Utilizzo del CRISPR-Cas9 nella terapia genica delle malattie mitocondriali: trattamento della MERRF

Scuola Superiore di Studi Avanzati – SSAS
Seminario disciplinare «Medicina molecolare»
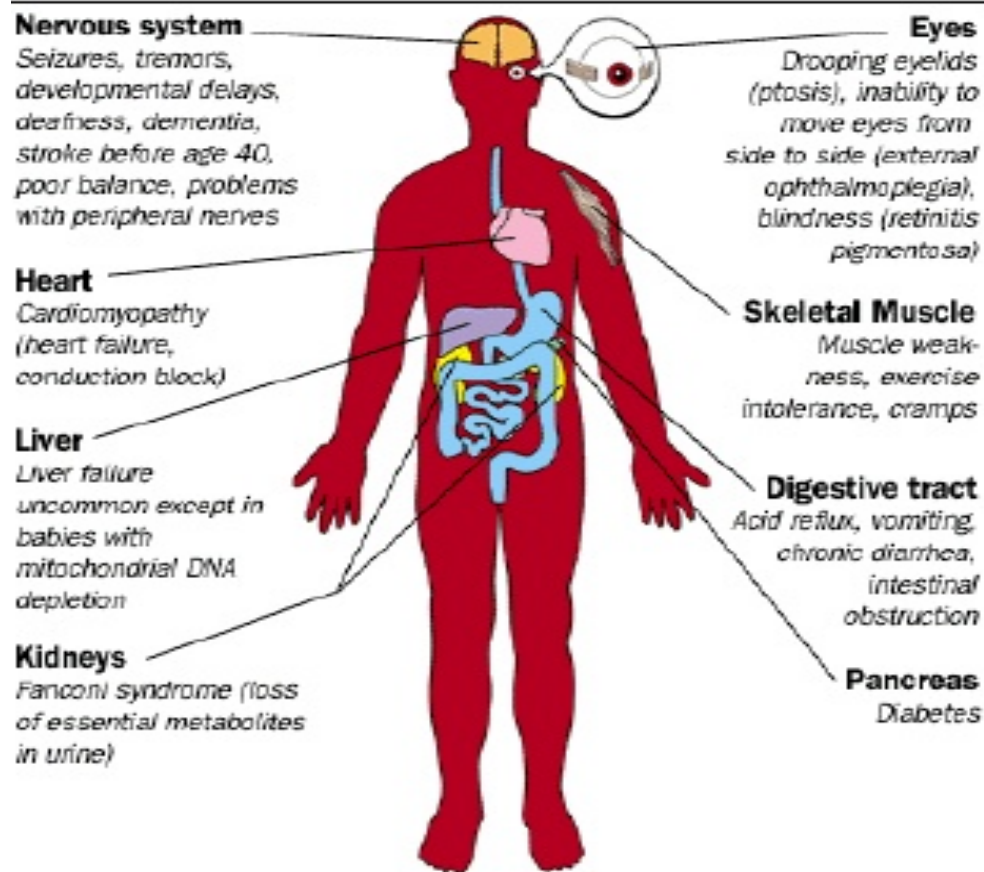Classe di Scienze della Vita
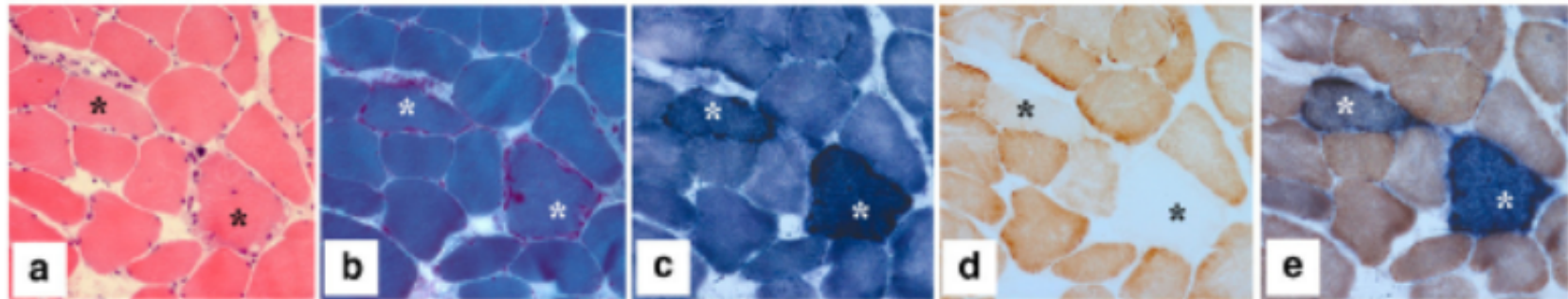Chiara Cataldi, Sara D'Uva, Federica Mosti, Vittorio Padovano

MERRF:
Epilessia mioclonica con
fibre rosse raggrinzite

Nervous system
Seizures, tremors, developmental delays, deafness, dementia, stroke before age 40, poor balance, problems with peripheral nerves

Heart
Cardiomyopathy (heart failure, conduction block)

Liver
Liver failure uncommon except in babies with mitochondrial DNA depletion

Kidneys
Fanconi syndrome (loss of essential metabolites in urine)

Eyes
Drooping eyelids (ptosis), inability to move eyes from side to side (external ophthalmoplegia), blindness (retinitis pigmentosa)

