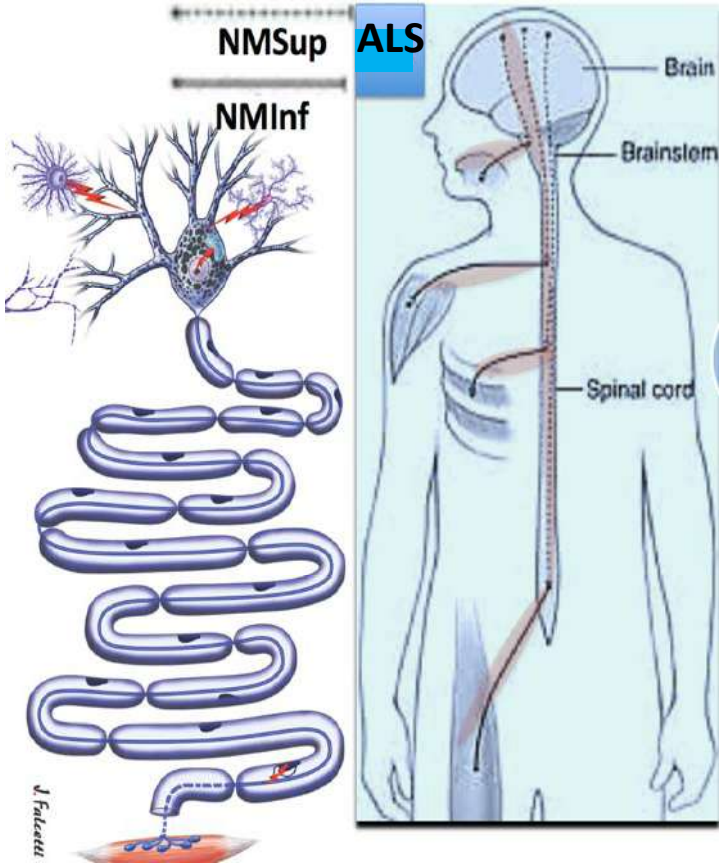
www.projetoelabrasil.com.br

Human iPS-derived motor neurons for sporadic ALS patients

Gerson Chadi. MD. PhD.
Full Professor
Head of Translational Neurology Unit
Department of Neurology. FMUSP
gerchadi@usp.br

BIOMED D'OR. Rio de janeiro. 2017

Amyotrophic Lateral Sclerosis (ALS)



Age: 22 -75 y-old

Late diagnosis, no biomarkers

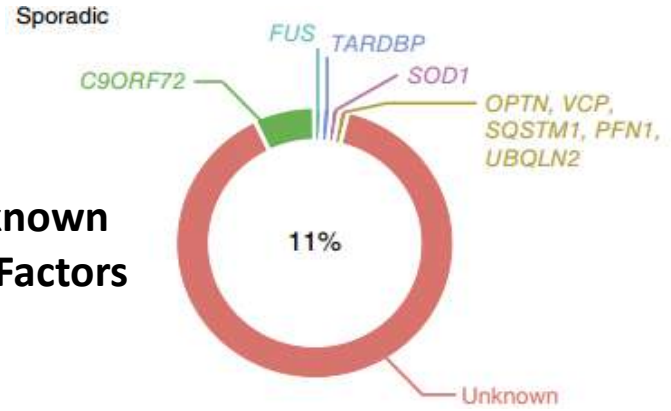
Severe disabilities, High morbidity

No specific treatments, No cure

Fast progression (2-5 y) (respiratory failure)

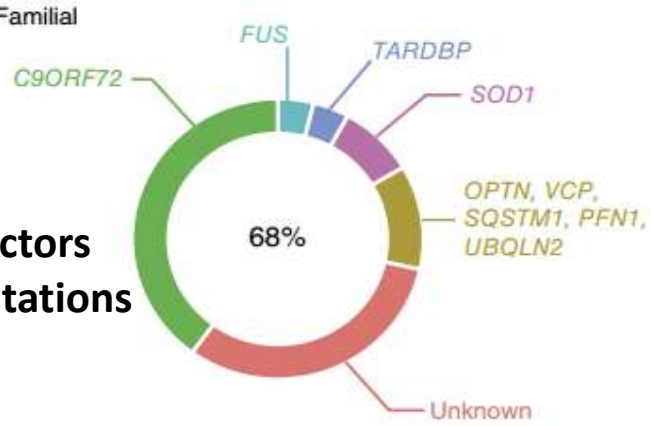
SPORADIC ALS (~95%)

Unknown Risk Factors



FAMILIAL ALS (~5%)

Risk Factors Gene Mutations



ALS Major Clinical Phenotypes

Inf MN

Muscle atrophy

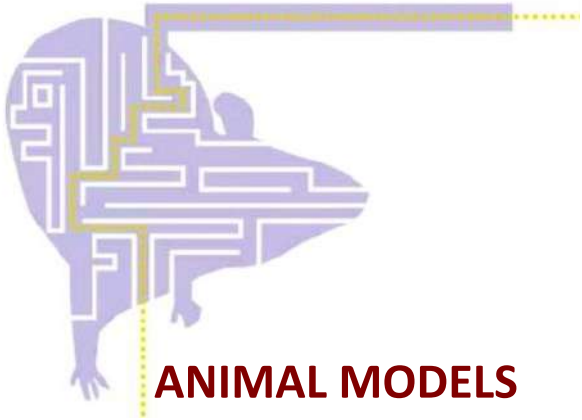
Refexes (hipo)

