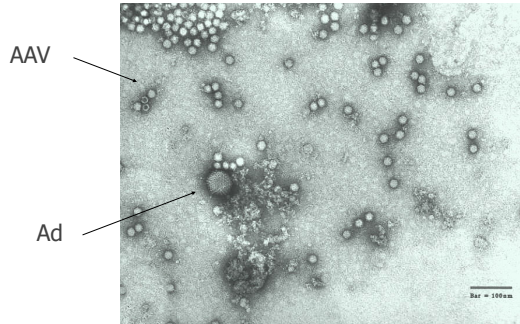


# Adeno-associated virus (AAV)

## Taxonomy

Family: Parvovirus  
Subfamily: Parvoviridae  
Genus: Dependovirus  
Type: AAV 1-9

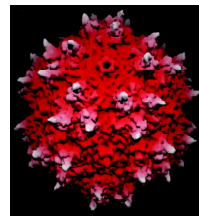


## Morphology

Particles are icosahedral, non-enveloped, 18-26 nm diameter, 50% protein (VP1-3) 50% DNA. Resistant to inactivation by solvents, pH and heat.

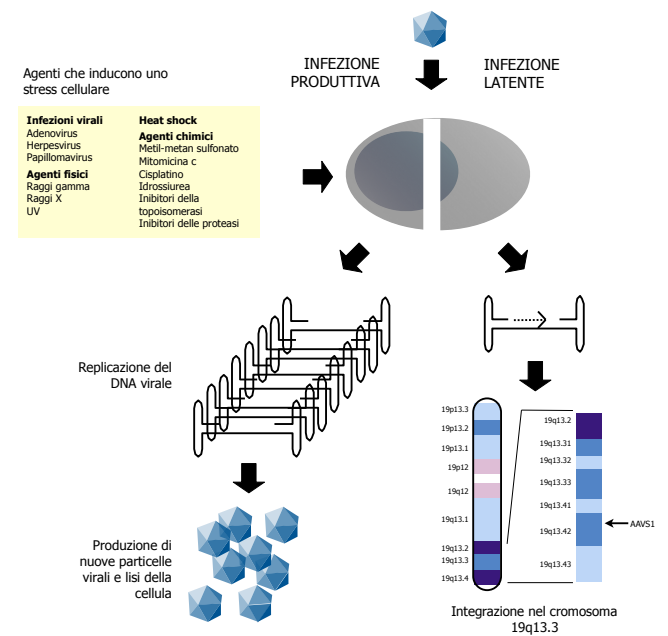
## Genome

Linear, non-segmented, ssDNA ~5 kb. AAVs package equal amounts of (+) and (-) strands.

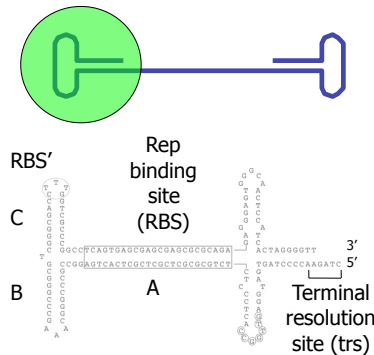
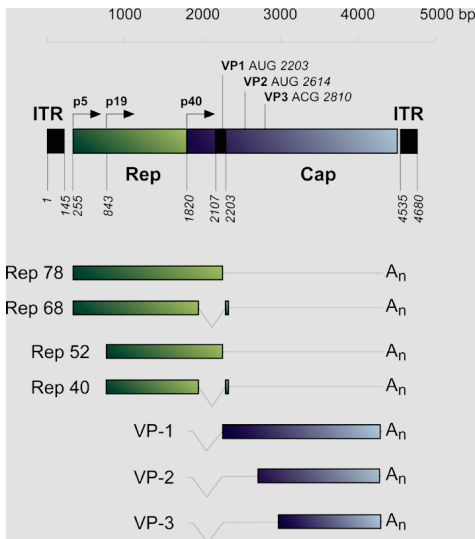


Xie et al. 2002

## Ciclo replicativo di AAV



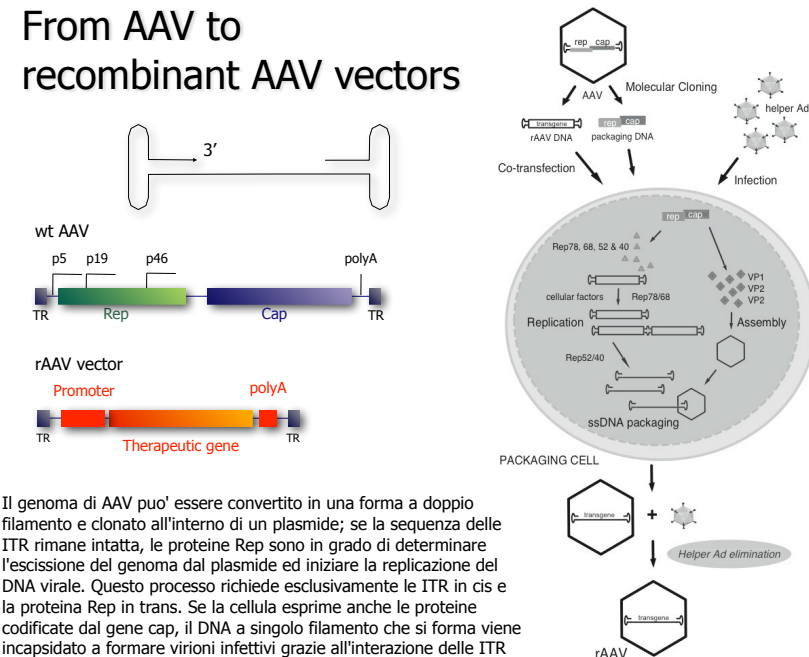
## Genetic organization and molecular structures of AAV DNA



5' and 3' Inverted Terminal Repeats have a predicted hammer-head like secondary structure

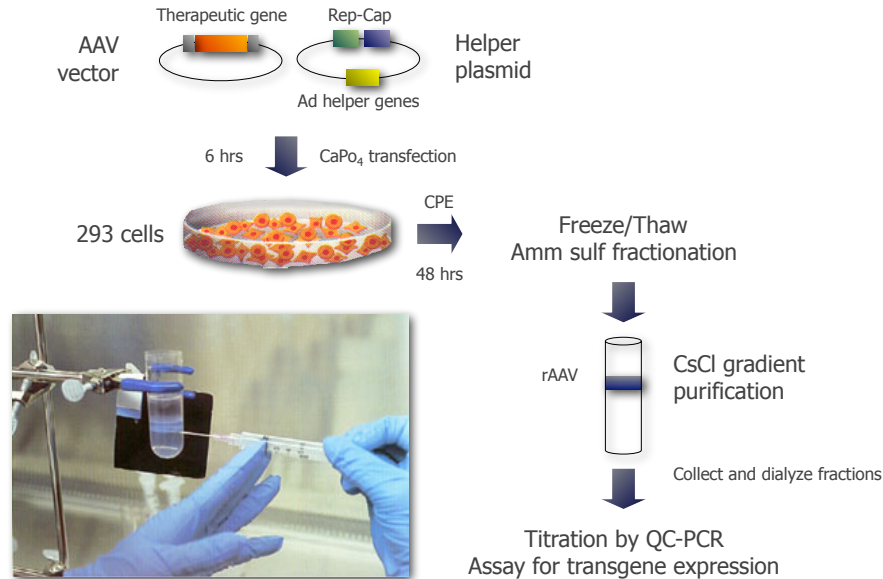
During AAV replication Rep bind the ITR and cleaves one strand

## From AAV to recombinant AAV vectors



Il genoma di AAV puo' essere convertito in una forma a doppio filamento e clonato all'interno di un plasmide; se la sequenza delle ITR rimane intatta, le proteine Rep sono in grado di determinare l'escissione del genoma dal plasmide ed iniziare la replicazione del DNA virale. Questo processo richiede esclusivamente le ITR in cis e la proteina Rep in trans. Se la cellula esprime anche le proteine codificate dal gene cap, il DNA a singolo filamento che si forma viene incapsidato a formare virioni infettivi grazie all'interazione delle ITR con le proteine VP1-3.

# Production of recombinant AAV



## AAV Vector Unit (AVU) facility at ICGEB Trieste

Small routine and large scale preparations of AAV vectors are generated at the AVU following double or triple transfection of subconfluent HEK293 cells in roller bottles, by AAV cis-plasmid encoding the gene of interest and AAV trans-plasmid/s containing AAV rep and cap genes, and adenovirus helper genes. Vector viral particles are purified by CsCl gradient and characterized by genome titration, infectivity and purity.



### Activities performed at the AVU

Plasmid amplification and purification  
 Vector packaging and purification from cellular lysates  
 Vector titration by real-time PCR  
 Vector characterization by infectivity assay and purity assessment



## AAV Vector Unit (AVU) facility at ICGEB Trieste: production of pre-clinical-grade AAV vectors with different serotypes as tools for in vivo investigation



<http://www.icgeb.org/avu-core-facility.html>

### Angiogenesis & Endothelial function

huVEGF-A<sup>165</sup>  
 huVEGF-A<sup>165</sup> (Tet-ON)  
 huVEGF-A<sup>165</sup>-HA  
 huVEGF-A<sup>121</sup>  
 muVEGF-A<sup>164</sup>  
 muVEGF-A<sup>120</sup>  
 huVEGF-D full length  
 huVEGF-D processed  
 huVEGF-B  
 VEGF/FGF4  
 Angiopoietin 1  
 Semaphorin 3A  
 PlGF  
 PDGF-B  
 TRAIL  
 HIF-1α-VP16  
 Netrin1

### Development & Regeneration

Islet-1  
 Msx-1  
 Notch ICD  
 Cripto  
 c-Myc  
 Klf4  
 Oct3/4  
 Sox2

### Cardiac & muscle function

IGF-1  
 Melusin  
 ZASP1  
 Ghrelin

### Cytokines & Chemokines

RANTES  
 Interleukin 1β  
 Interleukin 1β NG  
 RGMa  
 MCP-1  
 MIP-1a

### PET Imaging

HSV-1 TK  
 HSV-1 TKsl39  
 HIV-1 Tat-HSV-1 TK  
 HIV-1 Tat-HSV-1 TKSL39

### HIV-1

HIV-1 Tat  
 HIV-1 Tat-SP

### Apoptosis

Bcl-2

### shRNAs

FLK1  
 Zscaf6/hairpin  
 Sh-Amb1  
 Sh-Amb2  
 Zscaf6  
 GFP

### Inflammation

IκB-α

### Antibodies

ScFv-D18 (anti-Prp)  
 ScFv-TG (anti-TG)

### Immune function

CTLA-4  
 ADAMTS13

### Extracellular matrix

Timp-1  
 Pentraxin-3

### Cell cycle & Cancer

p27\*  
 Ras  
 Mutated Ras  
 cMyc  
 HPV E7

### Reporters

LacO binding site repeats  
 LacZ  
 GFP  
 GFP (Tet-ON)

### HPV

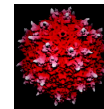
E6  
 E7

### Others

Cre-GFP  
 CFTR-YFP  
 DCN  
 Erb4DCF  
 NRG1IIIb3  
 Calpain1  
 Calpain2  
 PA3  
 PE8  
 Cap-YFP

**Mouse secretome collection**  
 ~1600 arrayed clones

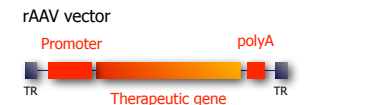
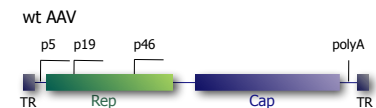
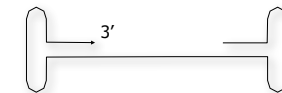
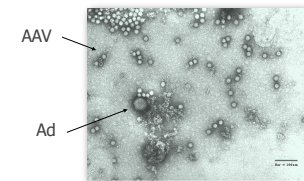
**Human microRNA collection**  
 ~800 arrayed clones



Xie et al. 2002

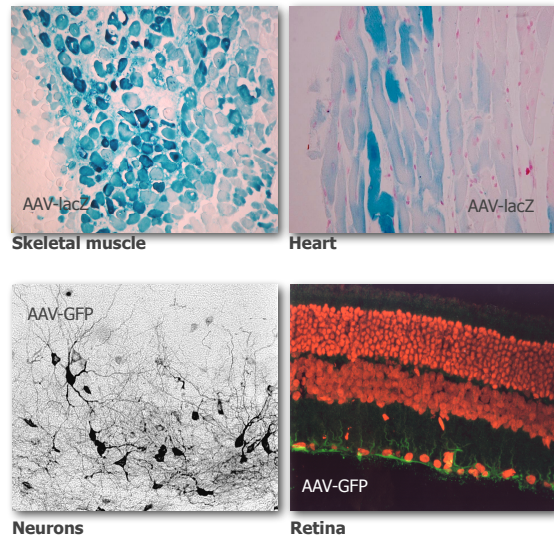
## Gene transfer using Adeno-Associated Virus (AAV) vectors

1. Based on a widely diffused, non pathogenic virus
2. Vectors retain less than 10% of the viral genome
3. Vectors do not express viral proteins (not inflammatory and not immunogenic); permit long term persistence in vivo
4. Expression of the therapeutic gene can be driven by any desirable promoter
5. High titer vector preparations can be obtained by virion purification
6. Cells are transduced at high multiplicity of infection; mixing of different rAAV preparations results in the simultaneous expression of gene combinations in vivo