Skeletal Muscle
Muscle weakness, exercise intolerance, cramps

Digestive tract
Acid reflux, vomiting, chronic diarrhea, intestinal obstruction

Pancreas
Diabetes

Koenig *et al,* 2008

A livello sistemico

A livello del tessuto muscolare

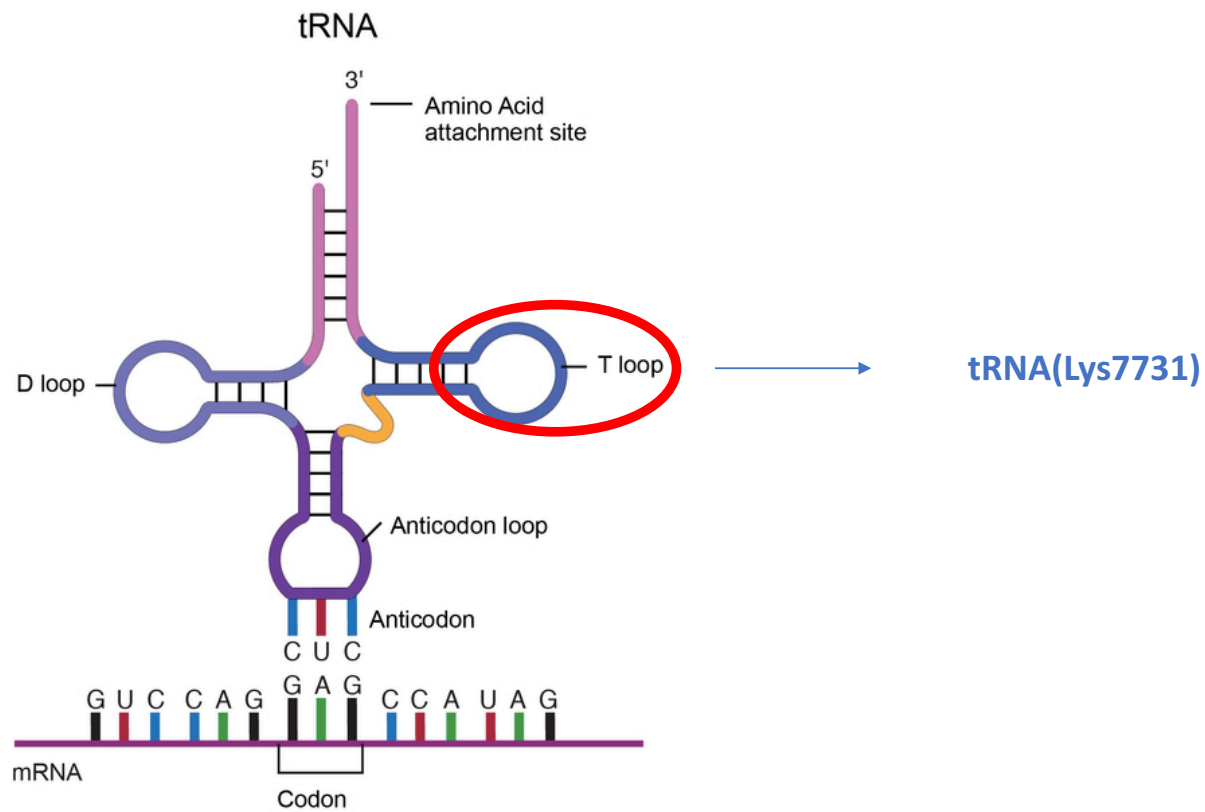| Controllo | Tricromica Gomori | SDH Succinato Deidrogenasi Compl. II | COX Citocromo C Ossidasi Compl. IV | Merge |

Tuppen *et al*, 2010

A livello genetico

Wikipedia
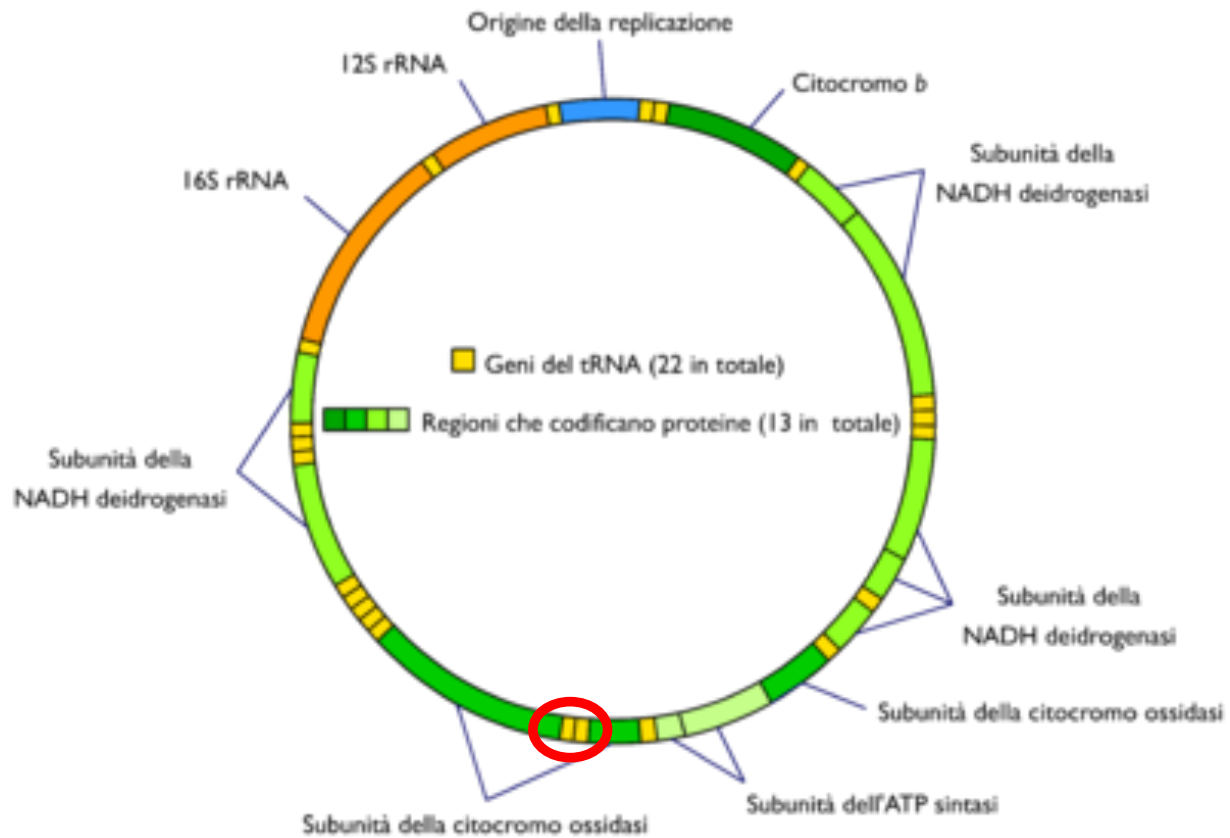
A livello molecolare

tRNA(Lys7731)

Trattamento – Gene Therapy

Nucleic acid delivery	Kolesnikova et al.[43], 2004
	Mahata et al.[41], 2005
	Mahata et al.[42], 2006
Peptide-mediated therapy	Chang et al.[50], 2013
	Chang et al.[51], 2013
	Muratovska et al.[53], 2001
	Perli et al.[49], 2016
	Taylor et al.[54], 1997
mitoTALENs	Bacman et al.[55], 2015
	Hashimoto et al.[56], 2015

COME?

Agresti *et al.,* 2018

Obiettivi e strategie

Obiettivi
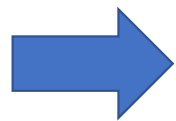
Strategie

➡️ Ottimizzare un sistema di targeting e delivery in miotubi

Nanoparticelle di PLGA rivestite di anticorpi specifici per marker di superficie dei miotubi
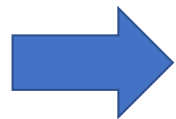
➡️ Sviluppare un approccio per abbassare la percentuale di eteroplasmia

CRISPR-mitoCAs9

Il nostro modello: mito-mouse tRNA (Lys7731)

Transmitochondrial mice as models for primary prevention of diseases caused by mutation in the *tRNA^Lys* gene

Akinori Shimizu[a], Takayuki Mito[a], Chisato Hayashi[a], Emi Ogasawara[a], Ryusuke Koba[a], Issei Negishi[a], Keizo Takenaga[b], Kazuto Nakada[a,c], and Jun-Ichi Hayashi[a,c,1]