Sup MN

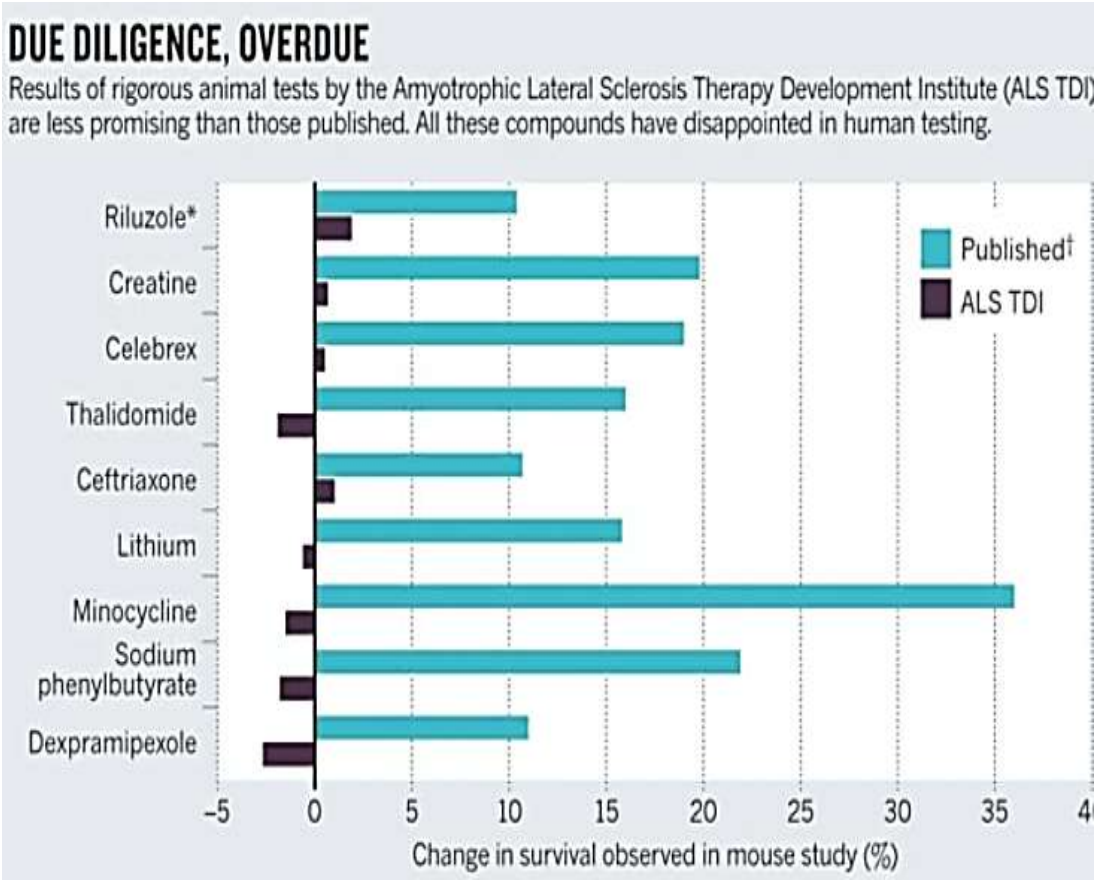
Refexes (hiper) **Clonus**

Fasciculation

THERAPEUTICAL TRANSLATIONAL FAILURE ON ALS.
WHERE IS THE PROBLEM?

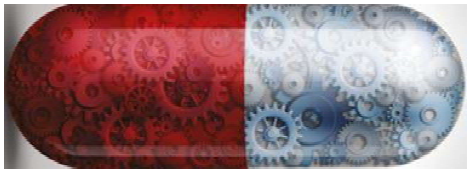


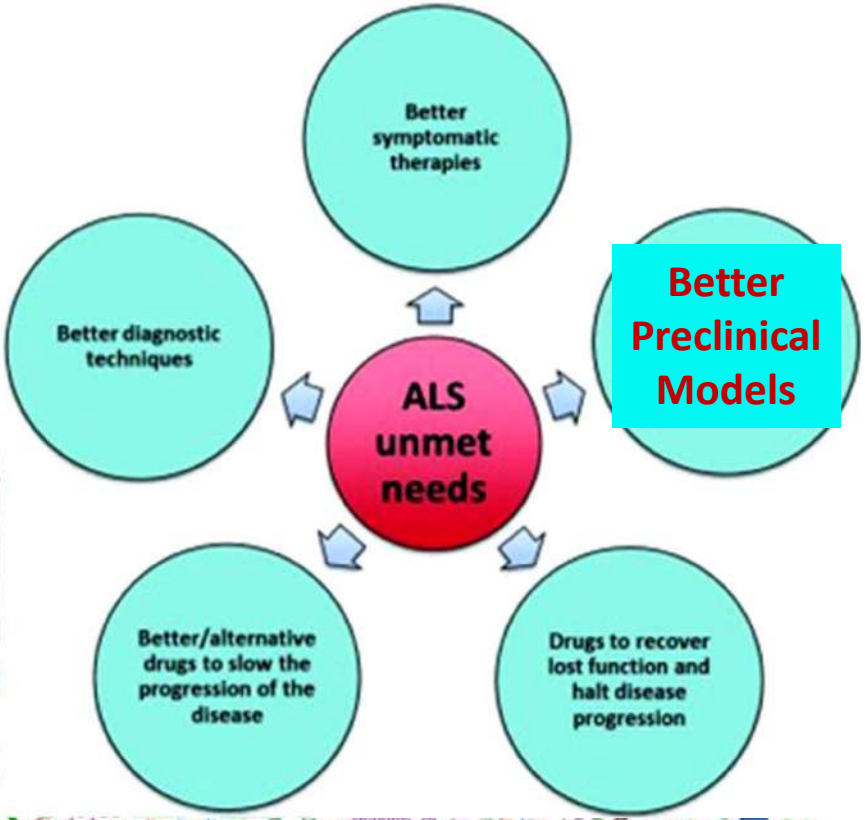
ANIMAL MODELS



ALS Therapy Development Institute (TDI) in Cambridge, Massachusetts

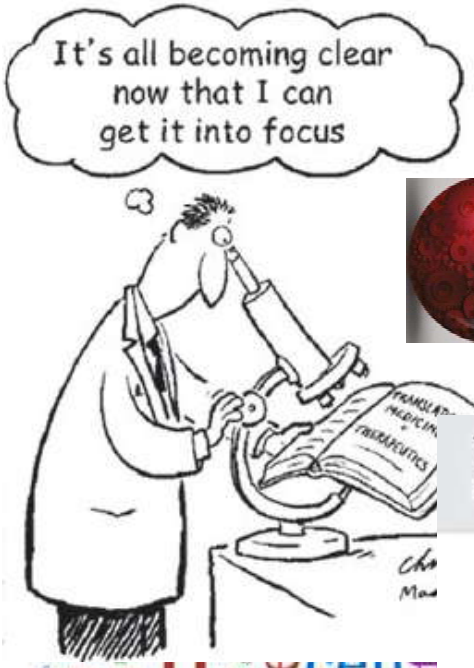
ALS Translational Neurology – What's the way?