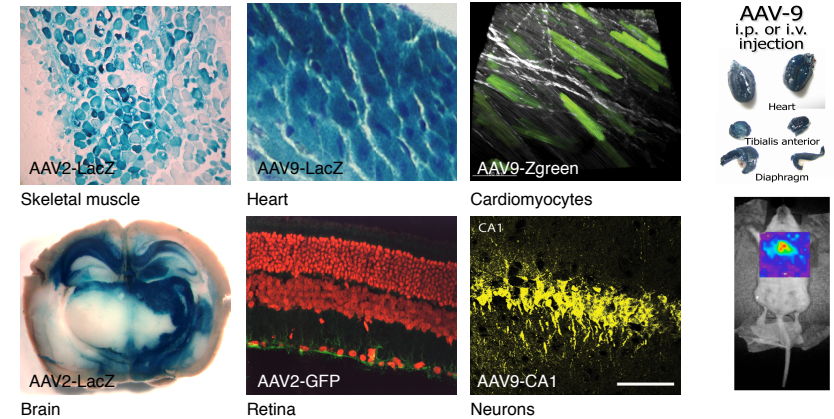




## Long-term persistence of rAAV transduction and gene expression in vivo is confined to post-mitotic cells

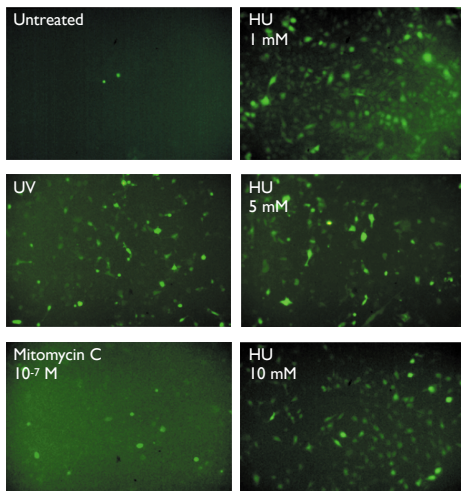


## AAV efficiently transduces permissive tissues and promotes persistent transgene expression



10

## Induction of rAAV-GFP transduction by genotoxic agents in wt CHO cells



## Helpers for AAV replication

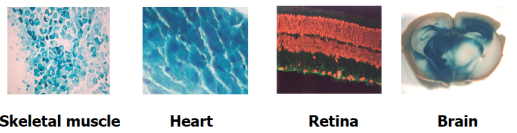
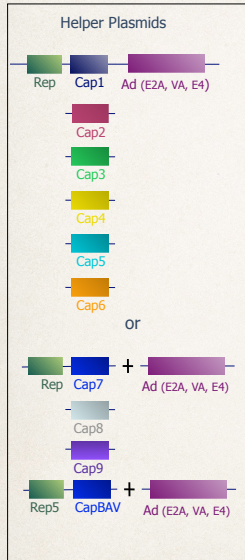
- Viruses**
  - Adenovirus (E4-ORF 6)
  - HSV-1
- Physical agents**
  - γ-radiation
  - X-ray
  - UV
  - Heat shock
- Chemical agents**
  - Methyl methan sulfonate
  - Mitomycin C
  - Cisplatinum
  - Hydroxyurea
  - Topoisomerase inhibitors (novobiocin, etoposide, camptotecin)
  - Protease inhibitors



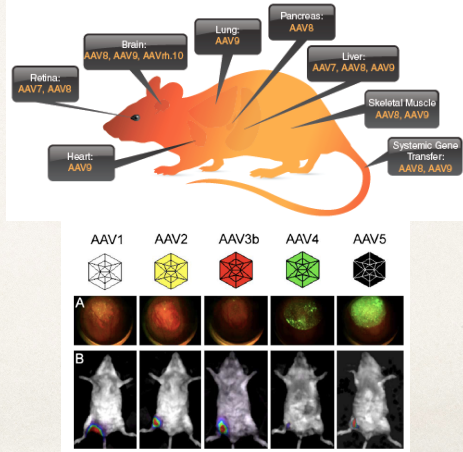
## Recettori di alcuni Parvovirus

Parvovirus	Recettore
AAV1	Acido sialico (legami α2-3-N e α2-6-N)
AAV2	Proteoglicani contenenti eparan-solfati (HSPG) Corecettori: integrina αvβ5, FGFR1, HGF-R
AAV3	Proteoglicani contenenti eparan-solfati (HSPG)
AAV4	Acido sialico (legami α2-3-O)
AAV5	Acido sialico (legami α2-3-O e α2-3-N) Recettore del PDGF (PDGFR)
AAV6	Acido sialico (legami α2-3-N e α2-6-N)
AAV7	Non noto
AAV8	Recettore della laminina (LamR)
AAV9	Non noto (LamR?)
Parvovirus B19	Antigene P dei globuli rossi
CPV (parvovirus canino)	Recettore della transferrina Acido sialico (acido N-glicolil-neuraminico, NeuGC)
FPV (parvovirus della panleucopenia felina)	Recettore della transferrina

# AAV efficiently transduces permissive tissues and promotes persistent transgene expression



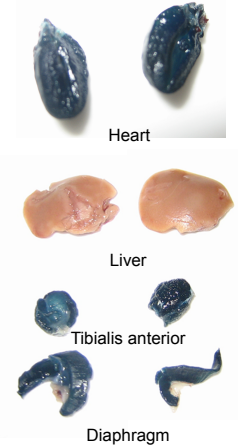
Optimal AAV serotypes for in vivo gene transfer to various tissues



# Tropismo d'organo dei sierotipi di AAV

Organo	Sierotipo (in ordine di efficienza)	
Fegato	AAV8	
Muscolo scheletrico	AAV1, AAV7, AAV6, AAV8, AAV9, AAV2, AAV3	
Sistema nervoso centrale	AAV5, AAV1, AAV4, AAV2	
Occhio	Epitelio pigmentato	AAV5, AAV4, AAV1, AAV6
	Fotorecettori	AAV5
Polmone	AAV5, AAV9	
Cuore	AAV9, AAV8	
Pancreas	AAV8	
Rene	AAV2	

# AAV-9 IP injection



# In vivo muscle and heart transduction using AAV-8

Direct injection, tibialis anterior

IP injection, diaphragm

b-galactosidase

IP injection, heart

Anti-b-gal antibody

# AAV-9 has high tropism for skeletal and cardiac muscle

$3 \times 10^{11}$  vgu AAV2/9-LacZ injected IP in 15 days old mice

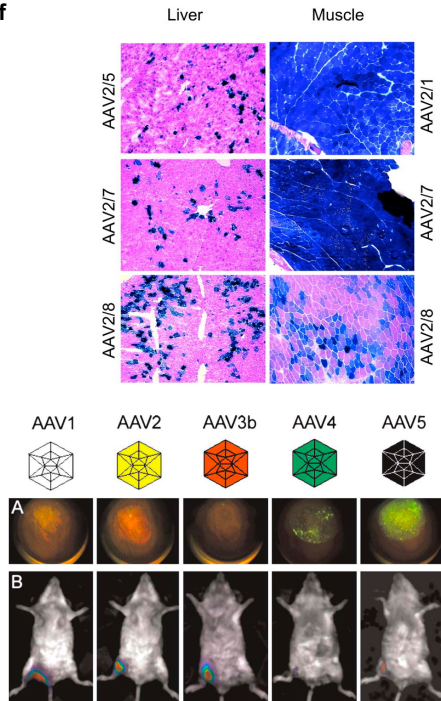
30 days

b-gal assay

Heart  
 Liver  
 Tibialis anterior  
 Diaphragm

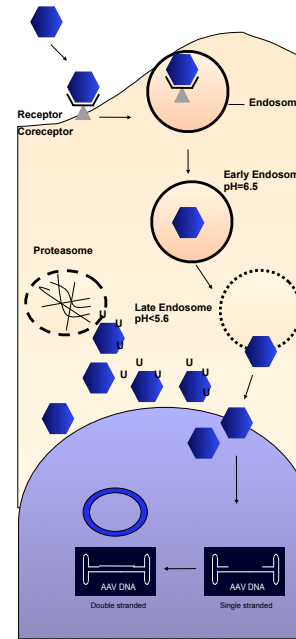


## Hierarchy of transduction efficiency of AAV serotype in different tissues



Tropism of AAV for post-mitotic cells in vivo.

Why?

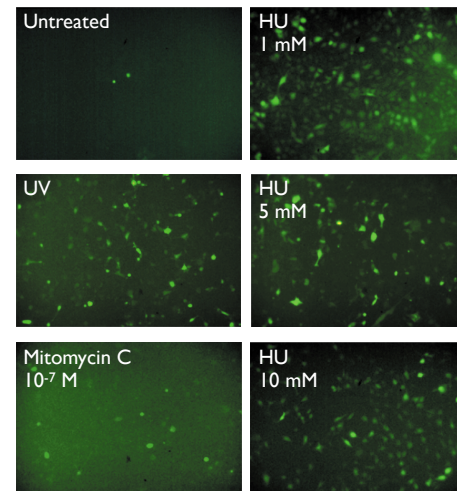


## Infectious entry pathways of AAV vectors

- Processing of AAV particles in endosomes
- AAV trafficking from the cell membrane to the nucleus
- Virion uncoating in the nucleus
- Genome conversion from single stranded to double stranded DNA and stability

The events that lead to efficient transduction of AAV vectors are still poorly understood. Tropism may depend on the ability of different cell types to support these events

## Induction of rAAV-GFP transduction by genotoxic agents in wt CHO cells



## Helpers for AAV replication

### Viruses

Adenovirus (E4-ORF 6)  
HSV-1

### Physical agents

$\gamma$ -radiation  
X-ray  
UV  
Heat shock

### Chemical agents

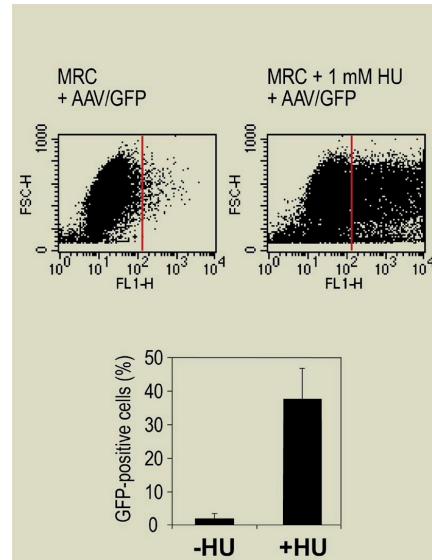
Methyl methan sulfonate  
Mitomycin C  
Cisplatinum  
Hydroxyurea  
Topoisomerase inhibitors  
(novobiocin, etoposide, camptotecin)  
Protease inhibitors



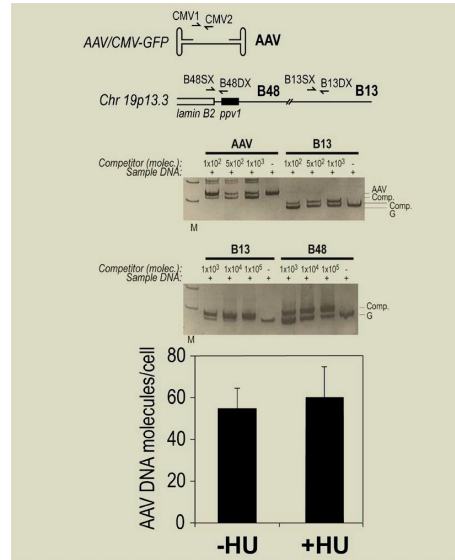


# Hydroxyurea induces permissivity to rAAV transduction through a post-entry mechanism

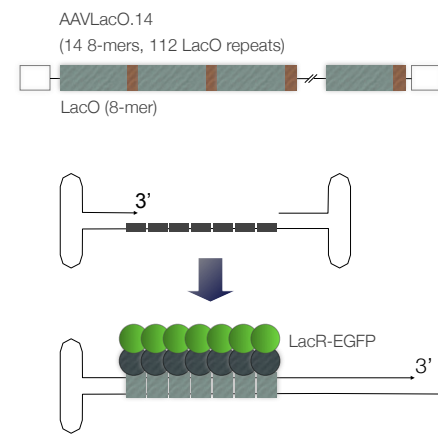
Efficiency of transduction



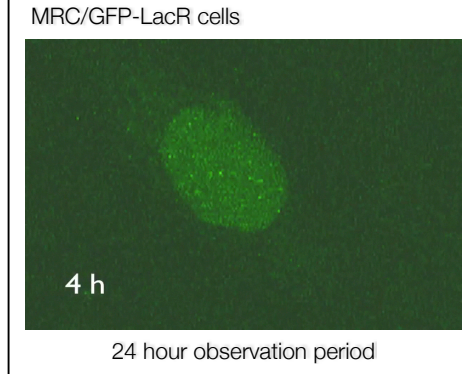
Quantification of intracellular DNA



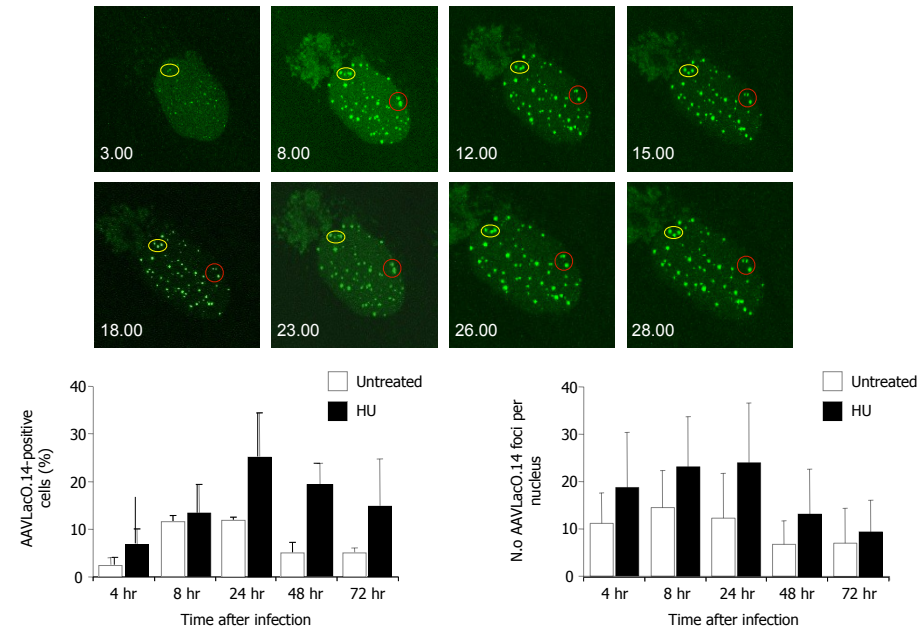
# An intracellular reporter to visualize nuclear dsAAV DNA



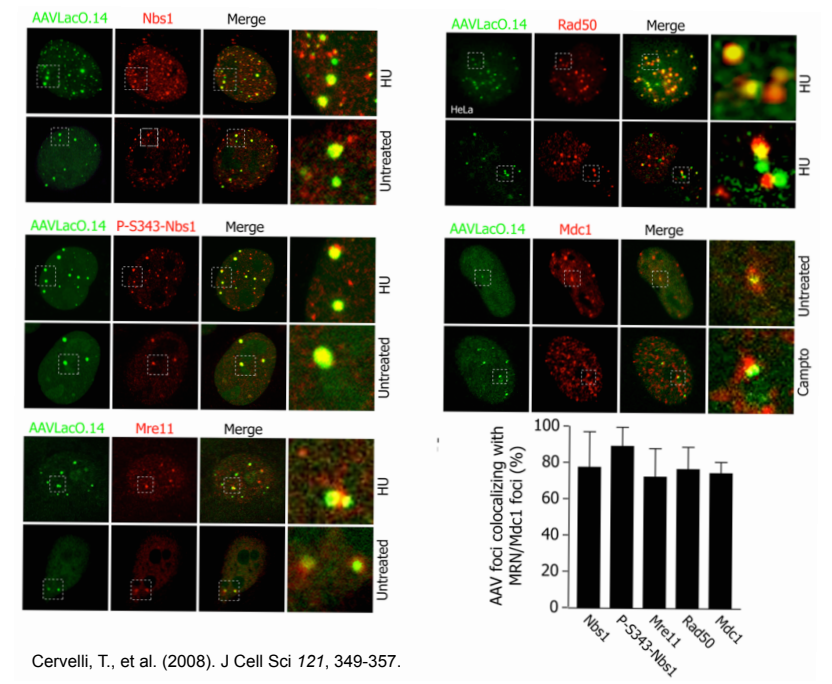
# Formation of nuclear dsDNA AAV foci after transduction of poorly permissive cells



# Kinetics of formation of AAVlacO.14 foci



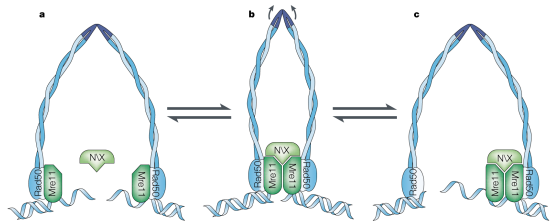
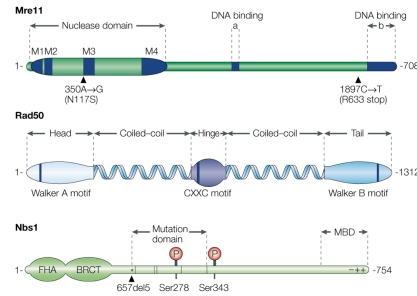
# AAVlacO.14 foci co-localize with DDR foci



Cervelli, T., et al. (2008). J Cell Sci 121, 349-357.