[a]Faculty of Life and Environmental Sciences and [c]International Institute for Integrative Sleep Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan; and [b]Department of Life Science, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan
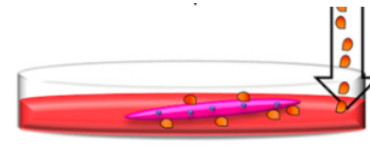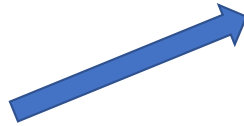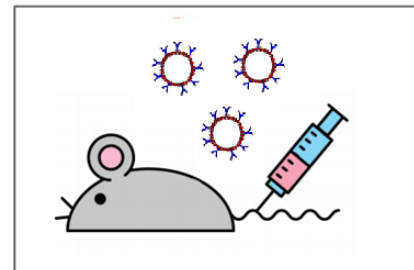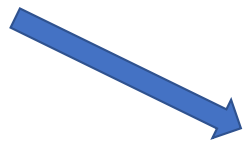
- **Genotipo**
  - mutazione **mtDNA G7731A**
  - percentuale di eteroplasmia: **76-84%**

MODELLO DI STUDIO *IN VITRO*
Miotubi da biopsia di
mito-mouse tRNA(Lys7731)

MODELLO DI STUDIO *IN VIVO*
mito-mouse tRNA(Lys7731)
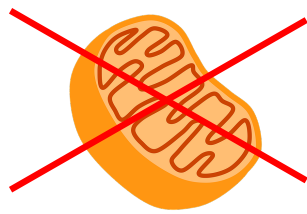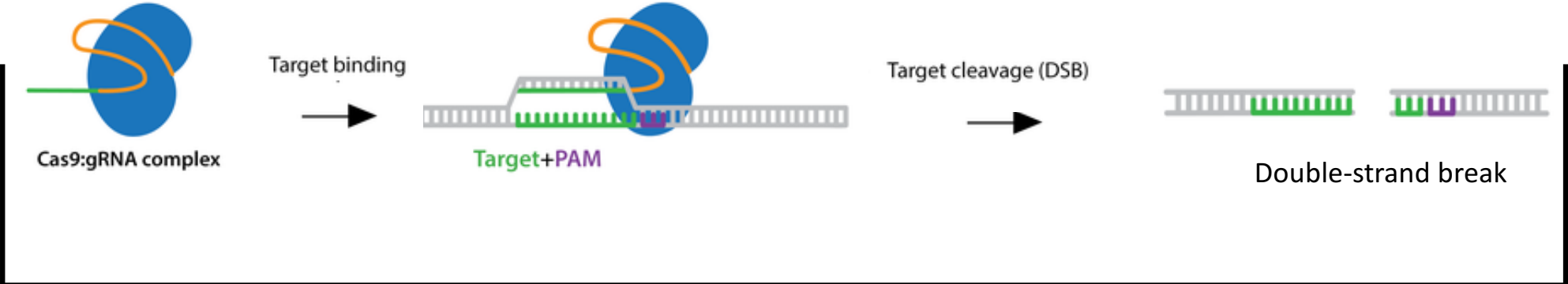
mito-mouse tRNA(Lys7731)

Piano Sperimentale

1. In vitro: Modello sperimentale → Miotubi di mito-mouse tRNA (Lys7731**)**

2. Ottimizzazione approccio sperimentale *in vitro*

3. In vivo: Modello sperimentale → Mito-mouse tRNA(Lys7731**)**
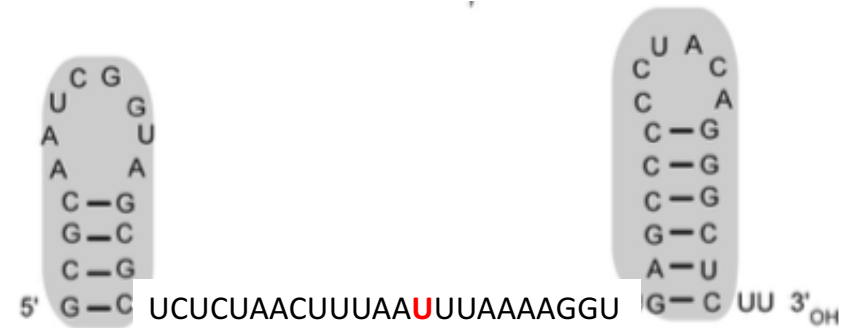
4. Restaurazione del fenotipo sano nel modello murino
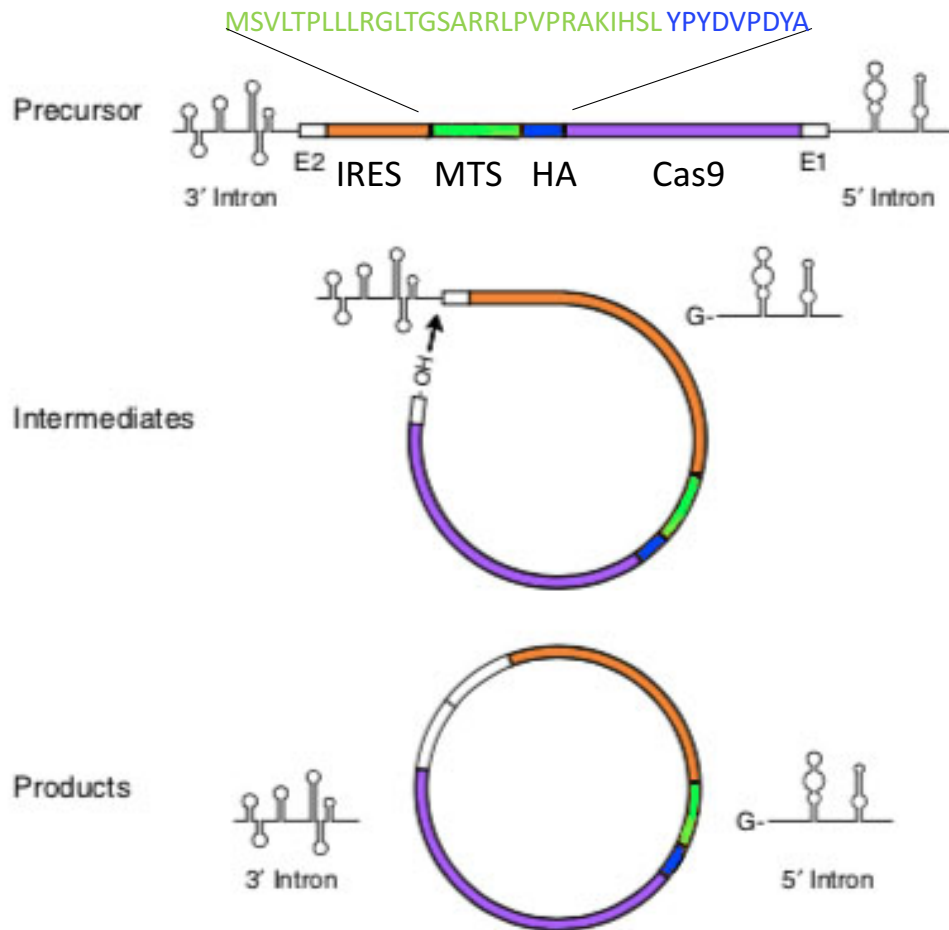
Meccanismo di azione del complesso Cas9-sgRNA nei mitocondri

Cas9:gRNA complex

Target binding

Target+PAM

Target cleavage (DSB)