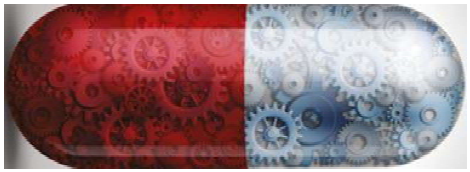




www.labnews.co.uk/features/overcoming-the-barrier-to-als

ALS Translational Neurology – What's the way?





Training laboratory and clinical investigators in team-based translation
Improving communications with new technologies and information systems

Better symptomatic therapies

Better Preclinical Models

Laboratory discoveries → Patient-centered research → Delivery to patients and communities

Drugs to recover lost function and halt disease progression

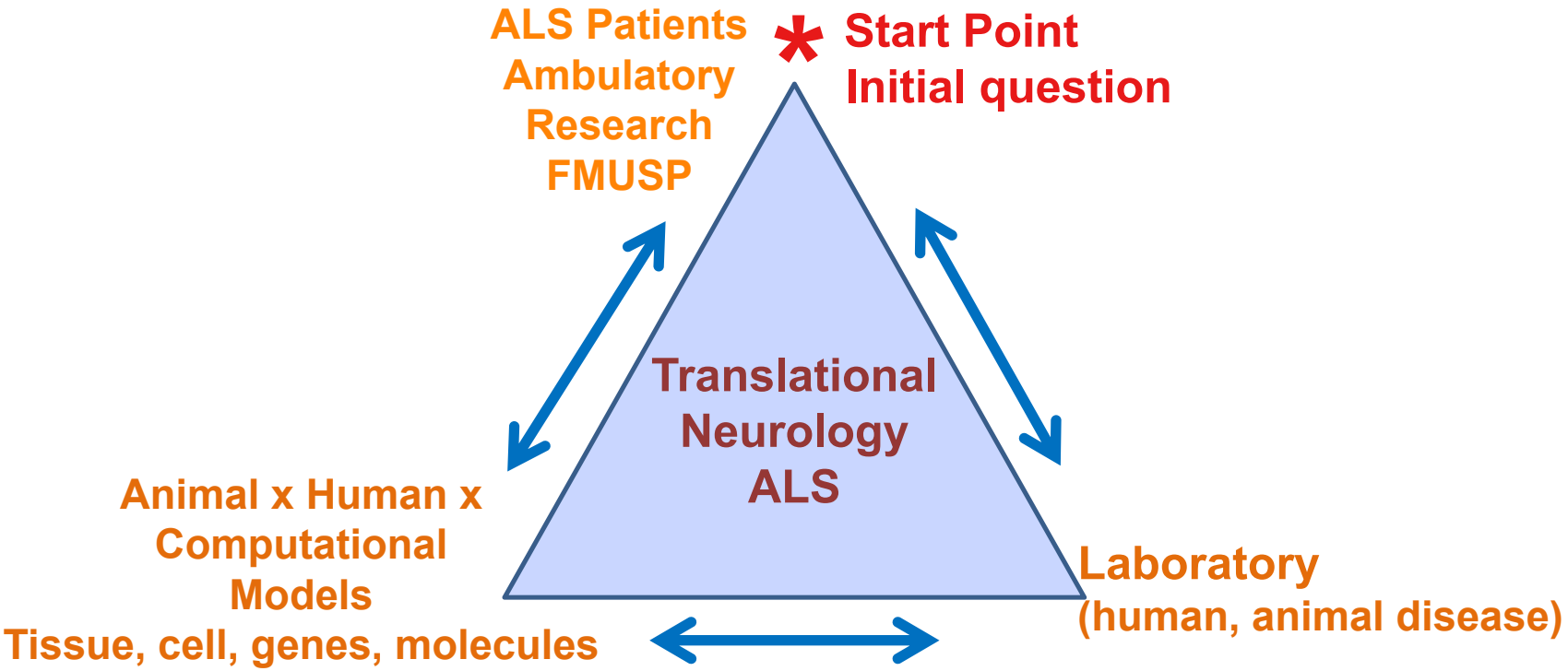
Bench → Bedside

www.labnews.co.uk/features/overcoming-the-barrier-to-als

ALS Translational Neurology

Translational Neurology Discipline and Unit at HC-FMUSP

Mechanisms related to neurodegeneration in neurodegenerative disorders are unknown



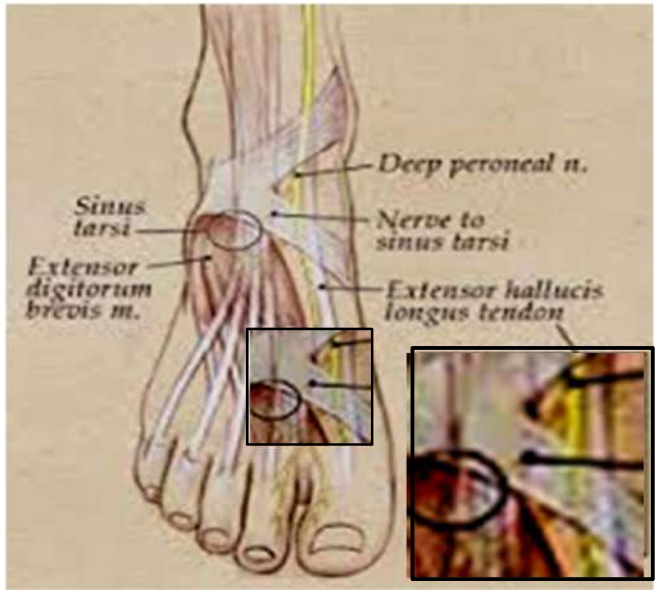
Systematic and interconnected studies → Increasing the expectation the discovery of therapeutic targets

ALS - Search for Molecular Signals

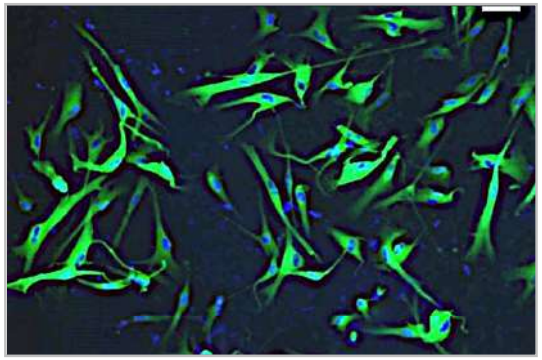
Motor Nerve from Human Sporadic ALS Form