# MRN complex

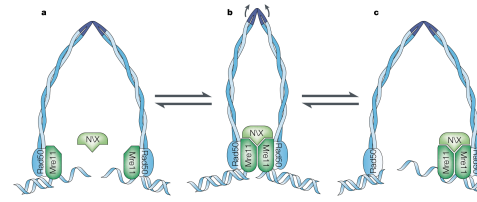
- Multisubunit nuclease composed of Mre11, Rad50 and Nbs-1
- Binds both ss and ds DNA and has pivotal role in sensing damaged or hairpin structured DNA



D'Amours and Jackson, Nat Rev Mol Cell Biol, 3, 317-327, 2002

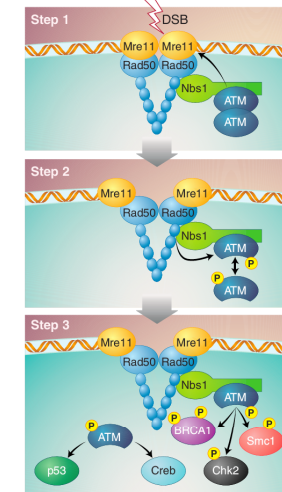
# MRN complex

- Multisubunit nuclease composed of Mre11, Rad50 and Nbs-1
- Binds both ss and ds DNA and has a pivotal role in sensing damaged or hairpin structured DNA and process it



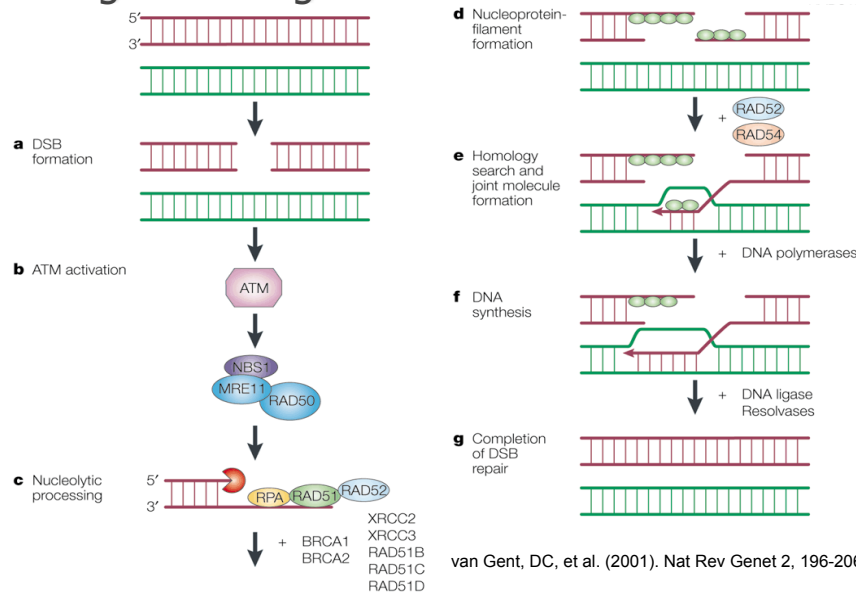
D'Amours and Jackson, Nat Rev Mol Cell Biol, 3, 317, 2002

# MRN complex activates ATM and checkpoint signalling



Lee JH, Paull TT, Science 308, 551, 2005

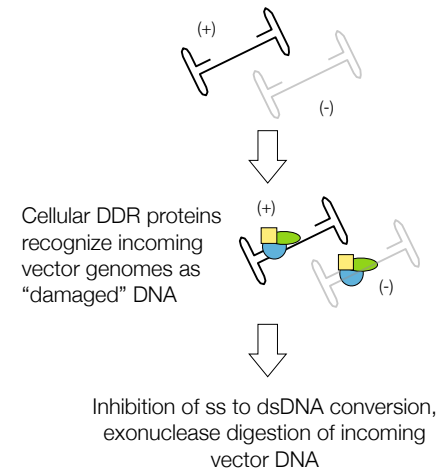
# Double-stranded DNA break repair through homologous recombination



van Gent, DC, et al. (2001). Nat Rev Genet 2, 196-206.

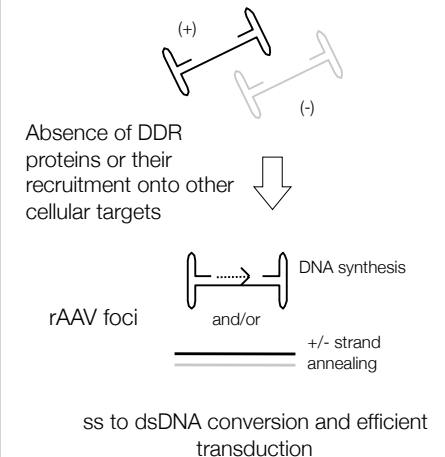
# Cellular DNA Damage Response (DDR) proteins restrict AAV transduction

*Non permissive cell*



"The DDR inhibition model"

*Permissive cell*



Zentilin L et al. 2001. J Virol 75, 12279  
Cervelli T et al. 2008. J Cell Sci 121, 349  
Schwartz PA et al. 2007. J Virol 81, 12936  
Lovric J et al. 2012. Mol Ther 20, 2087

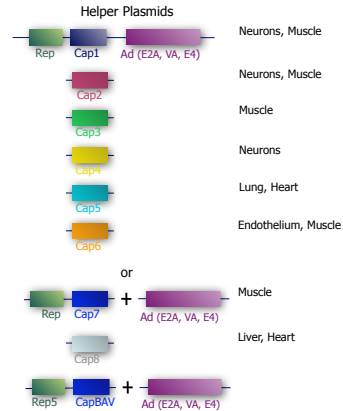


## AAV Vector Unit (AVU) facility at ICGEB Trieste: production of AAV vectors with different serotypes

www.icgeb.org/  
RESEARCH/TS/  
COREFACILITIES/  
AVUTRC.htm

The AVU produces, purifies, titers and characterizes research-grade recombinant AAV vectors

Both rAAV2 or hybrid vectors pseudotyped with capsid of serotypes from 1 to 9 are reproducibly obtained at a sterility and purity grade suitable for in vitro and animal experimentation



### Activities performed at the AVU

Plasmid amplification and purification  
Vector packaging and purification from cellular lysates  
Vector titration by real-time PCR

### Characteristics of final vector preparation

Yield:  $1 \times 10^3$  vg / cell  
Physical Titer:  $1 \times 10^{12}$  to  $1 \times 10^{13}$  vg / ml  
Final Volume of a standard vector batch: 3 ml in PBS



## AAV gene therapy clinical trials

- Melanoma
- Prostate cancer
- Cystic fibrosis
- Hemophilia B
- Muscular dystrophies
- Amyotrophic lateral sclerosis
- Canavan disease
- Alpha 1-antitrypsin deficiency
- Healthy subjects (aerosol)
- Rheumatoid arthritis
- Parkinson disease
- Retinal degeneration



## Gene therapy of muscle

For inherited disorders of muscle

For the release of therapeutic proteins in the blood

(To improve muscle function in adults)  
(Gene doping?)

Gene Therapy (2008) 15, 329-337  
© 2008 Nature Publishing Group All rights reserved 0969-7128/08 \$30.00  
www.nature.com/jgt

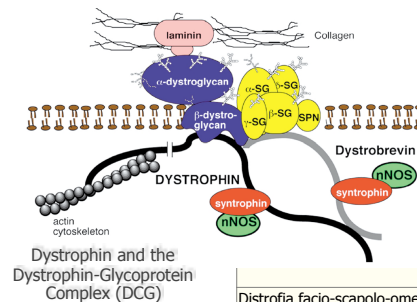
### REVIEW

*Progress and prospects: gene therapy for performance and appearance enhancement*

M Kiuru and RG Crystal  
Department of Genetic Medicine, Weill Medical College of Cornell University, New York, NY, USA



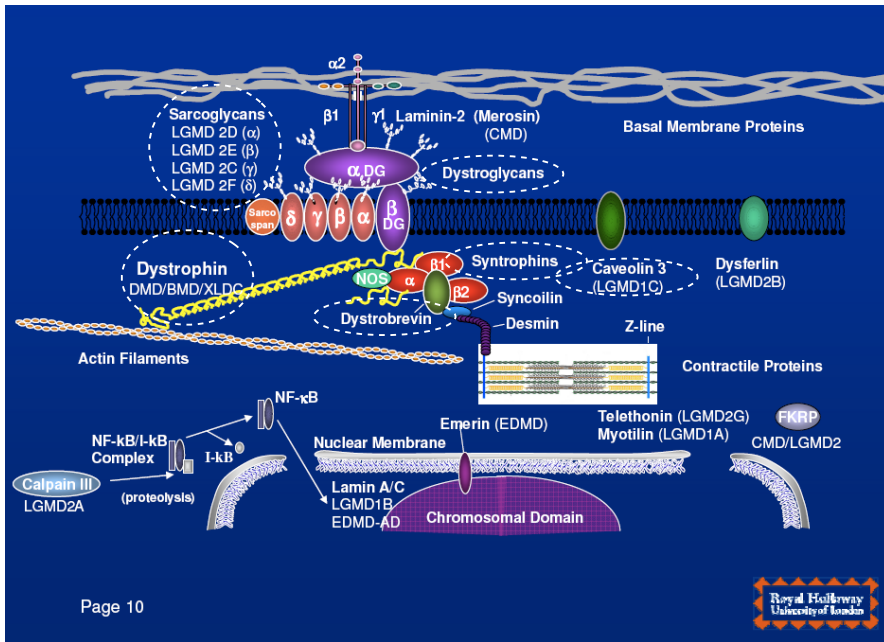
## Distrofie muscolari



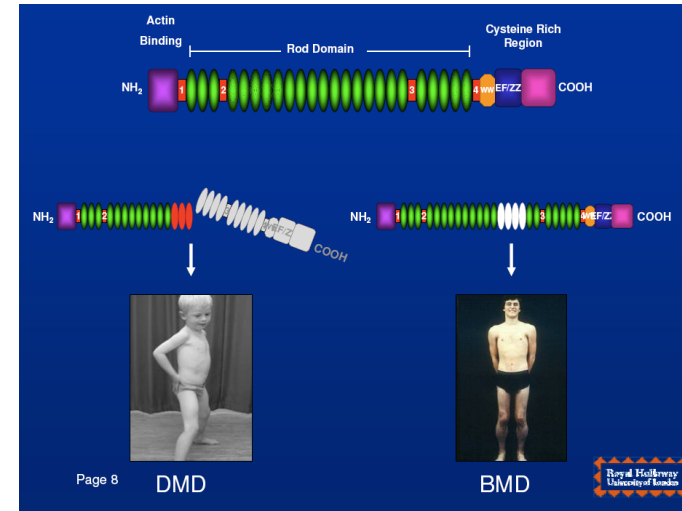
Malattia	Gene mutato
Distrofia muscolare di Duchenne (DMD)	Distrofina; incidenza di circa 1/3500 bambini maschi
Distrofia muscolare di Becker (BMD)	Distrofina; incidenza di circa 1/20.000 bambini maschi
Distrofia muscolare di Emery-Dreifuss	Emerina, lamina A o lamina C
Distrofia dei cingoli (LGMD)	Piu' di 15 geni diversi  autosomiche dominanti LGMD 1A: miotilina LGMD 1B: lamina A/C LGMD 1C: caveolina 3 ed altre  autosomiche recessive LGMD 2A: calpaina-3 LGMD 2B: disferlina LGMD 2C: gamma-sarcoglicano LGMD 2D: alpha-sarcoglicano LGMD 2E: beta-sarcoglicano LGMD 2F: delta-sarcoglicano ed altre
Distrofia facio-scapolo-omeroale o di Lnadouzy-Dejerine (FSHD)	Non noto
Distrofia miotonica o malattia di Steinert (MMD)	DMPK (DM1) e ZNF9 (DM2)
Distrofia oculo-faringea (OPMD)	Poly(A)-binding protein nuclear 1 (PABPN1)
Distrofia muscolare distale (DD)	Almeno 8 geni diversi (disferlina, titina, desmina ed altri)
Distrofia muscolare congenita (CMD)	Geni diversi (Laminina alpha-2 - merosina, fukutina, collagene di tipo VI, integrina a7, ed altri)



# Molecular defects in muscular dystrophies



# Molecular defects in dystrophin lead to Duchenne and Becker muscular dystrophy



427 kDa, gene 2.4 Mbp with 70 exons, mRNA 14 kb (coding region 11 kb) - transcription lasts 16 hrs  
 4 structural domains (N-terminal, rod, cysteine-rich and C-terminal)  
 N-terminal: binds actin, Cysteine rich: binds b-dystroglycan

## DMD: il problema clinico

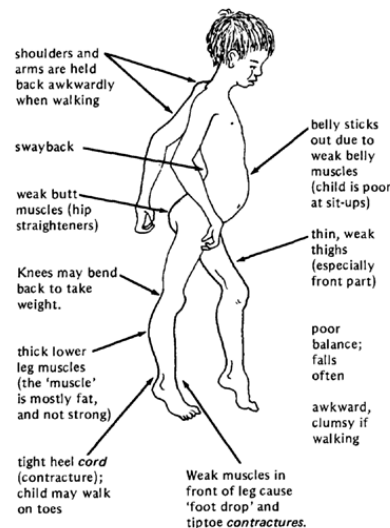
La DMD ha un decorso ingravescente e devastante (esaurimento delle cellule satelliti).