Double-strand break

Degradation of mitochondria with mutated mtDNA

Adattata da Addgene.org

RNA Circolare e sgRNA

MSVLTPLLLRGLTGSARRLPVPRAKIHSL YPYDVPDYA

Precursor

E2 IRES MTS HA Cas9 E1

3' Intron   5' Intron

Intermediates

Products

3' Intron   5' Intron
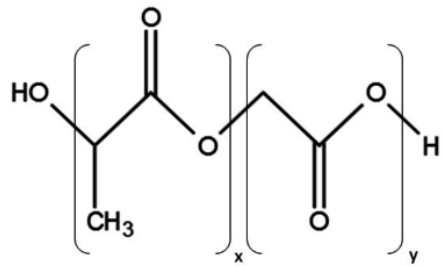
UCUCUAACUUUAAUUUAAAAGGU

sgRNA with mitochondrial import signal hairpins

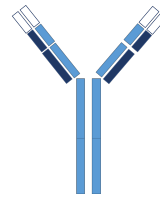Adattata da Wesselhoeft et al., 2018
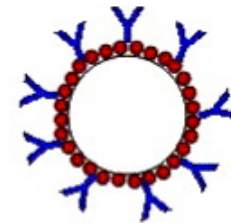
Adattata da Tonin et al., 2014

Delivery nei miotubi

Poly(lactide-co-glycolide acid)

PLGA

+

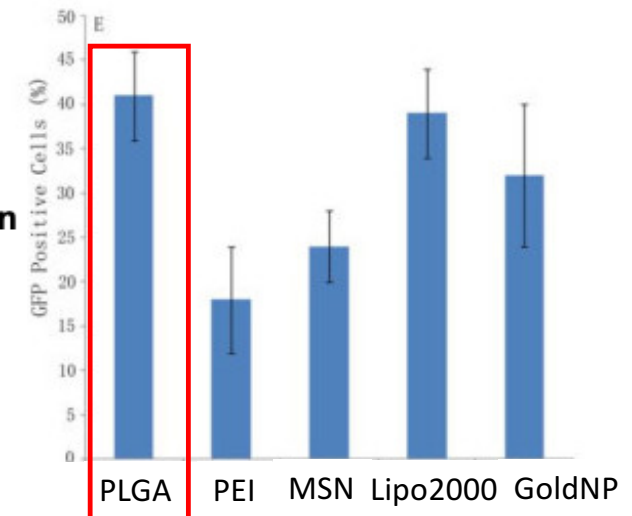**Anticorpo contro marker di superficie dei miotubi**

=

Nanoparticella di PLGA specifica per i miotubi

Uptake and intracellular distribution of different types of nanoparticles in primary human myoblasts and myotubes.

Guglielmi V[1], Carton F[1], Vattemi G[2], Arpicco S[3], Stella B[3], Berlier G[4], Marengo A[3], Boschi F[5], Malatesta M[6].

Liang et al., 2011

Risultati

In vitro system

Isolamento dei miotubi murini ΔG7731A

Dissezione   Digestione   Risospensione   Crescita dei mioblasti 3-5 gg   gg 7-9

Centrifuge at 500xg

coating di Matrigel   coating di collagene

1°,2°,3°,4° round…

coating di Matrigel   coating di Matrigel

Mioblasti   Miotubi (DM)

Shanini *et al.,* Stem Cell Research 2018
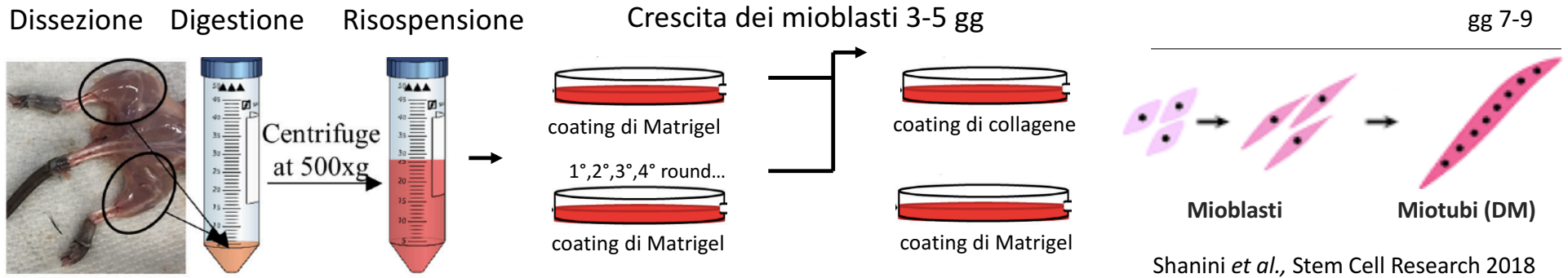
Miotubi WT
MYH1

✓ Efficienza di isolamento mioblasti →**90%**
✓ Purezza dei miotubi con un round di pre-plating →**98%**

50 μm

Myf5   MyoD   MyoG   MRF4

Expression Level Normalized to Polr2b

Myoblats   Myotubes

Determinazione della patogenicità dei miotubi ΔG7731A

COX citochemistry
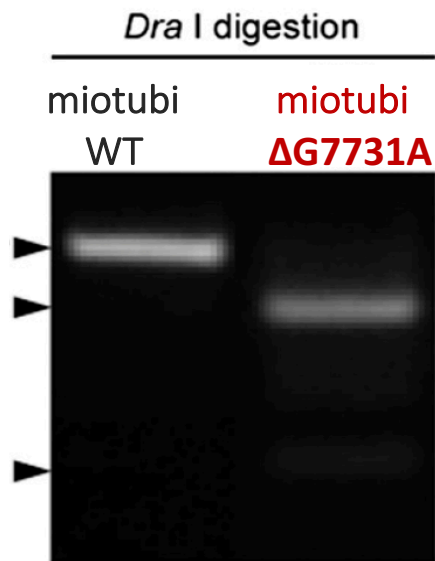
Adattato da Shimizu *et al.,* PNAS, 2018.
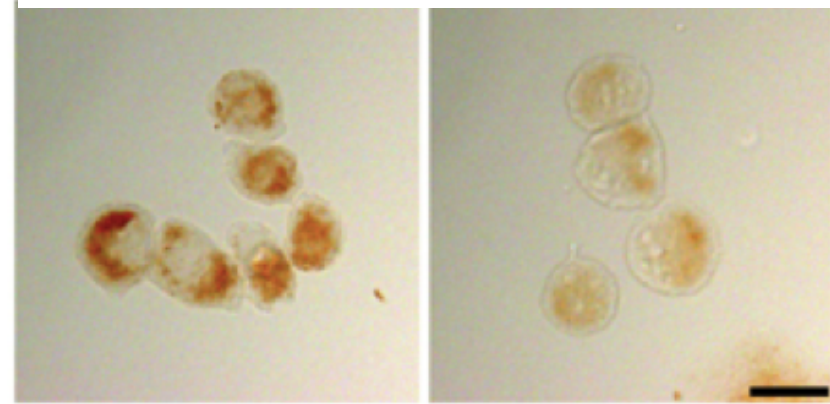
Adattato da Shimizu *et al.,* Biochemical and Biophysical Research Communications, 2015.

