18, 2012;
doi:10.1182/blood-2012-04-423715

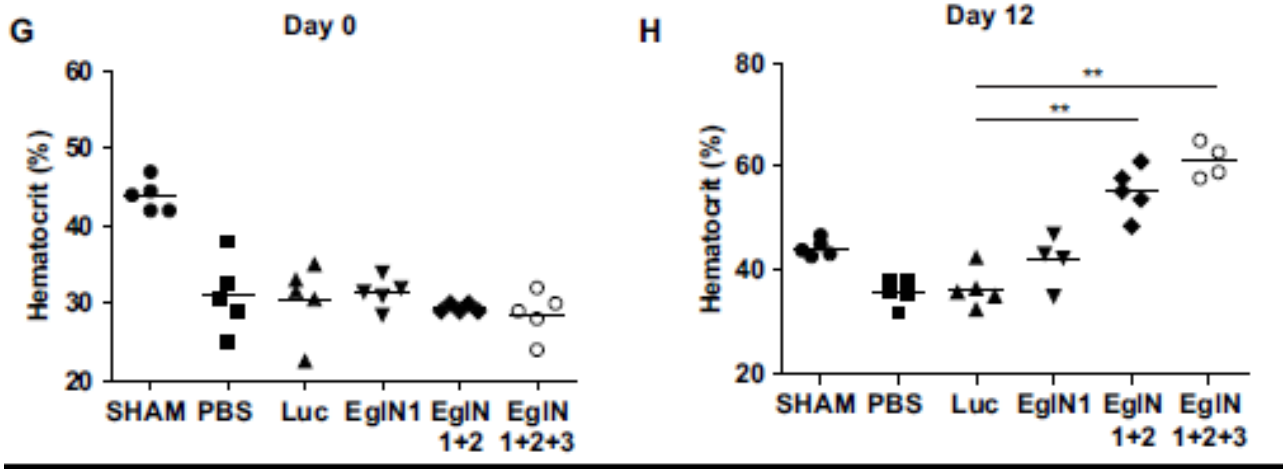
Treatment of erythropoietin deficiency in mice with systemically administered siRNA

William Querbes, Roman L. Bogorad, Javid Moslehi, Jamie Wong, Amy Y. Chan, Elena Bulgakova, Satya Kuchimanchi, Akin Akinc, Kevin Fitzgerald, Victor Koteliansky and William G. Kaelin, Jr.

- Transcription of the *EPO* is controlled by the transcription factor HIF (Hypoxia-Inducible Factor) and hence intimately linked to oxygen delivery to the kidneys, which are normally borderline hypoxic at rest and poised to respond to further decrements in oxygen delivery.
- HIF consists of an unstable alpha subunit and a stable beta subunit. In the presence of oxygen HIFα becomes prolyl hydroxylated by members of the EglN family of 2-oxoglutarate-dependent dioxygenases, leading to its polyubiquitination and proteasomal degradation. As oxygen levels fall EglN activity is diminished, leading to HIF stabilization and activation.
- There are three EglN family members although EglN1 is the primary regulator of HIF, with EglN2 and EglN3 playing compensatory roles.
- Ability of lipid nanoparticles to deliver siRNAs specifically to the liver as a means of inactivating hepatic EglN activity, thus reactivate hepatic erythropoietin production.

EglN siRNA Ameliorates Anemia in Preclinical Models



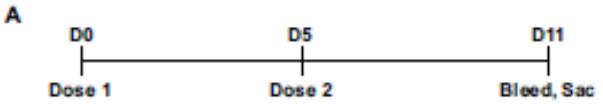


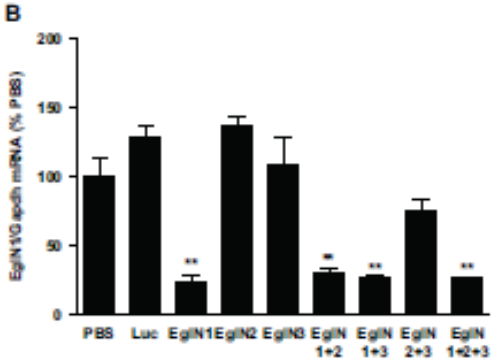
I

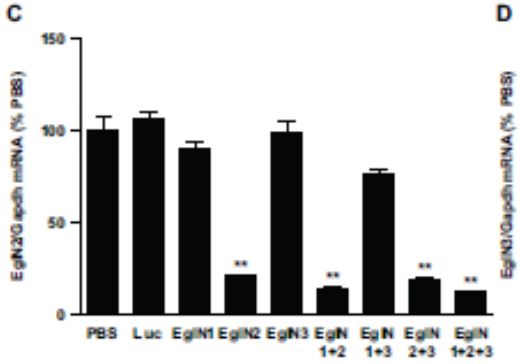
	RBC (x10⁶ cells/μL)	HGB (g/dL)	HCT %	RETIC %
SHAM	9.3	13.2	44.3	4.1
PBS	7.9	10.7	36.0	3.6
Luc	7.8	10.4	36.4	4.6
EglN1	8.8	12.0	41.9	6.3
EglN1+2	10.7	16.0	55.3	9.3
EglN1+2+3	11.1	17.4	61.1	11.8