✓ Transcriptome analysis – Schwann Cells and Differentiated Motor Neurons

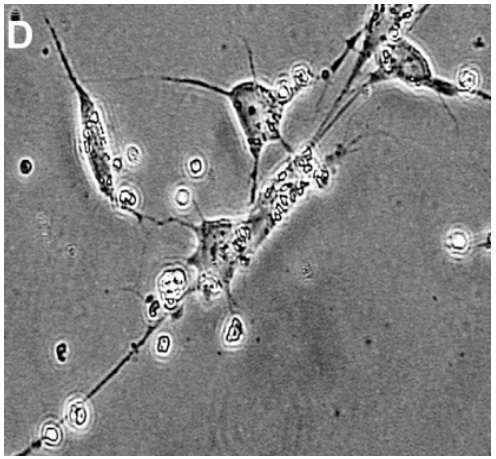
extensor hallucis brevis nerve



Human Schwann Cells from Motor Nerve

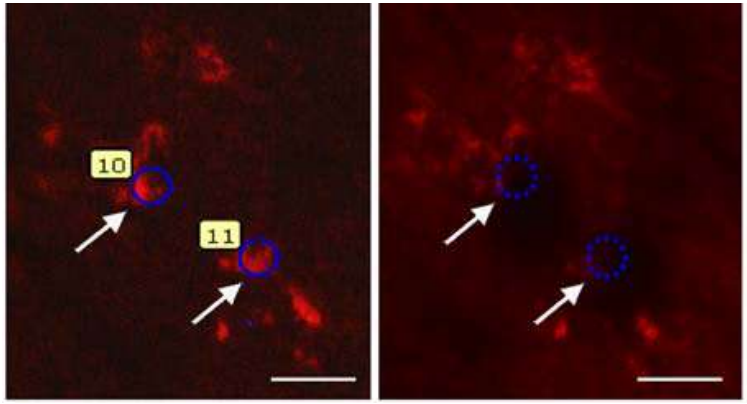


Transformed Motor Neurons

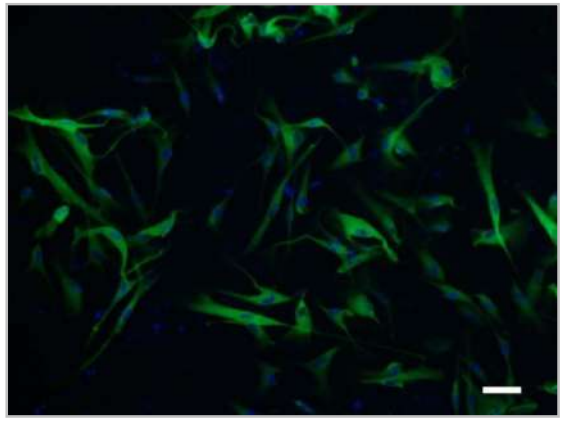


Schwann Cell Enrichment

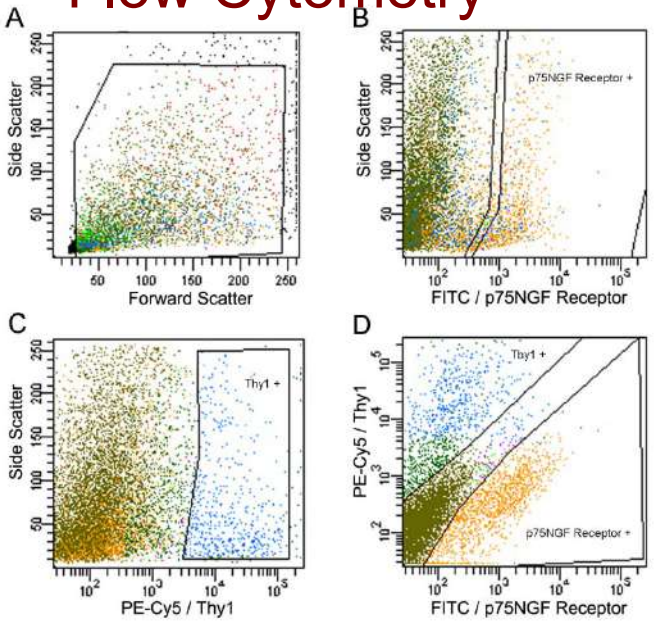
Laser Microdissection

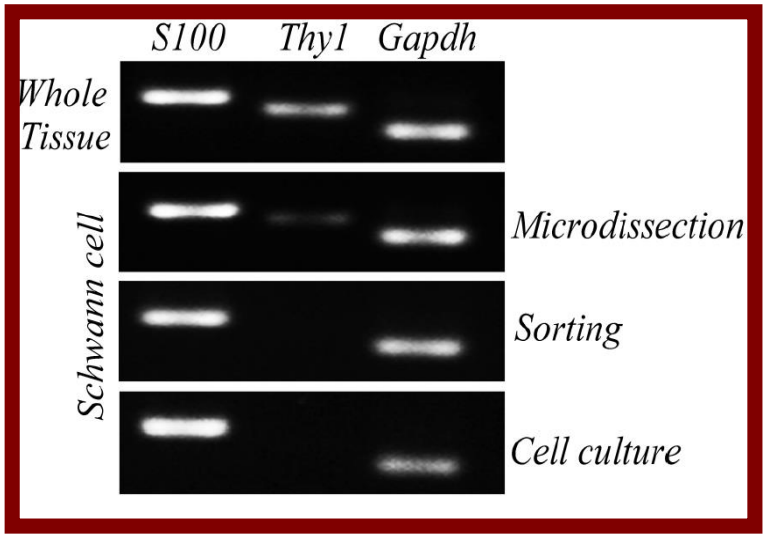


Cell Culture



Flow Cytometry





Transcriptome. Slide-based DNA microarrays

- **Molecular**
- **Biological**
- **Downstream**
- **Workflow overview data**

* **Pathway Analysis**
* Model Characterization
* Classifiers/Predictive Models
* **Drug-Analysis (Dose/Time/Class)**
* Integration Analysis