In vitro system

Determinazione della patogenicità dei miotubi ΔG7731A
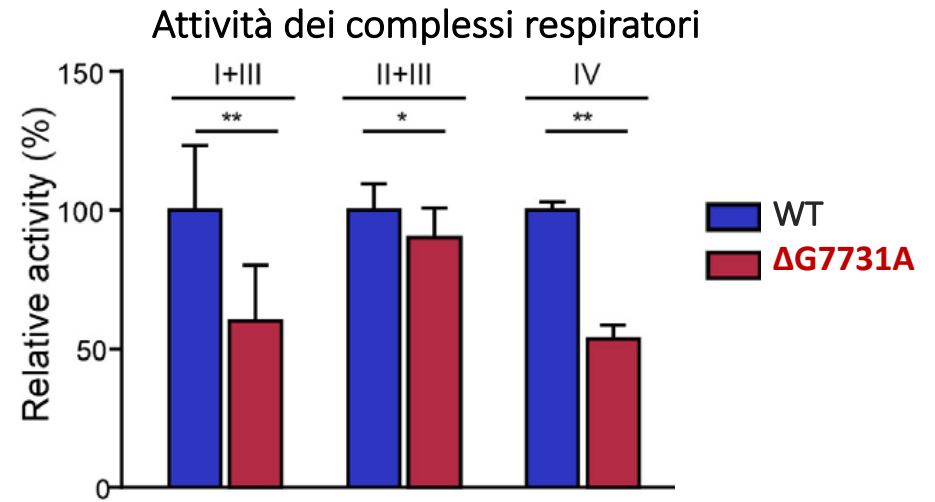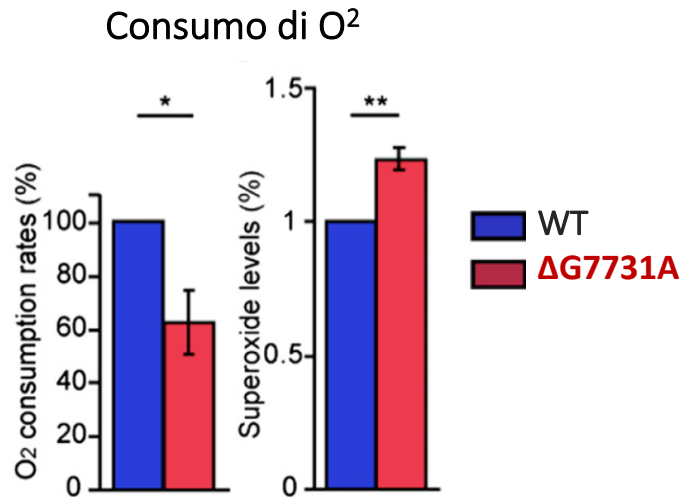
Consumo di O²

Attività dei complessi respiratori

Piruvato/malato

Contenuto di ATP

Adattato da Shimizu *et al.,* Biochemical and Biophysical Research Communications, 2015.

Incubazione dei miotubi murini con NP e mitoCas9

MSVLTPLLLRGLTGSARRLPVPRAKIHSL YPYDVPDYA

Precursor

G-OH

sgRNA

E2 IRES MTS HA Cas9 E1

3' Intron 5' Intron

PLGA nanocarrier + **mCas9 + sgRNA**

Miotubi da mito-mouse tRNA^{lys}
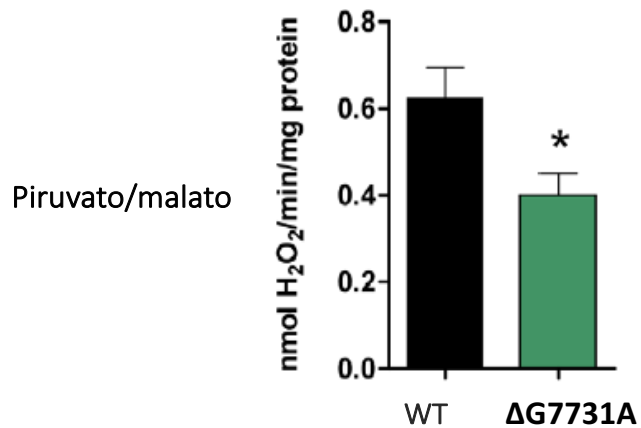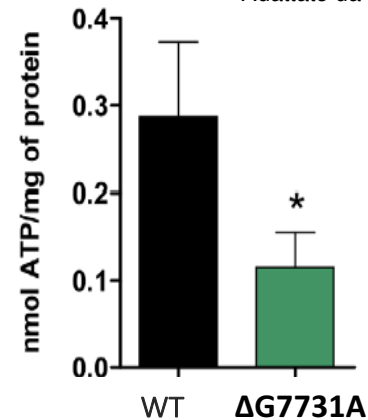
In vitro system

Incubazione dei miotubi murini con NP e mitoCas9

PLGA NP + circRNA mitoCas9 + sgRNA

Miotubi da mito-mouse tRNA^{lys}
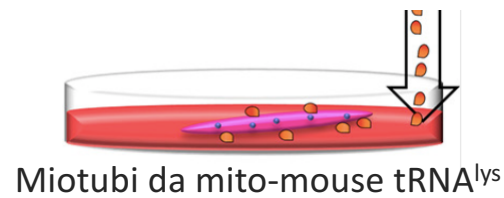
PLGA NP
DAPI
ACTINA

H

I
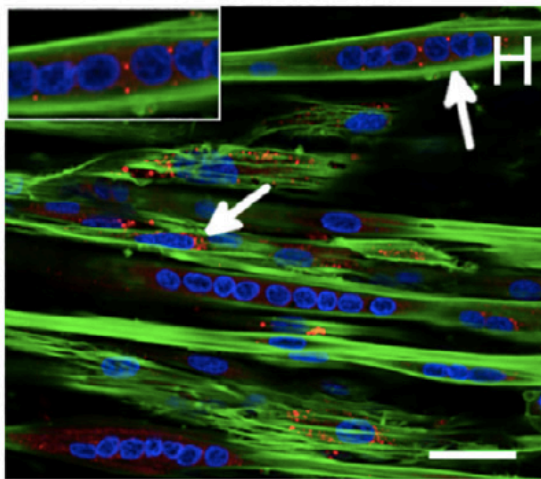
24 ore
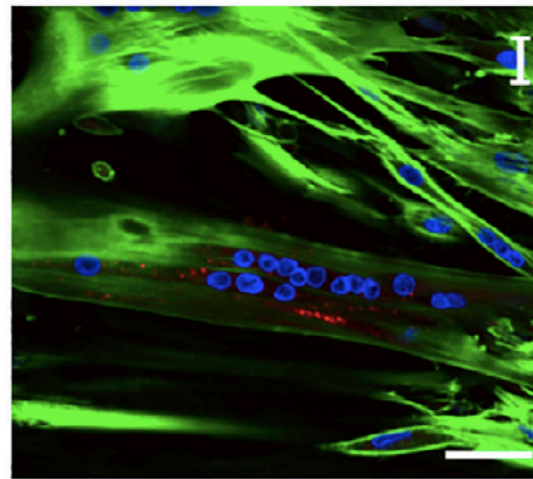
72 ore