Alla nascita, i bambini maschi affetti sembrano normali, ed i primi sintomi insorgono tra i 3 ed i 5 anni di vita sotto forma di blanda debolezza muscolare, che si manifesta con la difficoltà nel salire le scale, alzarsi nella posizione seduta o con l'incespicare di frequente. Con il passare del tempo, la muscolatura si indebolisce progressivamente.

Solitamente entro i 10 anni di vita gli individui affetti sono costretti sulla sedia a rotelle, e molti decedono entro il 20° anno di età.

Non esistono attualmente terapie per la malattia, se non quelle di supporto.

Oltre al muscolo scheletrico, i pazienti con DMD mostrano un interessamento più o meno marcato del cuore che spesso evolve in una forma franca di cardiomiopatia dilatativa.

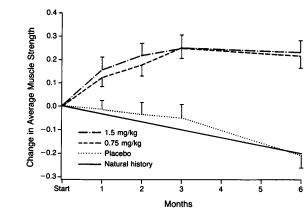


## DMD: current treatment options

Only glucocorticoids have consistently demonstrated efficacy in DMD

1592 THE NEW ENGLAND JOURNAL OF MEDICINE June 15, 1989  
**RANDOMIZED, DOUBLE-BLIND SIX-MONTH TRIAL OF PREDNISONE IN DUCHENNE'S MUSCULAR DYSTROPHY**  
 J.R. MENDELL, R.T. MOXLEY, R.C. GRIGGS, M.H. BROOKS, G.M. FENICHEL, J.P. MILLER, W. KING, L. SIGNORE, S. PANDYA, J. FLORENCE, J. SCHERBECKER, J. ROBISON, K. KAISER, S. MANDELL, C. ARFREEN, AND B. GLEDER

At 6 months: improvement in muscle strength, pulmonary function, time to rise from supine to standing, to walk 9m, to climb 4 stairs.



Neurology® 2011;77:444-452

## Randomized, blinded trial of weekend vs daily prednisone in Duchenne muscular dystrophy

**Conclusions:** Weekend dosing of prednisone is equally beneficial to the standard daily dosing of prednisone. Analysis of side effect profiles demonstrated overall tolerability of both dosing regimens.

## Side effects:

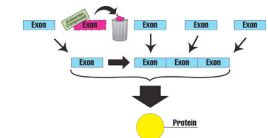
- weight gain with cushingoid appearance, high risk for hypertension, cataract, loss of bone density, vertebral compression fractures and long bone fractures
- long term administration limited by steroid-induced behavioral problems

# DMD: emerging drugs or small molecule therapies

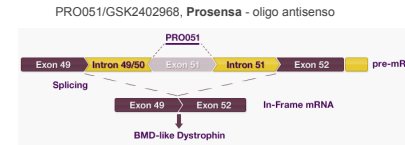
- Exon skipping
- Mutation suppression
- Gene therapy
- Muscle building strategy

## Exon skipping

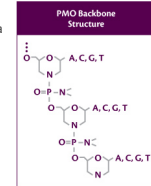
Exon skipping is targeted at the pre-mRNA level, allowing one or more exons to be omitted to restore the dystrophin reading frame. This is accomplished with splice-switching oligomers (20-30 nt), complementary to sequences of the pre-mRNA transcript.



## 2 proof-of-principle clinical trials targeting exon51



AVI-4658/Eteplirsen - Avi Biopharma - morpholino oligomer



ORIGINAL ARTICLE

### Systemic Administration of PRO051 in Duchenne's Muscular Dystrophy

Nathalie M. Goemans, M.D., Mar Tulinus, M.D., Ph.D., Johanna T. van den Akker, Ph.D., Brigitte E. Bunn, Ph.D., Peter F. Ekhart, M.Sc., Niik Heuvelmans, Tjeldine Holling, Ph.D., Anneke A. Janson, Gerard J. Platenburg, M.Sc., Jessica A. Sipkens, M.Sc., J.M. Ad Sitsen, M.D., Ph.D., Annemieke Aartsma-Rus, Ph.D., GerJan B. van Ommen, Ph.D., Gunnar Bayas, M.D., Ph.D., Niklas Darin, M.D., Ph.D., Jan J. Verschuuren, M.D., Ph.D., Giles V. Campion, M.D., Sijf J. de Kimpfe, Ph.D., and Judith C. van Deutekom, Ph.D.

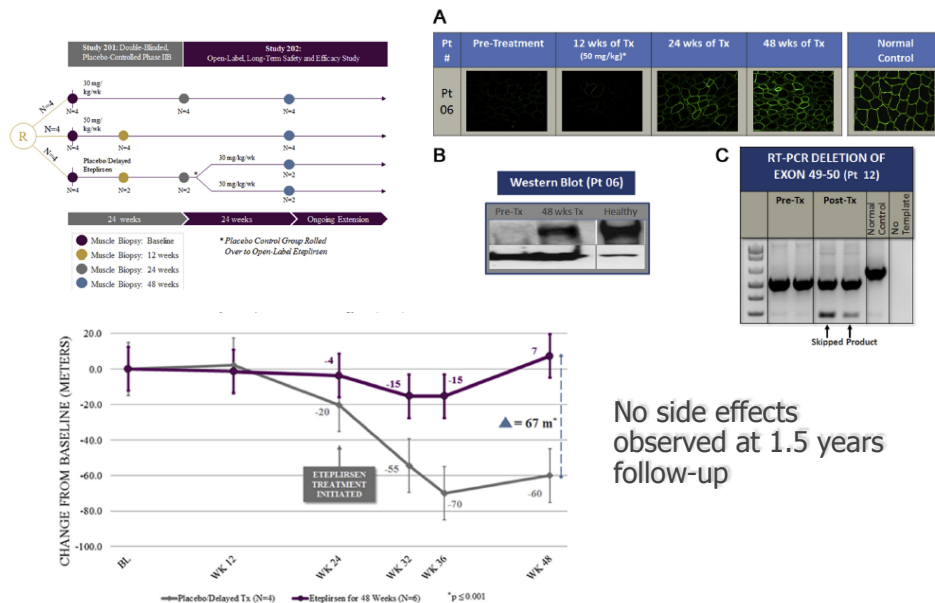
N Engl J Med 363:15-23, 2010

### Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study

Sabehzad Cosh, Virginia Anichenko-Gomez, Mahela Gugler, Lucy Feng, Silvio Torelli, Karen Anthony, Stephen Abbs, Marki Eless Gerardo, John Bourke, Dominic J.Wells, George Dickson, Matthew A Wood, Steve D Wilson, Walker Straub, Ryszard Kuc, Stephen B Shewsbury, Caroline Swery, Jennifer E Morgan, Kate Buckley, Francesco Montanini

www.thelancet.com Vol 378 August 13, 2011

## Molecular and functional efficacy Eteplirsen



No side effects observed at 1.5 years follow-up

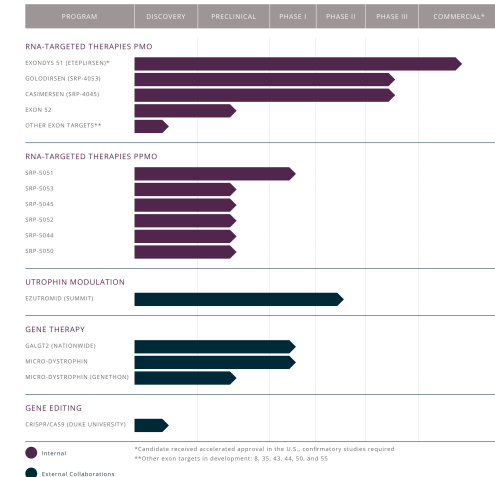
## Accelerated FDA approval of the exon-skipping drug eteplirsen

Approval to market eteplirsen was given in September 2016 to pharmaceutical company Sarepta Therapeutics. Eteplirsen will be the first disease-modifying drug on the market in the United States to treat DMD, and approximately 13 percent of DMD patients potentially may be eligible for treatment. Under the terms of the FDA's accelerated approval, Sarepta must conduct a clinical trial of eteplirsen to confirm clinical benefit. The approval is provisional, pending results of the ongoing phase III clinical trial.



"Our distinctive PMO-based platform chemistries allow us to address specific genetic diseases by altering the RNA transcription process and thereby modifying protein structure."

Gunnar J. Hanson, Ph.D., Senior Director of Research Chemistry



# EMA has not approved Eteplirsen as Duchenne MD Therapy in Europe



December 2016

Sarepta Therapeutics, Inc. (NASDAQ:SRPT), a commercial-stage developer of innovative RNA-targeted therapeutics, today announced that the European Medicines Agency (EMA) validated the previously submitted Marketing Authorization application (MAA) for eteplirsen to treat Duchenne muscular dystrophy amenable to exon 51 skipping. Sarepta is seeking conditional approval of eteplirsen in the EU through the centralized procedure. Validation of the MAA confirms that the submission is accepted and starts the formal review process by the EMA's Committee for Human Medicinal Products (CHMP). The standard review period is 210 days (plus additional time for applicant to respond to questions from the agency).

Under the brand name **Exondys 51**, eteplirsen was approved by the U.S. Food and Drug Administration (FDA) in September 2016, making it the first FDA-approved therapy specifically indicated to treat Duchenne MD. But **Exondys 51's** development and approval process was a prolonged one. The FDA decision also followed a campaign by muscular dystrophy advocacy groups urging access to a therapy offering some level of clinical improvement for a disease with no therapeutic options.

September 2018

## Duchenne secondo "no" europeo per eteplirsen

### Challenges to gene therapy for DMD

Gene therapy requires delivery of a new gene to the vast majority of muscles in the body and to the heart - a daunting challenge, since muscle tissues makes up **>40% of body mass**

The **dystrophin mRNA** is **14 kb** in size. A delivery vector must be identify that can carry this expression cassette; alternatively, truncated version of the gene can be used

Muscle transduction must not trigger toxic or immunological reactions that are harmful to the patient or that lead to further muscle damage

## DMD: emerging drugs or small molecule therapies

- Exon skipping
- Mutation suppression
- **Gene therapy**
- Muscle building strategy

## Duchenne muscular dystrophy - natural animal models

mdx mice (point mutation leading to a premature truncation)



xdm golden retriever dog (exon 7 skipping)



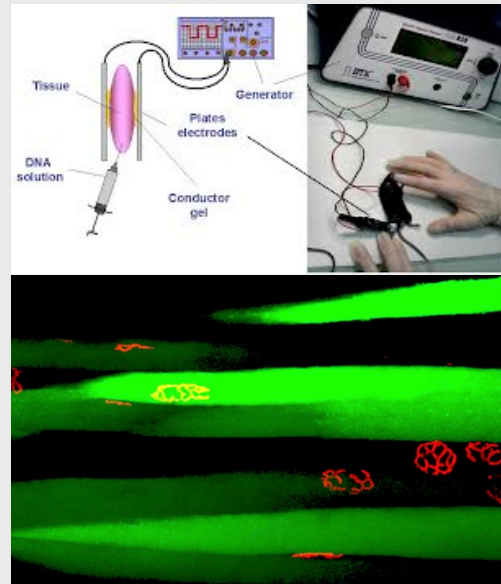


## Vectors for muscle gene therapy

- **Plasmid DNA:** displays a remarkable ability to transfer genes to muscle, specially if coupled with high pressure injection and/or electroporation

The first clinical trial, closed in 2006, entailed the injection of a plasmid containing the whole dystrophin cDNA under the control of the CMV promoter into the radialis muscle of 9 DMD/BMD patients. However, dystrophin expression resulted too low and not homogenous.

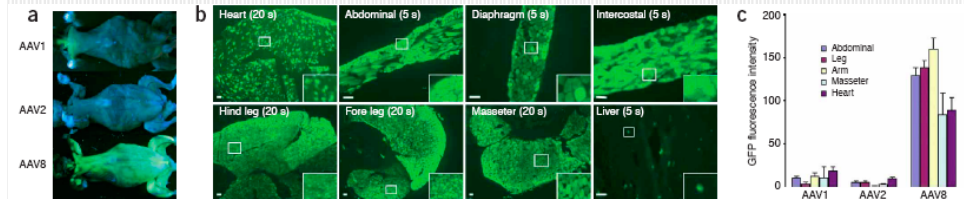
Different strategies can be used to increased transduction efficiency, including polymers, ultrasounds (with microbubbles), and **electroporation**



## Vectors for muscle gene therapy

### Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart

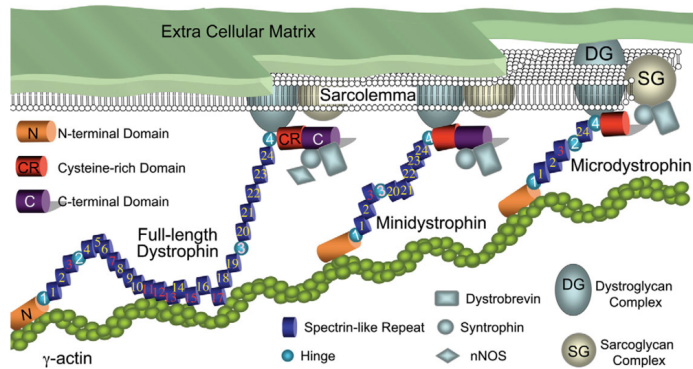
Zhong Wang<sup>1,3</sup>, Tong Zhu<sup>1,3</sup>, Chunping Qiao<sup>1</sup>, Liqiao Zhou<sup>1</sup>, Bing Wang<sup>1</sup>, Jian Zhang<sup>1</sup>, Chunlian Chen<sup>1</sup>, Juan Li<sup>1</sup> & Xiao Xiao<sup>1,2</sup>



**Figure 2** Systemic gene delivery to muscle of neonatal mice by different AAV serotypes via i.v. injection. (a) Whole-body fluorescent photography taken one month after i.v. (temporal vein) injection of  $2 \times 10^{11}$  v.g. of various dsAAV-CB-GFP at 3 d of age. (b) GFP expression seen in cryosections of heart, muscle and nonmuscle tissues one month after neonatal i.v. injection with  $2 \times 10^{11}$  v.g. of dsAAV8-CB-GFP. Numbers in parentheses denote microscopy exposure time in seconds (s). Scale bar, 100  $\mu$ m. Note the strong GFP expression in heart and muscles, and the minimal to undetectable GFP expression in nonmuscle tissues. (c) Quantitative analysis of average fluorescence intensity of cryosections from various muscles of AAV1, 2 and 8 treated mice (as shown in a).

## Distrofina e DGC

R254 Human Molecular Genetics, 2006, Vol. 15, Review Issue No. 2



**Figure 1.** Schematic outline of full-length dystrophin, minidystrophin and microdystrophin and their interaction with other cellular proteins. Spectrin-like repeats are numbered from 1 to 24 (positively charged repeats are in red color, other repeats are in yellow color). Proline-rich hinges are numbered from 1 to 4. Hinge 3 is in pink color to indicate that it can be cleaved by viral protease. Hinge 2 to repeat 19 are deleted in minidystrophin. Repeat 4–23 and the C-terminal domain are deleted in microdystrophin. Not drawn to scale.