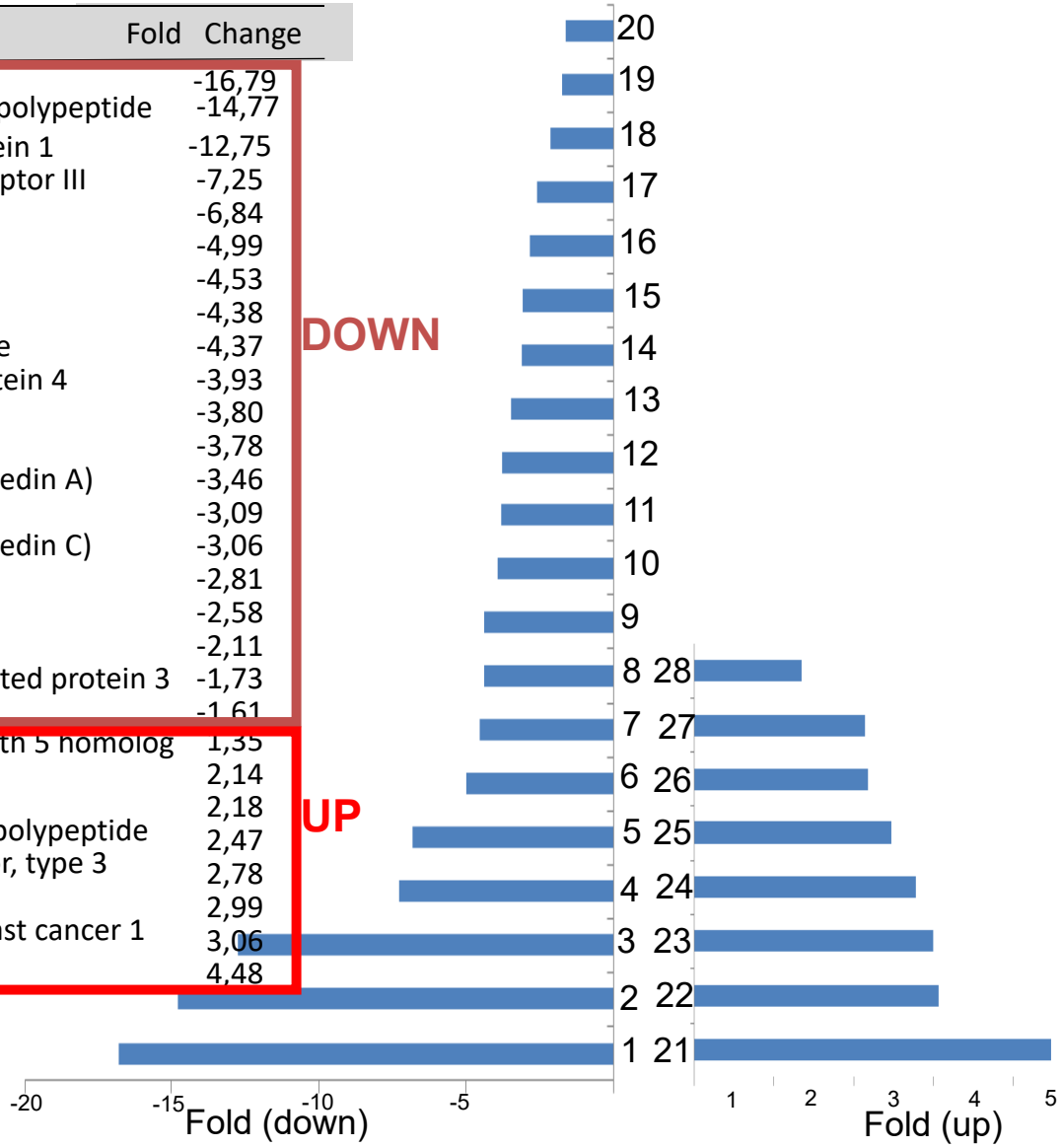
Enriched Schwann cells from ALS Functional Motor Nerves

Growth Factors

Numb	Gene Symbol	Description	Fold Change
1	FGF7	fibroblast growth factor 7	-16,79
2	PDGFRA	platelet-derived growth factor rec, α polypeptide	-14,77
3	FGFBP1	fibroblast growth factor binding protein 1	-12,75
4	TGFBR3	transforming growth factor, beta receptor III	-7,25
5	EGFR	epidermal growth factor receptor	-6,84
6	PGF	placental growth factor	-4,99
7	IGF2	insulin-like growth factor 2	-4,53
8	EGFR	epidermal growth factor receptor	-4,38
9	KGFLP1	fibroblast growth factor 7 pseudogene	-4,37
10	IGFBP4	insulin-like growth factor binding protein 4	-3,93
11	GDF15	growth differentiation factor 15	-3,80
12	FGF1	fibroblast growth factor 1 (acidic)	-3,78
13	IGF2	insulin-like growth factor 2 (somatomedin A)	-3,46
14	FGF11	fibroblast growth factor 11	-3,09
15	IGF1	insulin-like growth factor 1 (somatomedin C)	-3,06
16	GAS1	growth arrest-specific 1	-2,81
17	OGFRL1	opioid growth factor receptor-like 1	-2,58
18	FGFR2	fibroblast growth factor receptor 2	-2,11
19	HDGFRP3	hepatoma-derived growth factor, related protein 3	-1,73
20	VEGFA	vascular endothelial growth factor A	-1,61
21	USMG5	up-regul during skeletal muscle growth 5 homolog	1,35
22	TGFA	transforming growth factor, alpha	2,14
23	GAS8	growth arrest-specific 8	2,18
24	PDGFA	platelet-derived growth factor alpha polypeptide	2,47
25	NTRK3	neurotrophic tyrosine kinase, receptor, type 3	2,78
26	FGF12	fibroblast growth factor 12	2,99
27	GREB1	growth regulation by estrogen in breast cancer 1	3,06
28	GDF1	growth differentiation factor 1	4,48