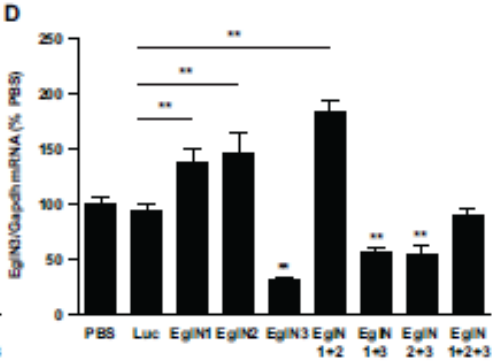
Figure 3. Targeting of *EglN* genes rescues anemia caused by renal failure. (A) Overview of 5/6 nephrectomy procedure and dosing schedule. (B-F) mRNA values at day 12 in mice treated with the indicated siRNAs as depicted in (A). HAMP1= hepcidin antimicrobrial peptide 1. mRNA levels were normalized to *actin* mRNA and then to corresponding sham mRNA level. Sham mice underwent sham surgery rather than 5/6 nephrectomy. (G-I) Baseline hematocrit (day 0)(G), day 12 hematocrit (H) and day 12 hematology parameters (I) in mice treated with the indicated siRNAs as depicted in (A). n=5. Error Bars represent 1 std. dev. *P<0.05, **P<0.01.

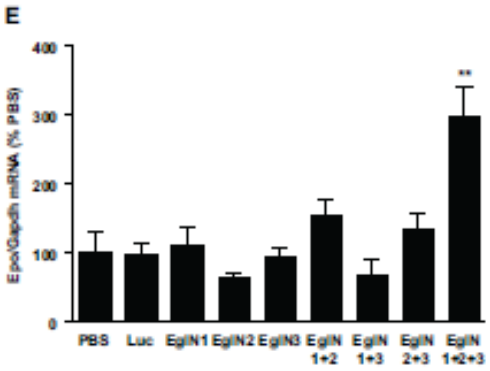
Combinatorial effects of EglN siRNAs on hepatic EPO production

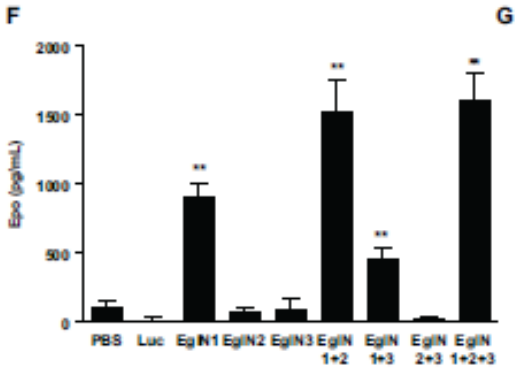








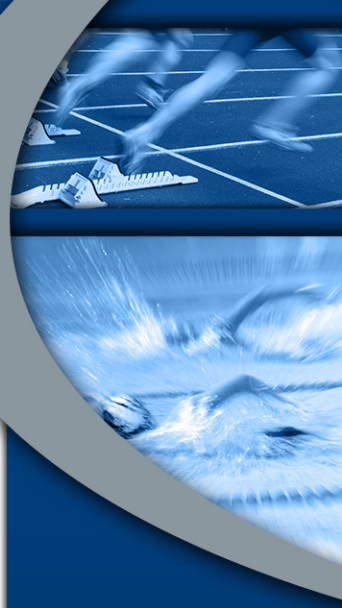




G

	RBC	HGB	HCT	RETIC
PBS	8.45	12.80	40.52	3.68
Luc	8.68	12.86	41.70	2.97
EglN1	10.63	15.43	52.28	8.66
EglN2	8.36	12.38	40.08	3.75
EglN3	8.31	12.32	40.04	3.63
EglN1+2	11.45	16.48	56.16	10.63
EglN1+3	10.02	14.82	49.30	7.60
EglN2+3	8.03	12.04	39.08	4.61
EglN1+2+3	11.87	16.93	58.53	12.21

Vivo-Morpholinos – Gene Tools



- Morpholinos are anti-sense oligonucleotide analogs that bind to complementary RNA sequences; overcome previous barriers
- Delivered systemically with intravenous (I.V.) intraperitoneal (I.P.) injection, localized delivery directly into the area of interest
- Ferguson DP, Schmitt EE, Lightfoot JT. **Vivo-morpholinos induced transient knockdown of physical activity related proteins.** PLoS One. 2013 Apr 22;8(4):e61472.