### minidistrofina (~6-7 kb) e microdistrofina (~4 kb)

Queste versioni ridotte della distrofina presentano delezioni comuni della regione centrale a **bastoncello** e nel dominio C-terminale della proteina parentale, lasciando intatti i domini funzionali essenziali della proteina, in particolare quello ricco in cisteine (CR)

## Safety Study of Mini-Dystrophin Gene to Treat Duchenne Muscular Dystrophy

Study Type: Interventional

Study Design: Treatment, Randomized, Double-Blind, Placebo Control, Single Group Assignment, Safety Study

Official Title: Phase 1 Clinical Trial of rAAV2.5-CMV-Mini-Dystrophin Gene Vector in Duchenne Muscular Dystrophy

Primary Outcome Measures:

- Safety

Secondary Outcome Measures:

- mini-dystrophin gene expression at the site of gene transfer
- muscle strength evaluated by Maximal Volume Isometric Contraction Testing

This phase I randomized double blind dose escalation study investigates the safety and efficacy of the mini-dystrophin gene transferred to the **biceps** muscle for **Duchenne muscular dystrophy** patients, **ages 5 to 12 years** of age, using a recombinant adeno-associated virus. Eligible participants must have a known dystrophin gene mutation and may be concurrently treated with corticoid steroids. The mini-dystrophin gene or a placebo agent (normal saline or empty viral capsids) are injected directly into both biceps muscles while under conscious sedation. Following the gene transfer, patients are admitted to the hospital for 48 hours of observation followed by weekly outpatient visits at the Columbus Children's Hospital Neuromuscular Clinic. A **bilateral muscle biopsy** is performed following 6 weeks with long term follow up will consisting of bi-annual visits for the next 2 years.

Principal Investigator:

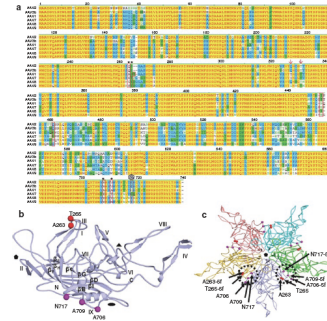
Jerry R. Mendell, MD  
Nationwide Children's Hospital

## Phase 1 Gene Therapy for Duchenne Muscular Dystrophy Using a Translational Optimized AAV Vector

Dawn E Bowles<sup>1</sup>, Scott WJ McPhee<sup>2</sup>, Chengwen Li<sup>3</sup>, Steven J Gray<sup>4</sup>, Jade J Samulski<sup>2</sup>, Angeleque S Camp<sup>5</sup>, Juan Li<sup>6</sup>, Bing Wang<sup>6</sup>, Paul E Monahan<sup>7</sup>, Joseph E Rabinowitz<sup>8</sup>, Joshua C Grieger<sup>9</sup>, Lakshmanan Govindasamy<sup>7</sup>, Mavis Agbandje-McKenna<sup>2</sup>, Xiao Xiao<sup>3</sup> and R Jude Samulski<sup>1</sup>

<sup>1</sup>Department of Surgery, Division of Surgical Sciences, Duke University Medical Center, Durham, North Carolina, USA; <sup>2</sup>Asklapio Biopharmaceutical Inc., Chapel Hill, North Carolina, USA; <sup>3</sup>Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA; <sup>4</sup>Joint Vector Core, University of North Carolina, Chapel Hill, North Carolina, USA; <sup>5</sup>Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina, USA; <sup>6</sup>Center for Translational Medicine, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, USA; <sup>7</sup>Department of Biochemistry and Molecular Biology, Center for Structural Biology, McKnight Brain Institute, University of Florida, Gainesville, Florida, USA

www.moleculartherapy.org vol.20 no.2 feb. 2012



**Table 2 The clinical data in patients with AAV2.5/minidystrophin muscular delivery**

Subject	Age	Sex	Weight (kg)	Height (cm)	Genotype	Capid dose (copies)	Empty capsid	Capid dose (copies)	Empty capsid	Revertant fibers			
1	8	Male	28.7	128.7	1:2	3.0 × 10 <sup>12</sup>	Empty capsid	High	D43	2.56	ND	0-11	
2	9	Male	30.0	130.0	1:100	3.0 × 10 <sup>12</sup>	Empty capsid	High	D90	0.08	ND	ND	
3	0	Male	15.8	75.1	1:2	3.0 × 10 <sup>12</sup>	Empty capsid	High	D43	1.42	weak	1-25	
4	5	Male	49-54	15.8	N	<1:2	3.0 × 10 <sup>12</sup>	Saline	Intermediate	D43	2.56	ND	0-9
5	11	Male	3-17	57.1	Y	1:100	3.0 × 10 <sup>12</sup>	Empty capsid	High	D90	0.08	ND	0-9
6	9	Male	46-52	28.7	Y	1:2	3.0 × 10 <sup>12</sup>	Empty capsid	High	D43	1.42	weak	1-25

Abbreviations: AAV, adeno-associated virus; ND, none detected; qPCR, quantitative PCR.

\*AAV2.5 minidystrophin vector genome dose (vector genomes/patient). <sup>b</sup>Total capsid dose (minidystrophin + empty capsid in subject's 5 and 6) capsid particles/patient. <sup>c</sup>Vector genome copy number isolated per nucleus as determined by qPCR (skeletal muscle cells are multinucleated).

## ARTICLE

Received 18 Aug 2016 | Accepted 30 May 2017 | Published 25 Jul 2017

DOI: 10.1038/ncomms16105

OPEN

## Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy

Caroline Le Guiner<sup>1,2</sup>, Laurent Servais<sup>3</sup>, Marie Montus<sup>2</sup>, Thibaut Larcher<sup>4</sup>, Bodvaël Fraysse<sup>1</sup>, Sophie Moulic<sup>5</sup>, Marine Allais<sup>1</sup>, Virginie François<sup>1</sup>, Maeva Dutilleul<sup>4</sup>, Alberto Malerba<sup>6</sup>, Taeyoung Koo<sup>6</sup>, Jean-Laurent Thibaut<sup>7,8</sup>, Béatrice Matot<sup>7</sup>, Marie Devaux<sup>1</sup>, Johanne Le Duff<sup>1</sup>, Jack-Yves Deschamps<sup>5</sup>, Inès Barthelemy<sup>8,9</sup>, Stéphane Blot<sup>8,9</sup>, Isabelle Testault<sup>10</sup>, Karim Wahbi<sup>11</sup>, Stéphane Ederhy<sup>12</sup>, Samia Martin<sup>2</sup>, Philippe Veron<sup>2</sup>, Christophe Georger<sup>2</sup>, Takis Athanasopoulos<sup>6,13,†</sup>, Carole Masurier<sup>2</sup>, Federico Mingozzi<sup>2</sup>, Pierre Carlier<sup>7</sup>, Bernard Gjata<sup>2</sup>, Jean-Yves Hogrel<sup>14</sup>, Oumeya Adjali<sup>1</sup>, Fulvio Mavilio<sup>2</sup>, Thomas Voit<sup>15,\*</sup>, Philippe Moullier<sup>1,16,\*</sup> & George Dickson<sup>6,\*</sup>



Duchenne muscular dystrophy (DMD) is an incurable X-linked muscle-wasting disease caused by mutations in the dystrophin gene. Gene therapy using highly functional microdystrophin genes and recombinant adeno-associated virus (rAAV) vectors is an attractive strategy to treat DMD. Here we show that locoregional and systemic delivery of a rAAV2/8 vector expressing a canine microdystrophin (cMD1) is effective in restoring dystrophin expression and stabilizing clinical symptoms in studies performed on a total of 12 treated golden retriever muscular dystrophy (GRMD) dogs. Locoregional delivery induces high levels of microdystrophin expression in limb musculature and significant amelioration of histological and functional parameters. Systemic intravenous administration without immunosuppression results in significant and sustained levels of microdystrophin in skeletal muscles and reduces dystrophic symptoms for over 2 years. No toxicity or adverse immune consequences of vector administration are observed. These studies indicate safety and efficacy of systemic rAAV-cMD1 delivery in a large animal model of DMD, and pave the way towards clinical trials of rAAV-microdystrophin gene therapy in DMD patients.

**Table 2 | Levels of cMD1-positives fibres found after immunostaining analysis (NCL-DYSB) within the muscles of GRMD dogs injected by the LR route.**

Dog	Injected forelimb (n = 13 muscles)		Noninjected forelimb (n = 13 muscles)		Other muscles at distance (n = 17 muscles)	Heart	Diaphragm
	Mean of cMD1+ fibres	CV	Mean of cMD1+ fibres for the group	CV for the group			
LR1	51%	54%	50%	47%	3%	10%	13%
LR2	59%	30%			1%	10%	<0.5%
LR3	49%	58%			3%	11%	18%
LR4	43%	49%			1%	7%	1%
LR C1	<0.5%	NA	<0.5%	NA	<0.5%	<0.5%	<0.5%
LR C2	<0.5%	NA	<0.5%		<0.5%	<0.5%	<0.5%
LR C3	<0.5%	NA	<0.5%		<0.5%	<0.5%	<0.5%

CV, coefficient of variation.  
For the complete list of muscles and tissues sampled at the time of killing, see Supplementary Table 2 in ref. 33.

shedding of the vector after release of the tourniquet

Atlantic Gene Therapies

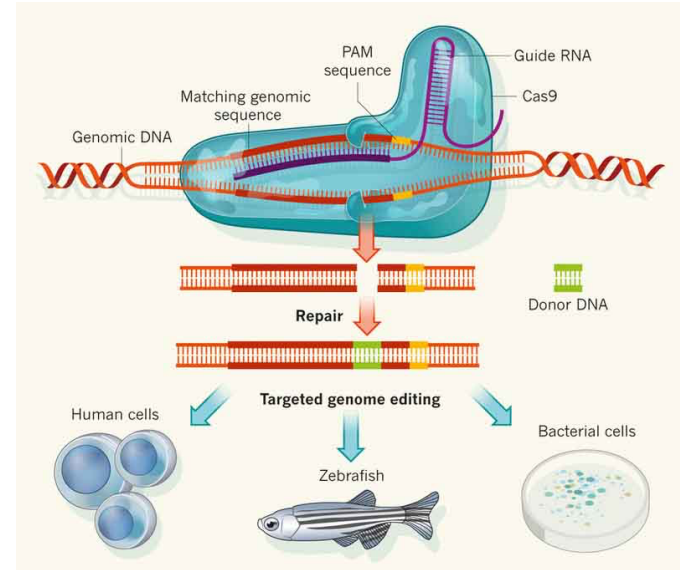
6 month-old GRMD dog

Untreated

# DMD: emerging drugs or small molecule therapies

- Exon skipping
- Mutation suppression
- Gene therapy
- Muscle building strategy
- **Genome editing**

# CRISPR/Cas9 and Targeted Genome Editing: A New Era in Molecular Biology



The functions of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes are essential in adaptive immunity in select bacteria and archaea, enabling the organisms to respond to and eliminate invading genetic material.

## In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy

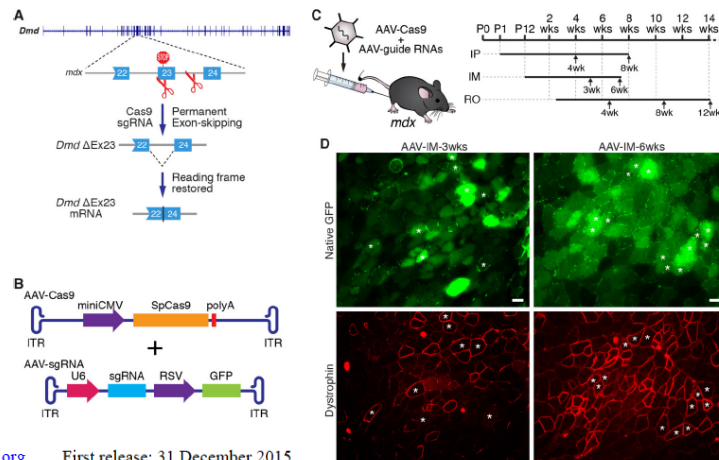
Christopher E. Nelson,<sup>1,2</sup> Chady H. Hakim,<sup>2</sup> David G. Ousterout,<sup>1,2</sup> Pratiksha I. Thakore,<sup>1,2</sup> Eirik A. Moreb,<sup>1,2</sup> Ruth M. Castellanos Rivera,<sup>1</sup> Sarina Madhavan,<sup>1,2</sup> Xiofang Pan,<sup>1</sup> F. Ann Ran,<sup>1,2</sup> Winston X. Yan,<sup>1,2</sup> Aravind Asokan,<sup>1</sup> Feng Zhang,<sup>1,2,3,4,5,6,7</sup> Dongsheng Duan,<sup>1,2</sup> Charles A. Gersbach<sup>1,2,3,4,5,6,7</sup>

## Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy

Chengzu Long,<sup>1,2,3,4</sup> Leonela Amoussi,<sup>1,2,3,4</sup> Alex A. Mireault,<sup>1,2,3,4</sup> John R. McAnally,<sup>1,2,3,4</sup> Hui L.J.,<sup>1,2,3,4</sup> Efrain Sanchez-Ortiz,<sup>1,2,3,4</sup> Samadrita Bhattacharyya,<sup>1,2,3,4</sup> John M. Shelton,<sup>1,2,3,4</sup> Rhonda Bassel-Duby,<sup>1,2,3,4</sup> Eric N. Olson<sup>1,2,3,4,5,6,7</sup>

## In vivo gene editing in dystrophic mouse muscle and muscle stem cells

Mohammadsharif Tabejborbar,<sup>1,2,3</sup> Kexian Zhu,<sup>1,2,3</sup> Jason K. W. Cheng,<sup>1</sup> Wei Leong Chew,<sup>1,2</sup> Jeffrey J. Widrick,<sup>2</sup> Winston X. Yan,<sup>1,2</sup> Claire Maesner,<sup>1</sup> Elizabeth Y. Wu,<sup>1</sup> Ru Xiao,<sup>1</sup> F. Ann Ran,<sup>1,2</sup> Le Cong,<sup>1,2</sup> Feng Zhang,<sup>1,2,3,4</sup> Luk H. Vandenberghe,<sup>1</sup> George M. Church,<sup>1</sup> Amy J. Wagers<sup>1,2</sup>



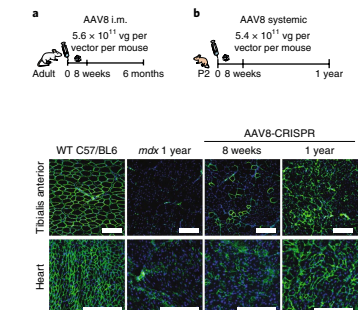
nature medicine LETTERS  
<https://doi.org/10.1038/s41591-019-0344-3>

## Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy

Christopher E. Nelson<sup>1,2</sup>, Yaoying Wu<sup>1</sup>, Matthew P. Gemberling<sup>1,2</sup>, Matthew L. Oliver<sup>1</sup>, Matthew A. Waller<sup>1,2</sup>, Joel D. Bohning<sup>1,2</sup>, Jacqueline N. Robinson-Hamm<sup>1,2</sup>, Karen Bulaklak<sup>1,2</sup>, Ruth M. Castellanos Rivera<sup>3</sup>, Joel H. Collier<sup>1</sup>, Aravind Asokan<sup>4,5</sup> and Charles A. Gersbach<sup>1,2,6,7</sup>

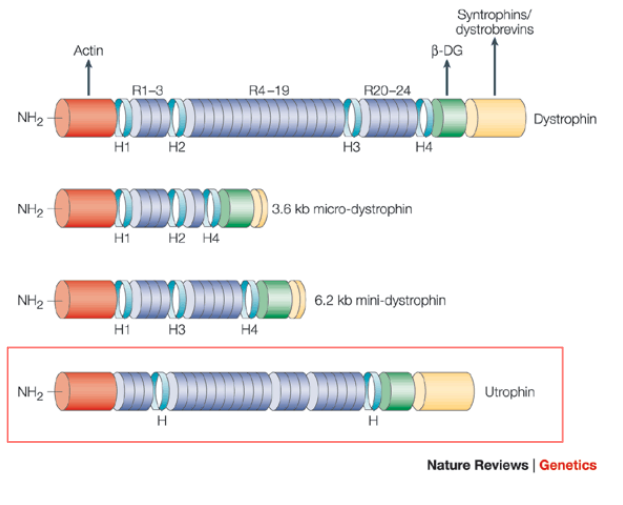
Duchenne muscular dystrophy (DMD) is a monogenic disorder and a candidate for therapeutic genome editing. There have been several recent reports of genome editing in pre-clinical models of Duchenne muscular dystrophy<sup>1-5</sup>, however, the long-term persistence and safety of these genome editing approaches have not been addressed. Here we show that genome editing and dystrophin protein restoration is sustained in the *mdx* mouse model of Duchenne muscular dystrophy for 1 year after a single intravenous administration of an adeno-associated virus that encodes CRISPR (AAV-CRISPR). We also show that AAV-CRISPR is immunogenic when administered to adult mice<sup>6</sup>; however, humoral and cellular immune responses can be avoided by treating neonatal mice. Additionally, we describe unintended genome and transcript alterations induced by AAV-CRISPR that should be considered for the development of AAV-CRISPR as a therapeutic approach. This study shows the potential of AAV-CRISPR for permanent genome corrections and highlights aspects of host response and alternative genome editing outcomes that require further study.

Genome editing is sustained for 1 year in neonatal mice treated by intravenous administration.





## Structural domains of the human full-length dystrophin, Becker mini- and micro-dystrophins, and utrophin.



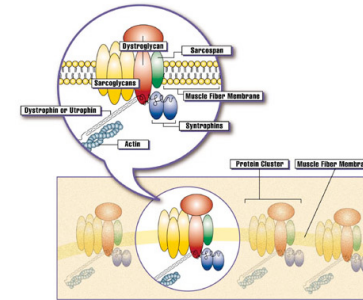
L'utrofina è una proteina, codificata da un gene posto sul cromosoma 6, omologa alla distrofina sia dal punto di vista strutturale. L'utrofina è normalmente presente alla giunzione neuromuscolare delle fibre muscolari mature, ed è anche espressa sul sarcolemma delle fibre muscolari fetali e del muscolo rigenerante. L'utrofina è anche espressa sul sarcolemma dei muscoli dei pazienti con DMD; tuttavia, i suoi livelli sono troppo bassi per vicariare la funzione della distrofina.

N-terminal domain (red) binds to F-actin; the cysteine-rich domain (green) binds to -Dystroglycan (-DG) and the C-terminal domain (yellow) binds to Dystrobrevins and syntrophins. The central coiled-coil rod domain (blue) contains 24 spectrin-like repeats (R1-R24) and 4 'hinge' regions (turquoise; H1-H4).

Nature Reviews | Genetics

## Successful Compensation for Dystrophin Deficiency by a Helper-dependent Adenovirus Expressing Full-length Utrophin

Jatinderpal R Deol<sup>1,2</sup>, Gawiyou Danielou<sup>1</sup>, Nancy Larochele<sup>1</sup>, Mylène Bourget<sup>1</sup>, Joon-Shik Moon<sup>1\*</sup>, An-Bang Liu<sup>1\*</sup>, Rénaud Gilbert<sup>1</sup>, Basil J Petrof<sup>2,3</sup>, Josephine Nalbantoglu<sup>1,2,3</sup> and George Karpati<sup>1,5</sup>

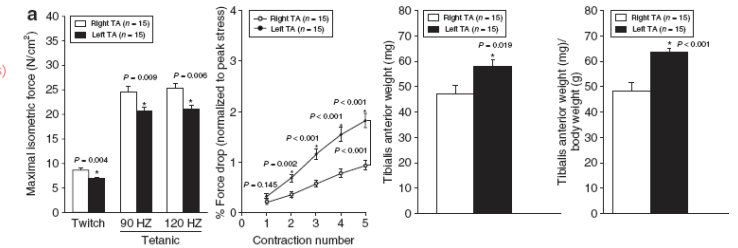


### First generation Ad vectors:

- good expression levels
- transient expression (10-15 days)
- impossible re-administration

### Gutless Ad vectors:

- tricky production
- not moved to the clinics yet



Molecular Therapy vol. 15 no. 10, 1767-1774 oct. 2007

## Summit Therapeutics Ends Development of Ezutromid Therapy for DMD After Trial Failure

### SMT C1100 (Ezutromid)

### A Small Molecule Utrophin Modulator for Duchenne

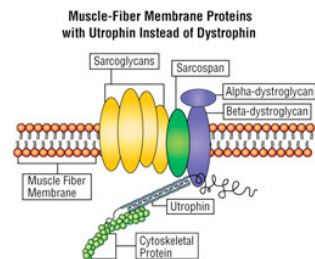
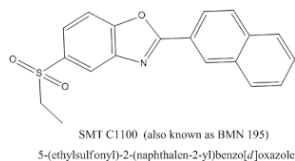


SMT C1100 has successfully completed a Phase 1 clinical trial in healthy male volunteers in 2012.

The Phase 1b dose-escalating trial was conducted in 12 patients with DMD aged between 5 and 11 years old. These preliminary results show that SMT C1100 was safe and well tolerated at all doses tested in the study, and that there were no issues with patient compliance. All the boys had variable blood plasma concentrations of SMT C1100 with only two of the boys achieving concentrations similar to those of the adult volunteers in the 2012 Phase 1 study. Initial evidence suggests that the variability in drug uptake may be due to differences in diet and to other disease-related factors.

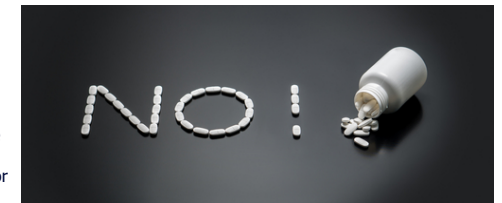
The non-placebo controlled trial also measured creatine kinase (CK) levels, an enzyme that is associated with muscle fibre damage and elevated in boys with DMD. In the majority of patients there was a reduction in CK levels during dosing with SMT C1100. These data are consistent with non-clinical in vivo efficacy studies in the mdx model of DMD that showed SMT C1100 reduced CK levels after only 15 days.

### Phase 2 trial initiated in 2016



48-week PhaseOut DMD trial which included 40 boys with DMD

Primary endpoint was the change from baseline in magnetic resonance parameters related to the leg muscles



Secondary endpoint consisted of biopsy measures evaluating utrophin and muscle damage, with patients having 2 biopsies, 1 at baseline and a second at either 24- or 48-weeks

Statistical decreases in developmental myosin and magnetic resonance T2 measures were seen after 24 weeks of treatment, but were not seen after 48 weeks

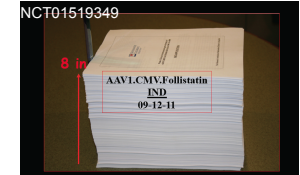
June 2018

## DMD: emerging drugs or small molecule therapies

- Exon skipping
- Mutation suppression
- Gene therapy
- Muscle building strategy

## Follistatin Gene Transfer to Patients With Becker Muscular Dystrophy and Sporadic Inclusion Body Myositis

Phase I Clinical Intramuscular Gene Transfer of rAAV1.CMV.huFollistatin (an antagonist to myostatin) Trial to Patients With Becker Muscular Dystrophy and Sporadic Inclusion Body Myositis.



Proc Natl Acad Sci USA  
Vol. 94, pp. 12457-12461, November 1997  
Genetics

### Double muscling in cattle due to mutations in the myostatin gene

ALEXANDRA C. MCPHERRON AND SE-JIN LEE\*

Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205



FIG. 2. A fullblood Belgian Blue bull showing the double muscling phenotype.



### Suppression of body fat accumulation in myostatin-deficient mice

Alexandra C. McPherron and Se-Jin Lee

Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Address correspondence to: Se-Jin Lee, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, Maryland 21205, USA. Phone: (410) 614-0198; Fax: (410) 955-0831; E-mail: spler@jhmi.edu.

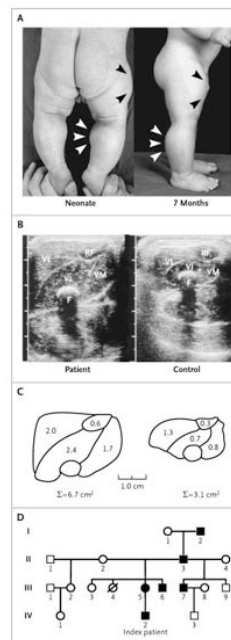
Received for publication June 19, 2001, and accepted in revised form January 30, 2002.

## Myostatin



Myostatin belongs to the TGF- $\beta$  superfamily of signal proteins, and it is normally made and secreted by skeletal muscle cells, providing negative feedback to limit muscle growth.

Small amounts of the protein can be detected in the circulation of adult humans, and it has been reported that the amount is raised in AIDS patients who show muscle wasting. Thus, myostatin may act as a negative regulator of muscle growth in adult life as well as during development. The growth of some other organs is similarly controlled by a negative-feedback action of a factor that they themselves produce.



Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Clinical Biochemistry 38 (2005) 959–965

Review

### Doping in the recombinant era: Strategies and counterstrategies<sup>☆</sup>

Hassan M.E. Azzazy<sup>a,\*</sup>, Mai M.H. Mansour<sup>a</sup>, Robert H. Christenson<sup>b</sup>

<sup>a</sup> Department of Chemistry and Science & Technology Research Center, The American University in Cairo, Cairo, Egypt

<sup>b</sup> Departments of Pathology and Medical & Research Technology, University of Maryland School of Medicine, Baltimore, MD 21201, USA

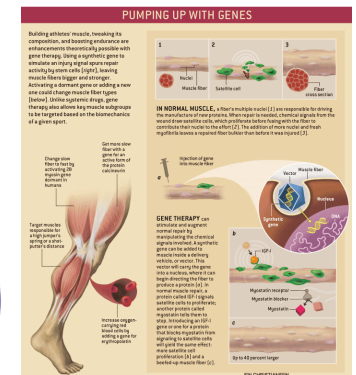
Received 21 July 2005; received in revised form 30 August 2005; accepted 2 September 2005



Table 1

#### Candidate genes for sports doping

Gene/product	System/organ targets	Gene product properties	Physiologic response
ACE	Skeletal muscles	Peptidyl dipeptidase	ACE-D is involved in fast twitch muscles ACE-I seems to correlate with endurance Involved in fast twitch muscles
ACTN3	Skeletal muscle	Actin-binding proteins related to dystrophin	
Endorphins	Central and peripheral nervous systems	Widely active peptides	Pain modulation
EPO	Hematopoietic system	Glycoprotein hormone	Increases RBC mass and oxygen delivery
HGH	Endocrine system	[91]-amino acid protein	Increases muscle size, power, and recovery
HIF	Hematologic and immune systems	Multisubunit protein	Regulates transcription at hypoxia response elements
IGF-1	Endocrine/metabolic/skeletal muscle	70-amino acid protein	Increases muscle size, power, and recovery by increasing regulator cells
Myostatin	Skeletal muscle	2-subunit protein	Regulates skeletal muscle. Inhibition increases muscle size, power, and recovery.
PPAR-delta	Skeletal muscle and adipose tissue	Nuclear hormone receptor protein	Promotes fat metabolism and increases number of slow twitch fibers
VEGF	Vascular endothelium	Glycosylated disulfide-bonded homodimers	Induces development of new blood vessels



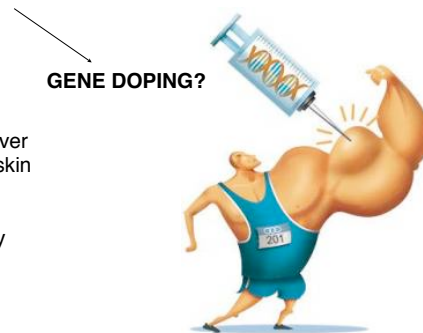
## GH as a performance-enhancing drug

The use of GH and IGF-I is banned by the World Anti Doping Agency not only because of the principle of fair play in competitive sports, but also because of the adverse effects of supraphysiological doses on health. In addition, although rhGH has been available since 1985, GH extracted from the pituitary glands of human cadavers is still available in some countries. Administration from this source carries a significant risk of infection of transmissible brain diseases.

The use of GH in amateur and professional sports seems to be widespread, although the evidence is quite strong that supraphysiological GH administration does not potentiate the effects of exercise on muscle mass and strength in healthy individuals. IGF-I use is probably more limited as it is less readily available than GH.