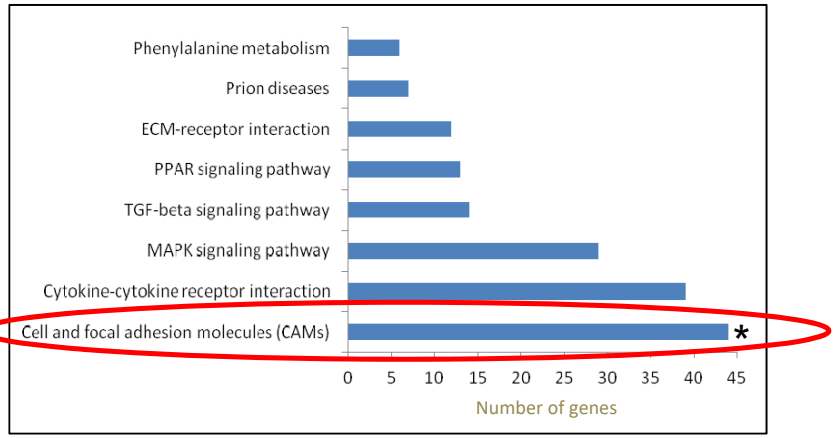
DOWN

UP

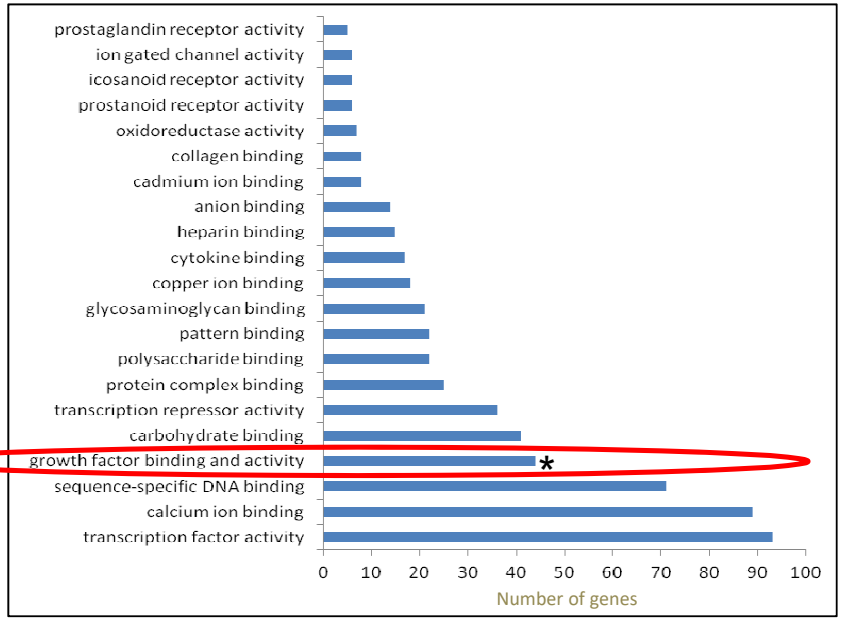


Peripheral Nerve – ALS Sporadic Patients

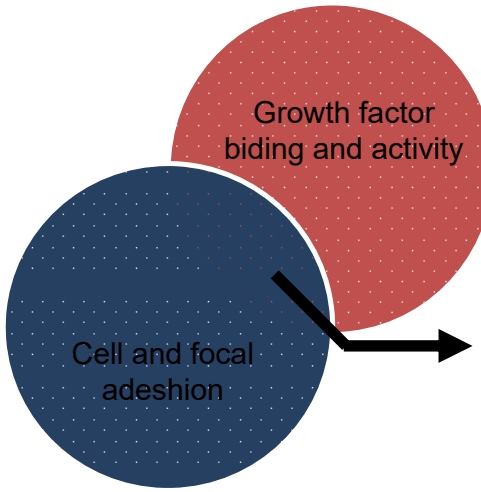
Signaling Pathways (KEGG)