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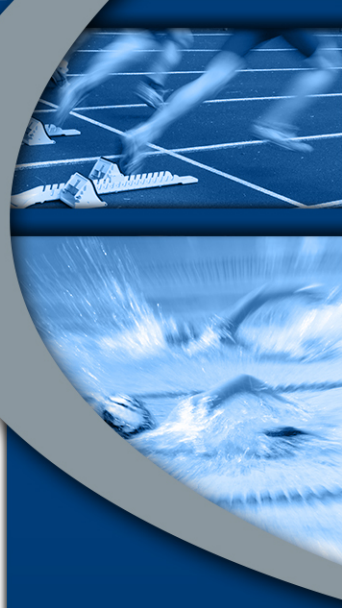
Lab to Reality: Barriers to Athletes



- RNA design & target-efficacy
- Safety & off-target effects
- Delivery system
- Local vs. systemic delivery
- Detectability
- Cost
- Will athletes go to the trouble?
- Rogue labs? Limit of specific publications?

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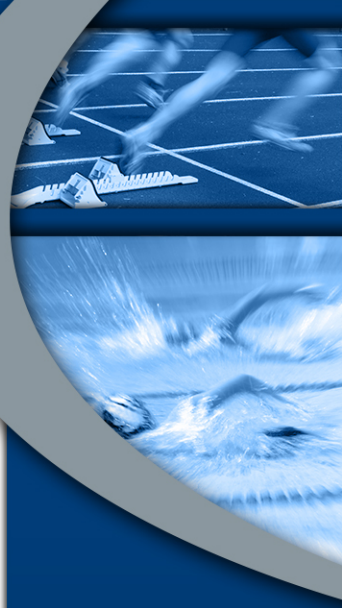
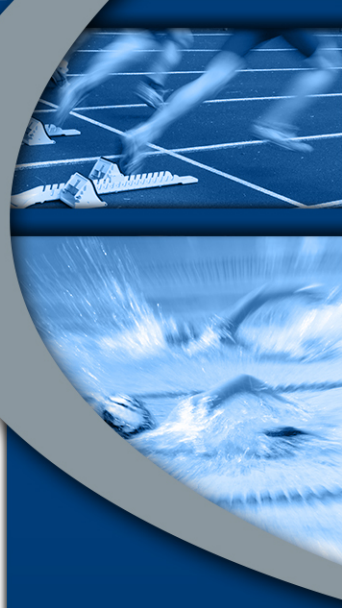
RNAi Leaders to watch



- Alnylam Pharmaceuticals (USA)
- Tekmira Pharmaceuticals (Canada)
- Silence Therapuetics (UK)
- Merck (Global)
- ZaBeCor (USA)
- Halo-Bio RNAi Therapeutics (USA)
- Sirnaomics (USA & China)

Product approvals imminent

Application to Anti-Doping



- Important to be aware of *in vivo,* pre-clinical and clinical advancements
- Communication strategy with biotechnology industry
- Knowledge of unpublished research
- Increase knowledge on bio-distribution, metabolism, half-life & degradation
- Detection strategy development
- Monitor relevance of siRNA targets to performance targets (*RNA interference for performance enhancement and detection in doping control.* Kohler et al. Drug Test Anal, 2011 (3):661-667.)
  - Semi-annual update to clinical trial status with relevance to anti-doping

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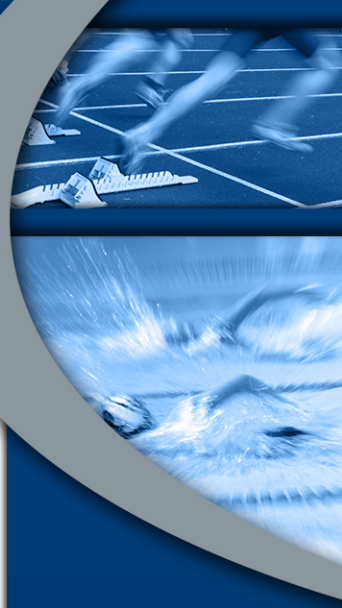
Potential Impact on Anti-Doping Organizations



- Detection methodology – development and implementation, cost, laboratory capability
- Sample collection – timing, biological matrix
- Test distribution plan
- Establishment of highest risk gene targets/doping strategies
- *Therapeutic Use Exemption* process
- Questions from athletes – gene & stem cell therapy
- Education programs to athletes, coaches, health professionals, media, & wider anti-doping/sport community
- Arbitration of gene doping cases
- Proactive contact with pharmaceutical industry

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