50 um
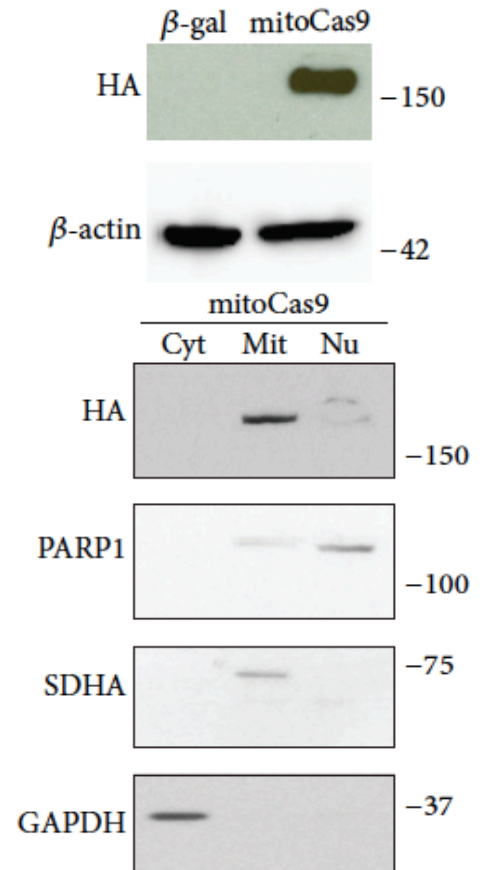
Guglielmi *et al.,* Internation Journal of Pharmaceutics, 2018.

Areum J. *et al.,* BioMed Research International, 2015.

In vitro system

Rescue del fenotipo dei miotubi ΔG7731A

Dra I digestion

G7731A   **ΔG7731A mCas9**

Adattato da Shimizu *et al.,* PNAS, 2018.

COX citochemistry

G7731A   **ΔG7731A mCas9**

Adattato da Shimizu *et al.,* Biochemical and Biophysical Research Communications, 2015.

Piruvato/malato

nmol H2O2/min/mg protein

WT   G7731A   G7731 mCAS9

Contenuto di ATP

mol ATP/mg protein

WT   G7731A   G7731 mCAS9

In vitro system

Rescue del fenotipo dei miotubi ΔG7731A

Consumo di O^2

Attività dei complessi respiratori

Adattato da Shimizu *et al.,* Biochemical and Biophysical Research Communications, 2015.

Esperimenti in vivo: mito-mouse tRNA(Lys7731)
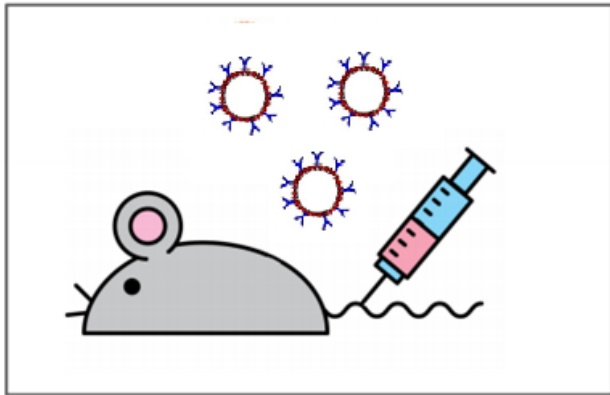
In vivo system

4 MESI: Iniezione IV

5 MESI: Sequenziamento e valutazione dell' heteroplasmic shift

8 MESI: Follow-up e raccolta dati
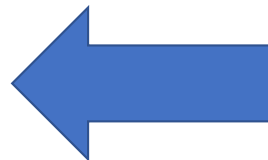
Risultati predittivi: heteroplasmic shift (a 5 mesi dalla nascita)
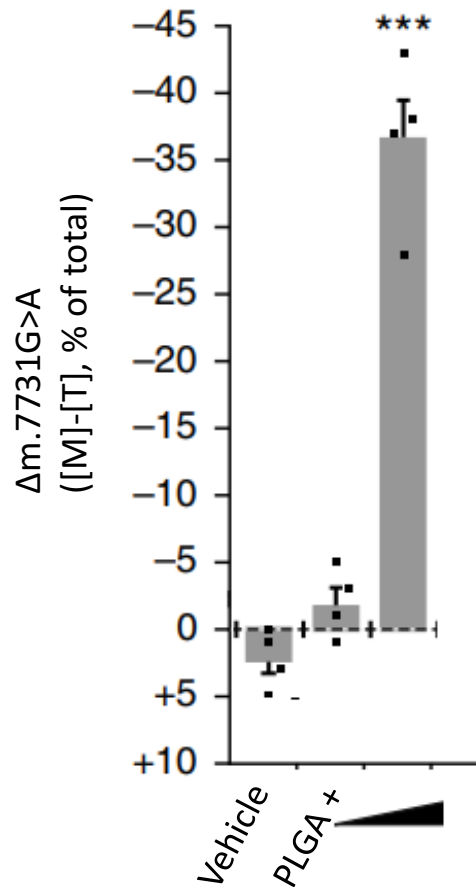
- **Analisi dati di pirosequenziamento da biopsie muscolari (M) per valutare lo shift della % eteroplasmia**

Adattato da Gammage et al., 2018

Risultati predittivi: follow-up 8 mesi (dalla nascita)
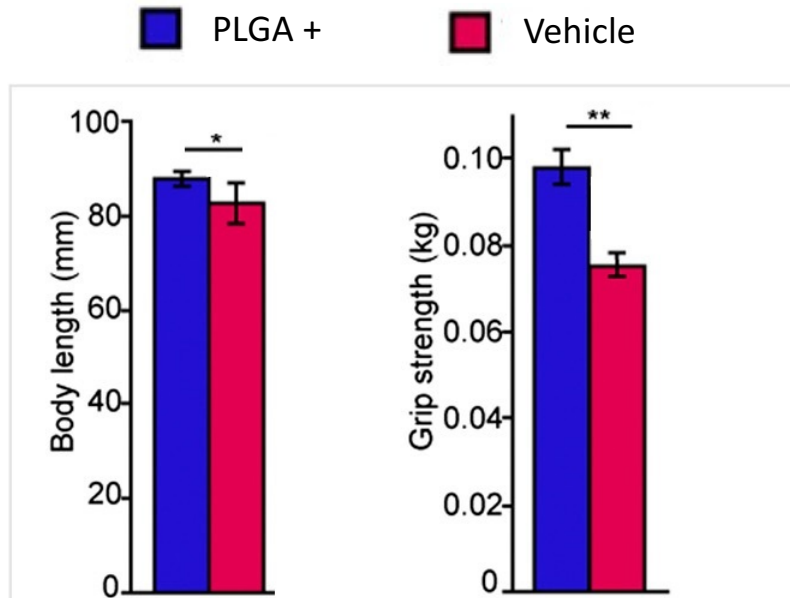
- **Lunghezza corporea e grip strength test (sopra)**
- **Saggio biochimico dell'attività relativa degli enzimi della catena respiratoria (dx)**
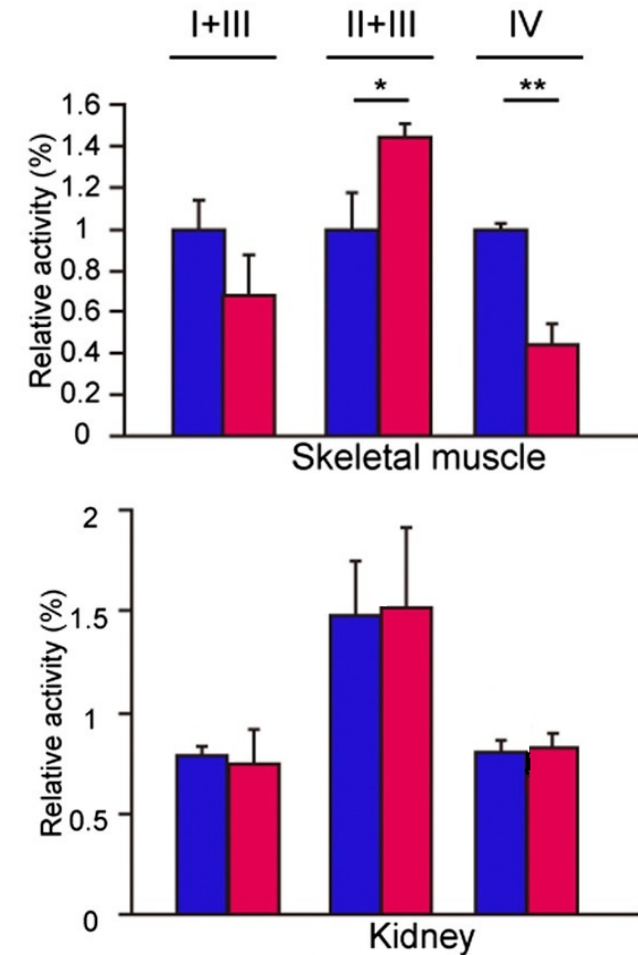
Dati adattati a scopo illustrativo da Shimizu et al, 2014