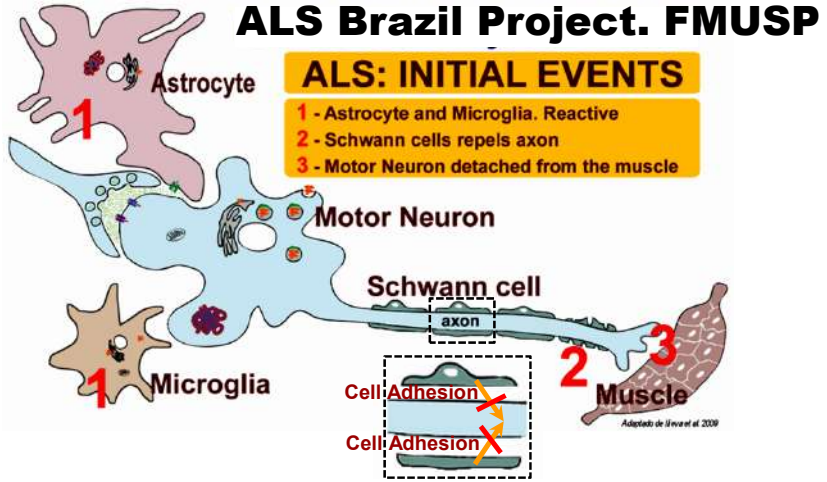
Molecular Function



Venn Diagram

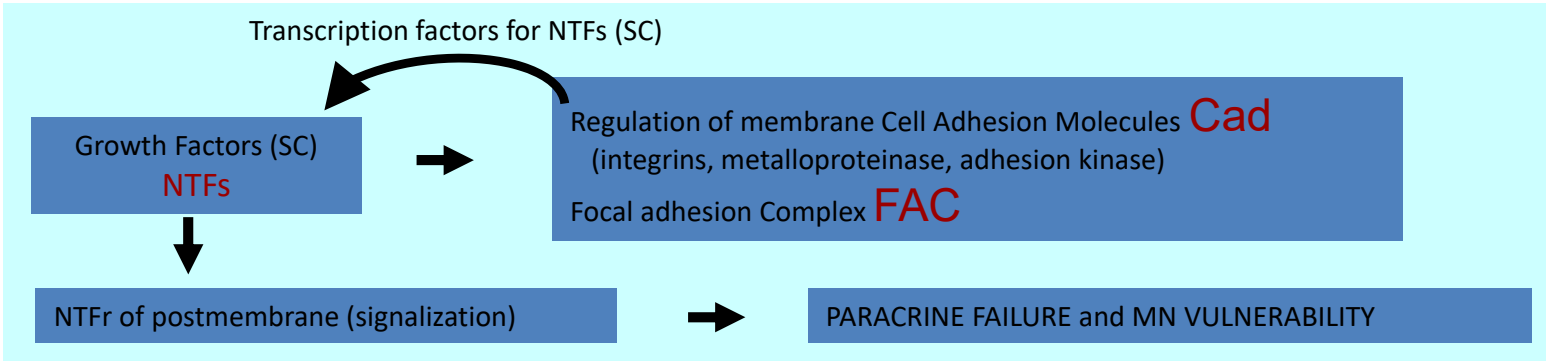


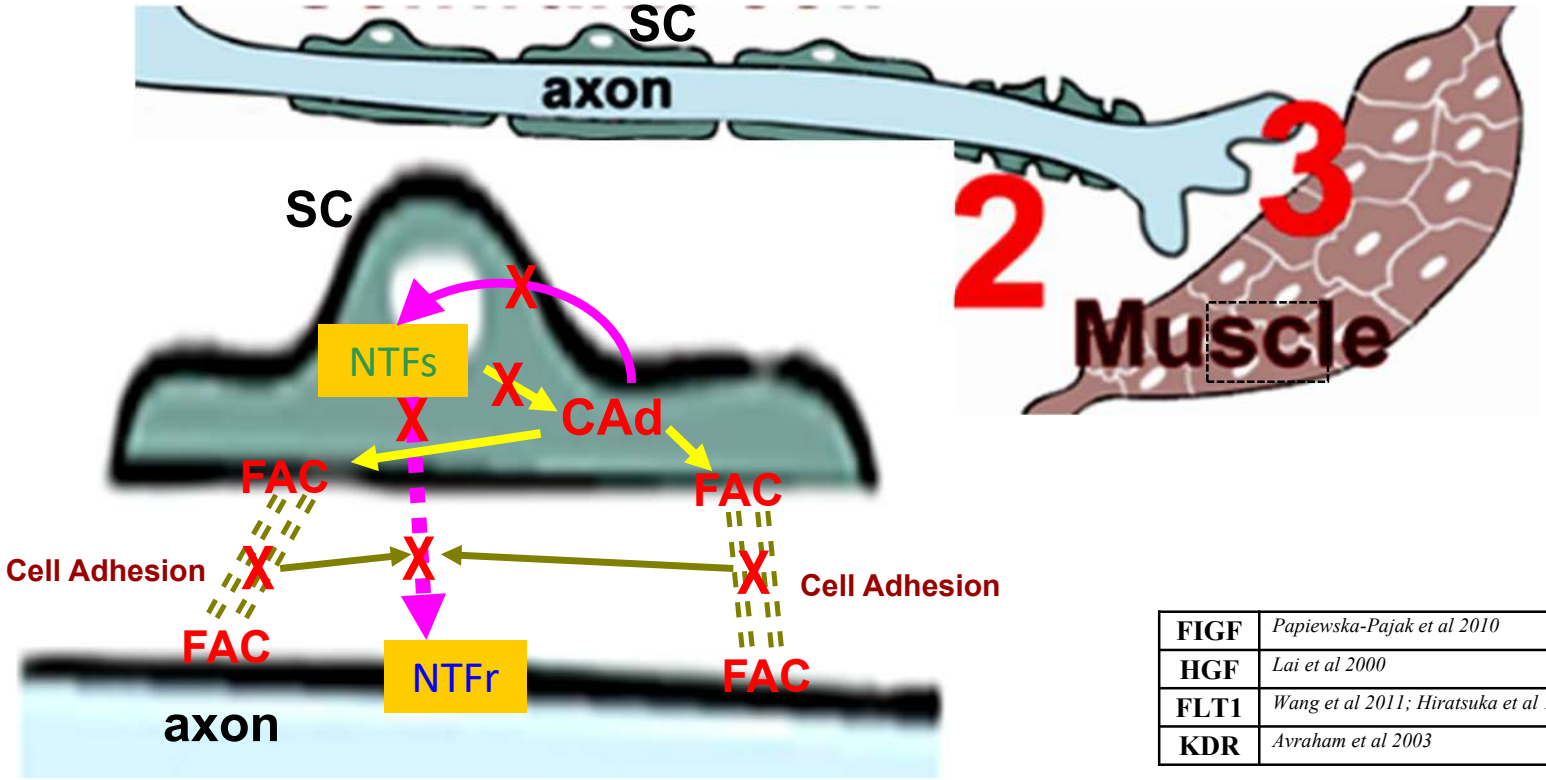
Genes	
FIGF +	PDGFRB +
FLT1 +	PGF +
HGF +	THBS1 -
KDR +	VEGFC +
PDGFB +	



Disrupted Focal Adhesion/Neurotrophic Factor Signaling impairs Paracrine Trophic Actions of SC to Motor Neurons in ALS