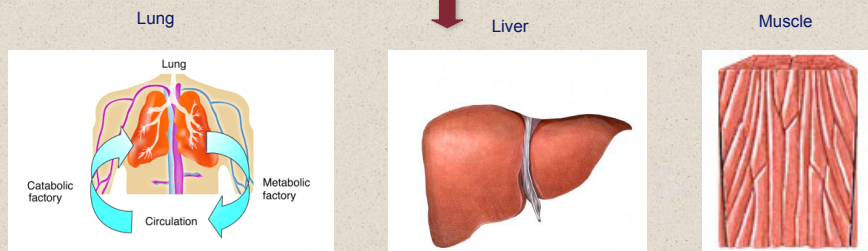
Attraction of GH abuse:

- GH is lipolytic
- GH has known effects on collagen and bone turnover
- GH has anecdotal side effects such as improving skin tone, eyesight and recovery time from injury
- combination of testosterone and GH leads to improved body composition and  $\text{VO}_2$  max in elderly men



## Genetically engineered metabolic factory to treat genetic diseases

Gene transfer by viral vectors

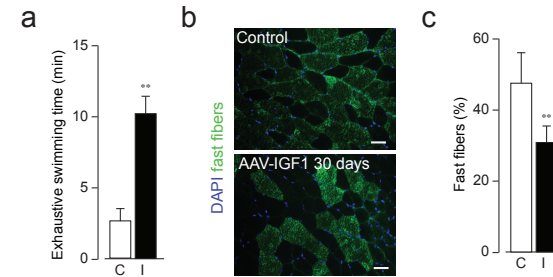


Ectopic production of genetically engineered protein products (Factor IX, Lysosomal enzymes, AAT-1, Epo, ecc)

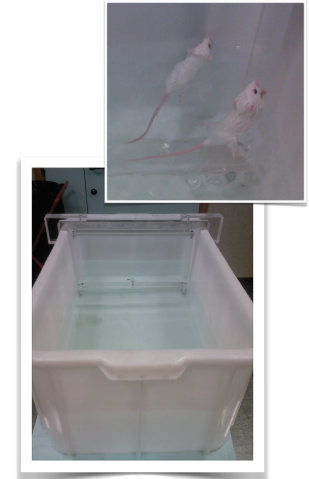
Delivery into the circulation

## Enhanced Athletic Performance on Multisite AAV-IGF1 Gene Transfer Coincides with Massive Modification of the Muscle Proteome

Antero Macedo,<sup>1,\*</sup> Manuela Moriggi,<sup>2,\*</sup> Michele Vasso,<sup>2,3</sup> Sara De Palma,<sup>2</sup> Mauro Sturnega,<sup>1</sup> Giorgio Friso,<sup>4</sup> Cecilia Gelfi,<sup>2,3</sup> Mauro Giacca,<sup>1</sup> and Serena Zacchignò



HUMAN GENE THERAPY 23:146–157 (February 2012)  
© Mary Ann Liebert, Inc.  
DOI: 10.1089/hum.2011.157



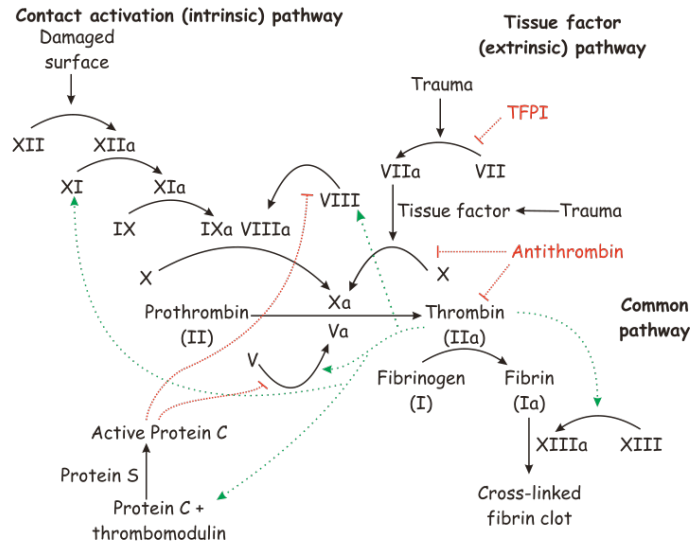
## Hemophilia



- Affects 1:5,000 males
  - 80% hemophilia A due to Factor VIII deficiency
  - 20% hemophilia B due to Factor IX deficiency
- Results in spontaneous bleeding, which can be fatal
- Treated with prophylactic or therapeutic infusion of the deficient factor
- Correction to 1% of normal activity would reduce spontaneous bleeding
- Correction to 10% of normal activity would eliminate most spontaneous bleeding



# Blood coagulation



## Historic Overview on Hemophilia Therapy

- Replacement therapy: blood transfusion since '70s
- Recombinant FVIII/FIX infusion since '90s

## Gene Therapy Approaches for Hemophilia:

- Existence of small (KO mice) and large (dog) animal models
- Easy assessment of efficacy (coagulation tests)
- Small correction should be sufficient (1%)
- Liver-directed, Muscle-directed
- Expensive therapy available

## Haemophilia gene transfer trials

1, 2, 5 - FVIII (>8 kb)  
3, 4 - FIX (1.4 kb)

Sponsor	Trial No.	n	Vector/Route	Factor Level†	Side Effects
Chiron	1—Phase I	13	Retrovirus/IV	0–1%	None
TKT	2—Phase I	6	Plasmid/omentum	0–4%	None
Avigen	3—Phase I	9	AAV2/IM	0–1%	None
Avigen	4—Phase I	6	AAV2/intrahepatic	3–12%	Elevated transaminase‡
Genstar	5—Phase I	3	Adenovirus/IV	0–1%	Elevated transaminase,‡ thrombocytopenia

Gutless vectors?

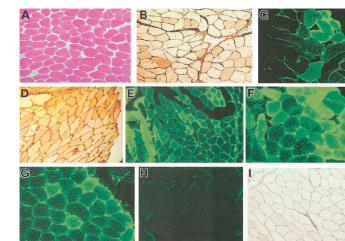
One patient so far.  
At 7 days: persistent elevation of transaminases = low therapeutic index  
No additional patients enrolled

### AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B

Catherine B. Manno, Amy J. Chew, Sylvia Hultchen, Peter J. Larson, Roland W. Herzog, Valdir R. Arruda, Shing-Jen Tai, Margaret V. Ragni, Arthur Thompson, Margresh Ozols, Linda B. Coult, Debra G. B. Leonard, Frederick A. Johnson, Alan McClelland, Charan Bhatia, Erik Skarsgard, Alan W. Flake, Mark A. Kay, Katherine A. High, and Bertil Glaser

Table 8. Bioactivity and efficacy studies in subjects treated with intramuscular AAV-hFIX

	PCR on muscle biopsy	Southern blot on muscle biopsy*	FIX immunohistochemistry	Max circ of FIX	Decrease in FIX infusion
A	Post	Neg	Neg	1–40%	50%
B	Post	ND	Pos	<1%	50%
C	Post	Pos (4)	Neg	<1%	None
D	Pos	Pos (1.5)	Pos	<1%	None
E	Neg	Neg	Pos	<1%	None
F	Neg	Neg	Pos	1%	None
G	Pos	Pos (0.5)	Pos	1%	None
H	Pos	Neg	Pos	<1%	None
I	Pos	Pos (0.5)	Pos	<1%	None



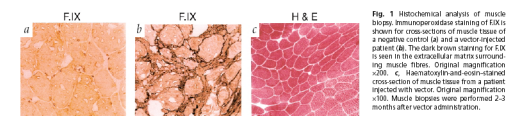
BLOOD, 15 APRIL 2003 • VOLUME 101, NUMBER 8

Phase I Trial: 8 adult men with severe hemophilia B (<1% baseline F.IX)

F.IX expression close to the site of injection up to 10 months after treatment; no evidence for inflammation

Circulating levels of F.IX were less than what is required for therapeutic effect

## Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector



	Patient A <sup>a</sup>		Patient B <sup>a</sup>	
	FIX	aPTT	FIX	aPTT
Baseline	<0.1%	82.9	<0.3%	102
Week 6	1%	61	0.3%	91.2
Week 8	1%	61	0.3%	102.3
Week 10	1.6%	48	0.3%	91.2
Week 12	1.4%	46.8	0.3%	102.3
Week 14	3.7%	41.0	3.0%	52.6 (post-FIX infusion)
Week 17	1.3%	50.6	0.4%	72
Week 18	0.8%	49.4	0.4%	107
Week 20	0.5%	54.1	0.4%	107
Week 22	0.9%	52.7	0.8%	65.5
Week 24	0.5%	52.1	0.8%	65.5

\*Unless otherwise noted, all data points were drawn at least 14 d after the most recent factor transfusion. <sup>a</sup>Data generated in CHOP Clinical Coagulation Laboratory. <sup>b</sup>Data generated in Stanford University Clinical Coagulation Laboratory.

Dose	Predicted levels of circulating FIX in humans			
	FIX level (in dog) <sup>a</sup>	FIX level (in human) <sup>b</sup>	Predicted level (in human) <sup>c</sup>	Predicted % normal levels in humans
2 × 10 <sup>11</sup> vg/kg	6 ng/ml	2–4 ng/ml	2–6 ng/ml	<0.1%
2 × 10 <sup>12</sup> vg/kg	60 ng/ml	16 ng/ml	16–60 ng/ml	0.3–1.2%
1 × 10 <sup>13</sup> vg/kg	300 ng/ml	80 ng/ml	80–300 ng/ml	1.3–6%

<sup>a</sup>Predicted plasma FIX level in mice based on mouse experimental data. <sup>b</sup>Predicted plasma FIX level in dogs based on canine experimental data. <sup>c</sup>Extrapolated from studies in animals.

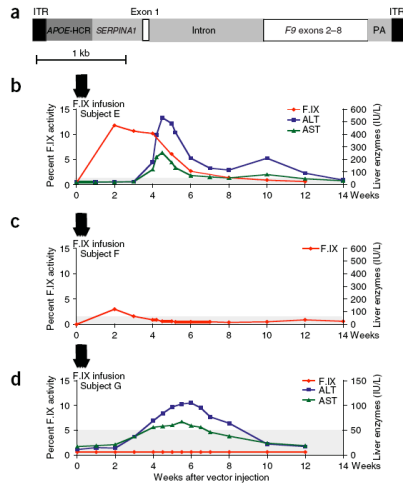
- Transient expression of FIX
- Partial correction of bleeding time
- Detection of anti-AAV2 antibodies

M. Kay, K. High

# Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response

MARCH 2006 NATURE MEDICINE

Catherine S Manno<sup>1,2,15</sup>, Glenn F Pierce<sup>3,15</sup>, Valder R Arruda<sup>1,2,15</sup>, Bertil Glader<sup>4,15</sup>, Margaret Ragni<sup>5</sup>, John J E Rasko<sup>6</sup>, Margareth C Ozelo<sup>7</sup>, Keith Hoots<sup>8</sup>, Philip Blatt<sup>9</sup>, Barbara Konkle<sup>10</sup>, Michael Dake<sup>4</sup>, Robin Kaye<sup>12</sup>, Mahmood Razavi<sup>1</sup>, Albert Zajko<sup>10</sup>, James Zehnder<sup>4</sup>, Pradip K Rustagi<sup>11</sup>, Hiroyuki Nakai<sup>4</sup>, Amy Chew<sup>13</sup>, Debra Leonard<sup>2,12</sup>, J Fraser Wright<sup>3</sup>, Ruth R Lessard<sup>3</sup>, Jürg M Sommer<sup>3</sup>, Michael Tigges<sup>3</sup>, Denise Sabatino<sup>1</sup>, Alvin Luk<sup>3</sup>, Haiyan Jiang<sup>3</sup>, Federico Mingozi<sup>1</sup>, Linda Couto<sup>3</sup>, Hildegund C Ertl<sup>1,13</sup>, Katherine A High<sup>1,2,14</sup> & Mark A Kay<sup>4</sup>



**F.IX activity assay and transaminase levels (AST, ALT) plotted as a function of time in weeks after vector administration in subjects E, F and G**

# Merry christmas for patients with hemophilia B.

ORIGINAL ARTICLE

## Adenovirus-Associated Virus Vector-Mediated Gene Transfer in Hemophilia B

Amit C. Nathwani, M.B., Ch.B., Ph.D., Edward G.D. Tuddenham, M.B., B.S., M.D., Savita Rangarajan, M.B., B.S., Cecilia Rosales, Ph.D., Jenny McIntosh, Ph.D., David C. Lynch, M.B., B.Chir., Pratima Chowdhary, M.B., B.S., Anne Riddell, B.Sc., Arnulfo Jaquimac Pie, B.S.N., Chris Harrington, B.S.N., James O'Beirne, M.B., B.S., M.D., Keith Smith, M.Sc., John Pasi, M.D., Bertil Glader, M.D., Ph.D., Pradip Rustagi, M.D., Catherine Y.C. Ng, M.S., Mark A. Kay, M.D., Ph.D., Junfang Zhou, M.D., Yunyu Spence, Ph.D., Christopher L. Morton, B.S., James Allay, Ph.D., John Coleman, M.S., Susan Sleep, Ph.D., John M. Cunningham, M.D., Deokumar Srivastava, Ph.D., Elena Basner-Tschakarjan, M.D., Federico Mingozi, Ph.D., Katherine A. High, M.D., John T. Gray, Ph.D., Ulrike M. Reiss, M.D., Arthur W. Nienhuis, M.D., and Andrew M. Davidoff, M.D.  
N Engl J Med 2011; 365:2357-2365 | December 22, 2011

Patients suffering from severe hemophilia B (< 1% FIX) were injected by peripheral vein administration with an AAV serotype 8 vector (AAV8) encoding a codon-optimized FIX

AAV8 can efficiently transduce hepatocytes, does not interact as efficiently with antigen-presenting cells as AAV2, and has limited cross-reactivity with preexisting anti-AAV2 antibodies

This scAAV design is more efficient possibly because it obviates the need for second-strand synthesis or reannealing of positive and negative AAV strands to generate transcription-competent double-stranded DNA templates

Subjects received low ( $2 \cdot 10^{11}$  vg/kg), intermediate ( $6 \cdot 10^{11}$  vg/kg), or high ( $2 \cdot 10^{12}$  vg/kg) scAAV8-FIX vector doses, with two participants in each cohort.

**All subjects expressed FIX above the 1% threshold for several months (FIX levels varied between 2% and 11%)**  
**Four discontinued FIX prophylaxis and remained free of spontaneous bleeding episodes, although most of these subjects required prophylaxis to prevent bleeding upon trauma**

One subject who received the highest vector dose developed grade III liver toxicity related to the vector itself, resulting in a significant increase in serum transaminase levels and a concomitant decrease of FIX levels from 7% to 3%. This was associated with the detection of AAV8 capsid-specific T-cells. The other subject had a slight increase in liver enzyme levels concomitant with an increase in AAV8 capsid-specific T-cells and a slight decrease in FIX level.

ORIGINAL ARTICLE

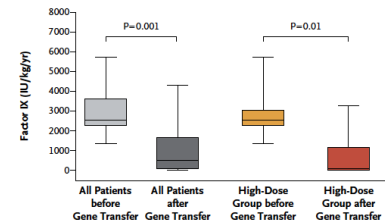
## Long-Term Safety and Efficacy of Factor IX Gene Therapy in Hemophilia B

N ENGL J MED 371:21 NEJM.ORG NOVEMBER 20, 2014

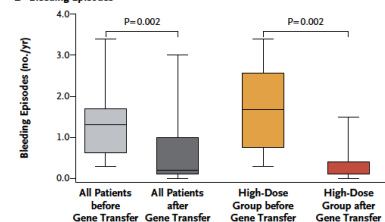
ABSTRACT

A.C. Nathwani, U.M. Reiss, E.G.D. Tuddenham, C. Rosales, P. Chowdhary, J. McIntosh, M. Della Peruta, E. Lheriteau, N. Patel, D. Raj, A. Riddell, J. Pie, S. Rangarajan, D. Bevan, M. Recht, Y.-M. Shen, K.G. Halka, E. Basner-Tschakarjan, F. Mingozi, K.A. High, J. Allay, M.A. Kay, C.Y.C. Ng, J. Zhou, M. Cancio, C.L. Morton, J.T. Gray, D. Srivastava, A.W. Nienhuis, and A.M. Davidoff

### A Annual Use of Factor IX Concentrate



### B Bleeding Episodes



### BACKGROUND

In patients with severe hemophilia B, gene therapy that is mediated by a novel self-complementary adeno-associated virus serotype 8 (AAV8) vector has been shown to raise factor IX levels for periods of up to 16 months. We wanted to determine the durability of transgene expression, the vector dose-response relationship, and the level of persistent or late toxicity.