Risultati predittivi: follow-up 8 mesi (dalla nascita)

Skeletal muscle

PLGA +   Vehicle

PLGA +   Vehicle
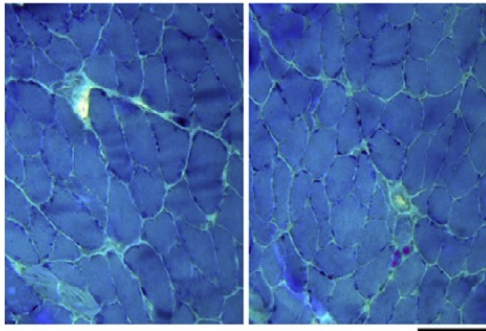
Gomori trichrome

- **Analisi morfologica ed istopatologica di muscolo scheletrico**

PLGA +   Vehicle
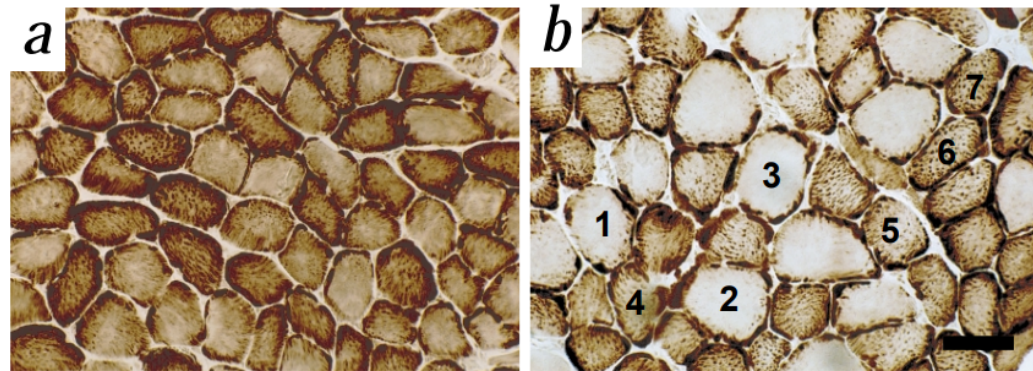
COX staining (brown)

Dati adattati a scopo illustrativo da Shimizu et al, 2015, Inoue et al, 2000

Pitfalls and solutions

- **Il circRNA e sgRNA potrebbero degradarsi**

- **L'iniezione IV non è efficiente per il delivery in muscolo scheletrico**

- **La somministrazione della terapia potrebbe essere troppo tardiva**

- **Utilizzo di un plasmide episomale e di una guida a DNA (necessità di trascrizione)**

- **Iniezione intramuscolare oppure miglioramento dell'efficienza dell'iniezione IV di nanoparticles (Burke et al 2011)**

- **Anticipare la somministrazione ad un periodo antecedente all'insorgenza dei sintomi**

Costi e tempistiche

Timeline:

- Primo anno
- Generazione RNA e Nanoparticelle
- Esperimenti in vitro
- Secondo anno
- Esperimenti in vivo
- Terzo anno
- Risultati

ThermoFisher SCIENTIFIC

SIGMA-ALDRICH

The Jackson Laboratory
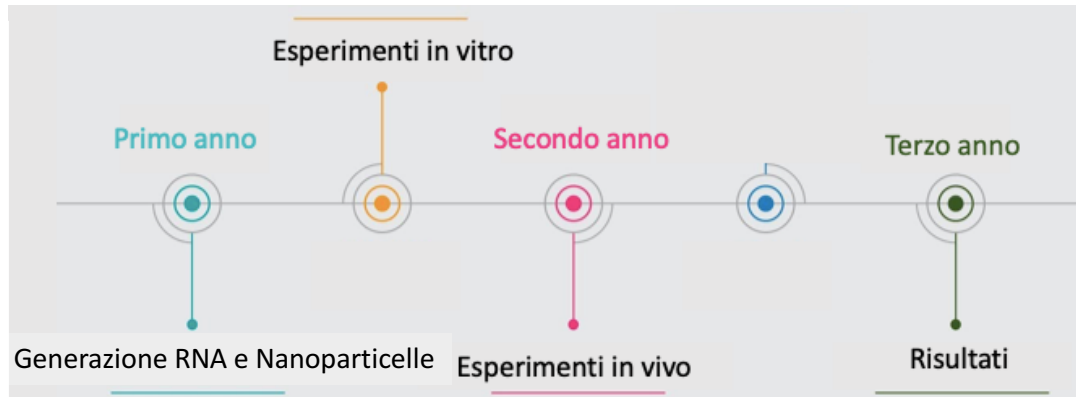
QIAGEN

- Molecular biology laboratory instruments: € 5.000

- Stabulation cost (each mouse): € 1.000 (x year) , approx. 20 mice

- Cell culture (each year): € 2000

- Antibody: € 400

- QIAGEN Multiplex PCR Kit (100): € 284,00

- MEGAscript™ T7 Transcription Kit (25 reactions): € 320

- Poly(Lactide-co-Glycolide) (PLGA) Copolymers: € 500

- DNA isolation (1 kit, 50 preps) Thermo Fisher: € 100

- NEW bioluminescence based ATP determination kit PRO, 10 ml: € 150

- Analisi cellulari di parametri metabolici: € 50

- Analisi citochimiche/biochimiche: € 500- €1000

- Mitocondrial DNA library pyrosequencing: € 500

- Histochemistry: € 500

- Grip strength test: € 80 (esperimento in triplice replicato)

Totale: 75044 €
(al netto dei salari dei ricercatori)

Bibliografia

- Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. Adv Drug Deliv Rev. 2013 Jan;65(1):36-48.

- Areum J, Sangwoo H, Gum H. Efficient Mitochondrial Genome Editing by CRISPR/Cas9. BioMed Research International. 2015:1-10.

- Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. PLGA-based nanoparticles: an overview of biomedical applications. J Control Release. 2012 Jul 20;161(2):505-22.

- Differentiation of control and ALS mutant human iPSCs into functional skeletal muscle cells, a tool for the study of neuromuscolar diseases.Lenzi et al., 2016. Stem Cell Res. 2016 Jul; 17(1): 140–147.

- Gammage PA, Viscomi C, Simard ML, Costa ASH, Gaude E, Powell CA, Van Haute L, McCann BJ, Rebelo-Guiomar P, Cerutti R, Zhang L, Rebar EJ, Zeviani M, Frezza C, Stewart JB, Minczuk M. Genome editing in mitochondria corrects a pathogenic mtDNA mutation in vivo. Nat Med. 2018 Nov;24(11):1691-1695.

- Guglielmi V, Carton F, Vattemi G, Arpicco S, Stella B, Berlier G, Marengo A, Boschi F, Malatesta M. Uptake and intracellular distribution of different types of nanoparticles in primary human myoblasts and myotubes. Int J Pharm. 2019 Feb 20;560:347-356. doi: 10.1016/j.ijpharm

- Hiona A, Sanz A, Kujoth GC, Pamplona R, Seo AY, Hofer T, Someya S, Miyakawa T, Nakayama C, Samhan-Arias AK, Servais S, Barger JL, Portero-Otín M, Tanokura M, Prolla TA, Leeuwenburgh C. Mitochondrial DNA mutations induce mitochondrial dysfunction, apoptosis and sarcopenia in skeletal muscle of mitochondrial DNA mutator mice. PLoS One. 2010 Jul 7;5(7):e11468.

- Inoue K, et al. (2000) Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. Nat Genet 26(2):176–181.

- Jo A, Ham S, Lee GH, Lee YI, Kim S, Lee YS, Shin JH, Lee Y. Efficient Mitochondrial Genome Editing by CRISPR/Cas9. Biomed Res Int. 2015;2015:305716.

- Liang GF, Zhu YL, Sun B, Hu FH, Tian T, Li SC, Xiao ZD. PLGA-based gene delivering nanoparticle enhance suppression effect of miRNA in HePG2 cells. Nanoscale Res Lett. 2011 Jul 12;6:447. doi: 10.1186/1556-276X-6-447

- Kauppila JHK, Baines HL, Bratic A, Simard ML, Freyer C, Mourier A, Stamp C, Filograna R, Larsson NG, Greaves LC, Stewart JB. A Phenotype-Driven Approach to Generate Mouse Models with Pathogenic mtDNA Mutations Causing Mitochondrial Disease. Cell Rep. 2016 Sep 13;16(11):2980-2990.

- Loutre R, Heckel AM, Smirnova A, Entelis N, Tarassov I. Can Mitochondrial DNA be CRISPRized: Pro and Contra. IUBMB Life. 2018 Dec;70(12):1233-1239.

- Noble GT, Stefanick JF, Ashley JD, Kiziltepe T, Bilgicer B. Ligand-targeted liposome design: challenges and fundamental considerations. Trends Biotechnol. 2014 Jan;32(1):32-45.

- Shahi PK, Hermans D, Sinha D, Brar S, Moulton H, Stulo S, Borys KD, Capowski E, Pillers DM, Gamm DM, Pattnaik BR. Gene Augmentation and Readthrough Rescue Channelopathy in an iPSC-RPE Model of Congenital Blindness. Am J Hum Genet. 2019 Feb 7;104(2):310-318.

- Shanini A, Vidiam K, Efficient and high yield isolation of myoblasts from skeletal muscle. Stem Cell Research. 2018,30:122-129.

- Shimizu A, Enoki S, Ishikawa K, Mito T, Obata K, Nagashima R, Yonekawa H, Nakada K, Hayashi J. Mouse somatic mutation orthologous to MELAS A3302G mutation in the mitochondrial tRNA(Leu(UUR)) gene confers respiration defects. Biochem Biophys Res Commun. 2015 Nov 27;467(4):1097-102.

- Shimizu A, Mito T, Hayashi C, Ogasawara E, Koba R, Negishi I, Takenaga K, Nakada K, Hayashi J. Transmitochondrial mice as models for primary prevention of diseases caused by mutation in the tRNA(Lys) gene. Proc Natl Acad Sci U S A. 2014 Feb 25;111(8):3104-9.

- Tang S, Wang J, Zhang VW, Li FY, Landsverk M, Cui H, Truong CK, Wang G, Chen LC, Graham B, Scaglia F, Schmitt ES, Craigen WJ, Wong LJ. Transition to next generation analysis of the whole mitochondrial genome: a summary of molecular defects. Hum Mutat. 2013 Jun;34(6):882-93.

- Tonin Y, Heckel AM, Vysokikh M, Dovydenko I, Meschaninova M, Rötig A, Munnich A, Venyaminova A, Tarassov I, Entelis N. Modeling of antigenomic therapy of mitochondrial diseases by mitochondrially addressed RNA targeting a pathogenic point mutation in mitochondrial DNA. J Biol Chem. 2014 May 9;289(19):13323-34.
- Wallace DC. Mouse models for mitochondrial disease. Am J Med Genet. 2001 Spring;106(1):71-93

- Wesselhoeft RA, Kowalski PS, Anderson DG. Engineering circular RNA for potent and stable translation in eukaryotic cells. Nat Commun. 2018 Jul 6;9(1):2629.

- Xia E, Zhang Y, Cao H, Li J, Duan R, Hu J. TALEN-Mediated Gene Targeting for Cystic Fibrosis-Gene Therapy. Genes (Basel). 2019 Jan 11;10(1).

- Xia E, Zhang Y, Cao H, Li J, Duan R, Hu J. TALEN-Mediated Gene Targeting for Cystic Fibrosis-Gene Therapy. Genes (Basel). 2019 Jan 11;10(1). pii: E39.

- Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, Maeder ML, Joung JK, Chen ZY, Liu DR. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat Biotechnol. 2015 Jan;33(1):73-80.

- Koenig, Mary Kay. "Presentation and diagnosis of mitochondrial disorders in children" Pediatric neurology vol. 38,5 (2008): 305-13.

- Helen A.L. Tuppen, Emma L.Blakely, Douglass M.Turnbull, Robert W.Taylor. "Mitochondrial DNA mutations and human disease" Biochimica et Biophysica Acta (BBA) – Bioenergetics Volume 1797, Issue 2, Feb 2010, Pages 113-128.

- Agresti CA, Halkiadakis PN, Tolias P. MERRF and MELAS: current gene therapy trends and approaches. J Transl Genet Genom 2018;2:9.