### METHODS

We evaluated the stability of transgene expression and long-term safety in 10 patients with severe hemophilia B: 6 patients who had been enrolled in an initial phase 1 dose-escalation trial, with 2 patients each receiving a low, intermediate, or high dose, and 4 additional patients who received the high dose ( $2 \cdot 10^{12}$  vector genomes per kilogram of body weight). The patients subsequently underwent extensive clinical and laboratory monitoring.

### RESULTS

A single intravenous infusion of vector in all 10 patients with severe hemophilia B resulted in a dose-dependent increase in circulating factor IX to a level that was 1 to 6% of the normal value over a median period of 3.2 years, with observation ongoing. In the high-dose group, a consistent increase in the factor IX level to a mean (±SD) of 5.1±1.7% was observed in all 6 patients, which resulted in a reduction of more than 90% in both bleeding episodes and the use of prophylactic factor IX concentrate. A transient increase in the mean alanine aminotransferase level to 86 IU per liter (range, 36 to 202) occurred between week 7 and week 10 in 4 of the 6 patients in the high-dose group but resolved over a median of 5 days (range, 2 to 35) after prednisolone treatment.

### CONCLUSIONS

In 10 patients with severe hemophilia B, the infusion of a single dose of AAV8 vector resulted in long-term therapeutic factor IX expression associated with clinical improvement. With a follow-up period of up to 3 years, no late toxic effects from the therapy were reported. (Funded by the National Heart, Lung, and Blood Institute and others; ClinicalTrials.gov number, NCT00979238.)

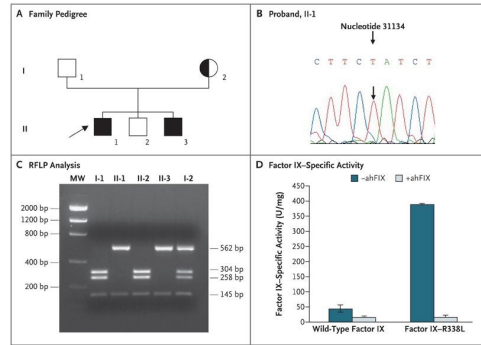
## Seven different pharma companies are currently developing gene therapy treatments for hemophilia:

- uniQure** (January 7th, 2016): UniQure presented results from an early study of AMT-060 (AAV5-hFIX). The first 2 hemophilia patients treated with AMT-060, about 12 and 20 weeks after treatment, are now producing 4.5 percent and 5.5 percent, respectively, of normal Factor IX. To put that in context, these patients have severe or moderately severe hemophilia, meaning they typically produce less than 1 to 2 percent of these levels, and rely on frequent infusions to get those numbers up.
- Baxalta** (June 24, 2015): Baxalta reported continued progress on the Phase 1/2 open-label clinical trial assessing the safety and optimal dosing level of BAX 335, an advanced AAV8-based gene therapy technology for FIX expression. Some FIX expression was observed in the lowest dosing cohort ( $2 \cdot 10^{11}$  vg/kg). In the second dosing cohort ( $1 \cdot 10^{12}$  vg/kg), two patients have experienced no bleeds without regular infusions of FIX and one of these patients has had sustained FIX expression levels of 20-25 % for 12 months. In the highest dose cohort ( $3 \cdot 10^{12}$  vg/kg), expression levels have peaked above 50 %, though the two patients in this cohort experienced an immune response which has led to decreased FIX expression, with one patient resuming regular FIX infusions.
- DIMENSION THERAPEUTICS**: AAV8 and AAVrh10 are two forms of AAV that selectively target liver cells and have been optimized to deliver missing intact genes in diseases associated with the liver.
- Spark Therapeutics**: With Pfizer we are developing novel bio-engineered AAV vectors utilizing a high-activity factor IX transgene and a treatment protocol designed to mitigate immune responses seen in other hemophilia B gene therapy trials, including our own, that have limited the duration of efficacy. We initiated a Phase 1/2 trial in June of 2015.
- BioMarin** (September 28, 2015): BioMarin Enrolls First Patient in Phase 1/2 Trial of Gene Therapy Drug Candidate BMN 270 for the Treatment of Hemophilia A. The Phase 1/2 study will evaluate the safety and efficacy of BMN 270 gene therapy in up to 12 patients with severe Hemophilia A. The primary endpoints are to assess the safety of a single intravenous administration of a recombinant AAV, human-coagulation Factor VIII vector and to determine the change from baseline of Factor VIII expression level at 16 weeks after infusion.
- Sangam Biosciences**: Sangam Biosciences is developing a ZFN-mediated genome editing approach to hemophilia A and B using our proprietary In Vivo Protein Replacement Platform (IVPRP).
- biogen idec**: Biogen Idec, Fondazione Telethon and Ospedale San Raffaele Announce Global Collaboration to Develop Gene Therapies for Hemophilia Programs to apply lentiviral vector technology to hemophilia A and B

Indication	Program	Target	Cell Type	Research	Pre-clinical	Phase 1	Phase 2	Phase 3
Hemophilia B	SB-FIX	Insert-FIX	Liver cells	█	█	█		
Hemophilia A	SB-FVIII	Insert-FVIII	Liver cells	█	█			

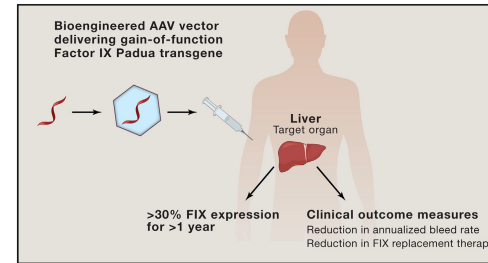
# Factor IX Padua variant

- single point mutation, R338L
- initially discovered in a thrombophilic patient
- possesses increased procoagulant activity due to an enhanced incorporation into the intrinsic tenase complex in the clotting cascade
- pre-clinical studies of FIX Padua in gene therapy confirmed its benefit

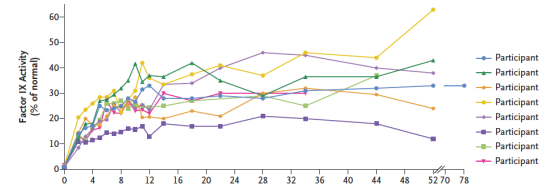


# Hemophilia B Gene Therapy with a High-Specific-Activity Factor IX Variant

L.A. George, S.K. Sullivan, A. Giermasz, J.E.J. Rasko, B.J. Samelson-Jones, J. Ducore, A. Cuker, L.M. Sullivan, S. Majumdar, J. Teitel, C.E. McGuinn, M.V. Ragni, A.Y. Luk, D. Hui, J.F. Wright, Y. Chen, Y. Liu, K. Wachtel, A. Winters, S. Tiefenbacher, V.R. Arruda, J.C.M. van der Loo, O. Zelenia, D. Takefman, M.E. Carr, L.B. Couto, X.M. Anguela, and K.A. High



- use of a gain-of-function FIX transgene, the Padua variant that results in a 7-fold increase in specific coagulant activity of the protein
- FIX levels not only sufficient to eliminate the risk of spontaneous bleeding but also to minimize the risk of bleeding from interventions and trauma
- persistence of FIX expression out to beyond one year - could a single administration of AAV-mediated gene therapy be curative for hemophilia?
- novel AAV vector that has bioengineered changes to the vector capsid to avoid pre-existing immunity



Spark Therapeutics N Engl J Med 2017;377:2215-27. DOI: 10.1056/NEJMoa1708538

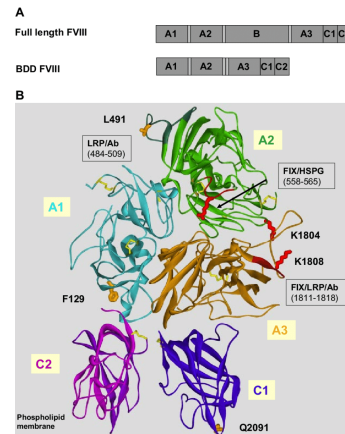
# AAV5–Factor VIII Gene Transfer in Severe Hemophilia A

Savita Rangarajan, M.B., B.S., Liron Walsh, M.D., Will Lester, M.B., Ch.B., Ph.D., David Perry, M.D., Ph.D., Bella Madan, M.D., Michael Laffan, D.M., Hua Yu, Ph.D., Christian Vettermann, Ph.D., Glenn F. Pierce, M.D., Ph.D., Wing Y. Wong, M.D., and K. John Pasi, M.B., Ch.B., Ph.D. [et al.](#)

**CONCLUSIONS** The infusion of AAV5-hFVIII-SQ was associated with the sustained normalization of factor VIII activity level over a period of 1 year in six of seven participants who received a high dose, with stabilization of hemostasis and a profound reduction in factor VIII use in all seven participants. In this small study, no safety events were noted, but no safety conclusions can be drawn. (Funded by BioMarin Pharmaceutical; ClinicalTrials.gov number, NCT02576795; EudraCT number, 2014-003880-38.)

**BIO MARIN** December 28, 2017 N Engl J Med 2017; 377:2519-2530

Infusion of a **single** intravenous dose of a codon-optimized adeno-associated virus serotype 5 (AAV5) vector encoding a B-domain–deleted human factor VIII (AAV5-hFVIII-SQ) in nine men with severe **hemophilia A (the most common type)**



## CASE STUDY - Jake Omer, 29

James lives in Billerica and is married with two children, aged 3 years and a baby of 5 weeks. Diagnosed at two years old, he has had frequent injections of factor VIII to prevent bleeds ever since. Before he was treated with the gene therapy, Jake would wake up early before work to inject three times a week as well as injecting whenever he had an injury to stop the bleeding. As a result of repeated bleeding Jake has arthritis in his ankles. His father is a Turk-Cypriot and as a child he and his family could never travel to visit relatives in case he needed medical care as the facilities wouldn't have been available.

"The gene therapy has changed my life. I now have hope for my future. It is incredible to now hope that I can play with my kids, kick a ball around and climb trees well into my kids' teenage years and beyond. The arthritis in my ankles meant I used to worry how far I would be able to walk once I turned 40. At 23 I struggled to run 100m to catch a bus; now at 29 I'm walking two miles every day which I just couldn't have done before having the gene therapy treatment. "It's really strange to not have to worry about bleeding or swellings. The first time I noticed a difference was about four months after the treatment when I dropped a weight in the gym, bashing my elbow really badly. I started to panic thinking this is going to be really bad, but after icing it that night I woke up and it looked normal. That was the moment I saw proof and knew that the gene therapy had worked."

The five UK trial sites included: The Royal London, Guys and St Thomas', Birmingham, Cambridge and Hampshire hospitals.



**Table 1. Active hemophilia A & B AAV gene therapy clinical studies (43)**

Sponsor	NCT	Study	AAV	Vector Dose (vg/kg)	Phase	Patients (target)	ED	AAV Ab	Status
SJCRH/UCL	NCT00979238	n/a	scAAV2/8-LP1-FIXco-wt	2e11, 6e11, 2e12	1	14	≥50	Negative	Not recruiting
Shire (Baxalta)	NCT01687608	AskBio009	AAV8.sc-TTR-FIXco-Padua	2e11, 1e12, 3e12	1, 2	30	n/a	Negative	Not recruiting
Uniqure	NCT02396342	AMT-060-01	AAV5-FIXco-wt	5e12, 2e13	1, 2	10	>-150	Negative	Not recruiting
	NCT03489291	AMT-061-01	AAV5-FIXco-Padua	2e13	2	3	>-20	Included	Not recruiting
	NCT03569891	AMT-061-02	AAV5-FIXco-Padua	2e13	3	56	>-150	Included	Recruiting
		(HOPE-B)							
Pfizer (Spark)	NCT02484092	SPK-9001-101	AAV-SPARK100-FIXco-Padua	5e11	2	15	≥50	Negative	Not recruiting
	NCT03307980	SPK-9001-LTFU-101		SPK-9001 extension study	2	20	n/a	n/a	Recruiting
	NCT03861273	BENEGENE-2	n/a	n/a	3	55	≥50	<1:1	Not yet recruiting
UCL Freeline	NCT03369444	FLT-180a	AAV53-FIXco-Padua	6e11, 2e12	1	18	>-150	Negative	Recruiting
	NCT03641703			FLT180a extension study	2, 3	50	n/a	n/a	Recruiting
Factor VIII Biomarin	NCT02576795	BMN-270-201	AAV5-FVIII-BDD	6e12, 2e13, 6e13	1, 2	15	>-150	Negative	Not recruiting
	NCT03392974	BMN-270-302		4e13	3	40	>-150	Negative	Recruiting
	NCT03370913	BMN-270-301		6e13	3	130	>-150	Negative	Recruiting
	NCT03520712	BMN-270-203		6e13	1, 2	10	>-150	Included	By invitation
Spark	NCT03003533	SPK-8011-101	AAV-SPARK200-FVIII-BDD	5e11, 1e12, 2e12	1, 2	30	>-150	Negative	Recruiting
	NCT02422520	SPK-8011-LTFU	n/a	SPK-8011 extension study	1, 2	100	n/a	n/a	By invitation
	NCT03734588	SPK-8016-101		Dose finding pre FVIII inhibitor study	1, 2	30	>-150	Negative	Recruiting
UCL	NCT03001830	GO-8	AAV2/8-HLP-FVIII-V3	6e11, 2e12, 6e12	1	18	>-50	Negative	Recruiting
Sangamo Shire (Baxalta)	NCT03061201	SB-525-1603	AAV2/6-FVIII-BDD	9e11, 2e12, 1e13, 3e13	1, 2	20	>-150	Negative	Recruiting
Bayer	NCT03370172	BAX-888	AAV8-FVIII-BDD	n/a	1, 2	10	>-150	<-1:5	Recruiting
	NCT03588299	BAV2599023 (DTX201)	n/a	n/a	1, 2	18	>-150	Negative	Recruiting

Ab, antibody; co, codon optimized; ED, exposure days to factor concentrate; sc, self-complementary; SJCRH, St. Jude Children's Research Hospital; UCL, University College London; vg, vector genomes; wt, wild-type; FVIII-BDD, B-domain deleted FVIII.