Current Theory of ALS Brazil Project. FMUSP

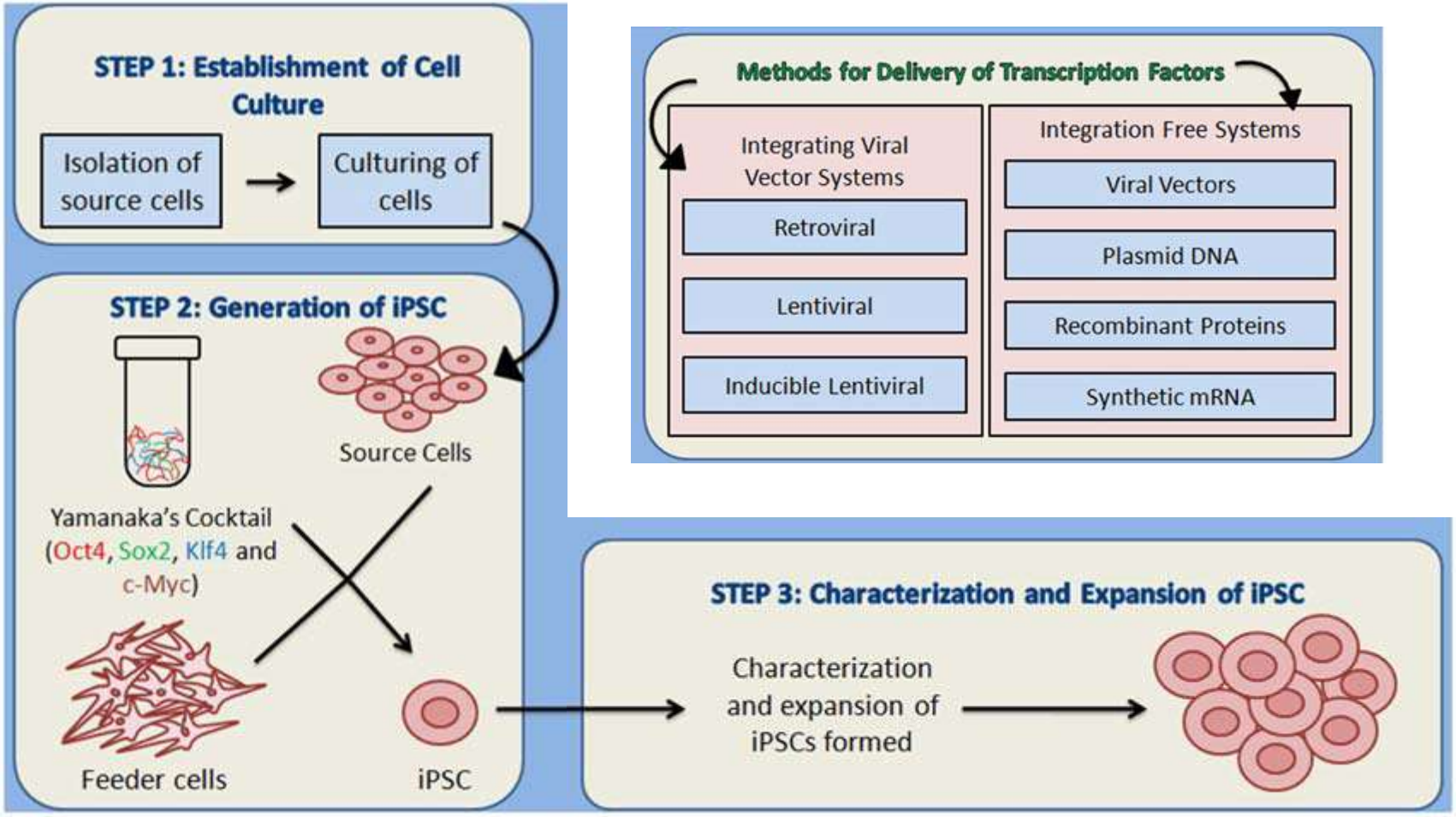




FIGF	*Papiewska-Pajak et al 2010*
HGF	*Lai et al 2000*
FLT1	*Wang et al 2011; Hiratsuka et al 1998*
KDR	*Avraham et al 2003*

ALS Translational Neurology

Generation of induced Pluripotent Stem Cells (iPS) from mature cells of ALS Patients

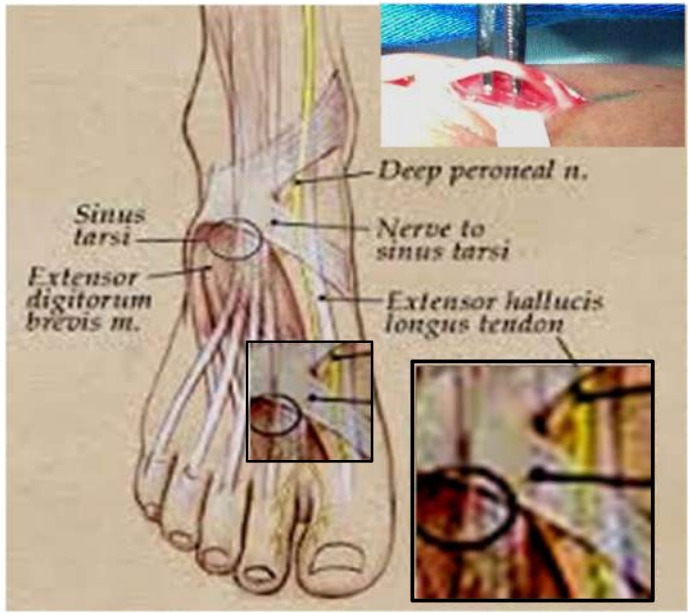


ALS - Search for Molecular Signals

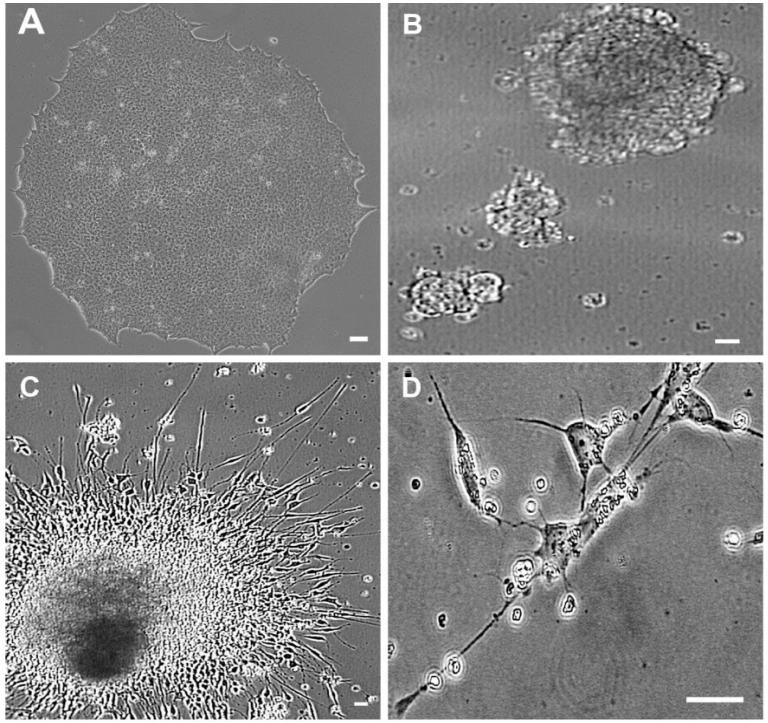
Biopsy Motor Nerve from ALS Sporadic Patients

✓ Microarray Analysis – Schwann Cells and Differentiated Motor Neurons

Extensor hallucis brevis nerve



Morphology



ALS - Search for Molecular Signals

ALS Sporadic Form

✓ Motor Neuron Differentiation from **Fibroblasts (Motor Nerve)**

-CytoTune iPS Reprogramming Kit containing Sendai virus vectors (Life Tech; A1378001)

-hSTEMCCA-loxP lentivirus

Control Patients

Nervus Accessorius

REPROGRAMING
(Oct4, Sox2, c-Myc, Klf4)

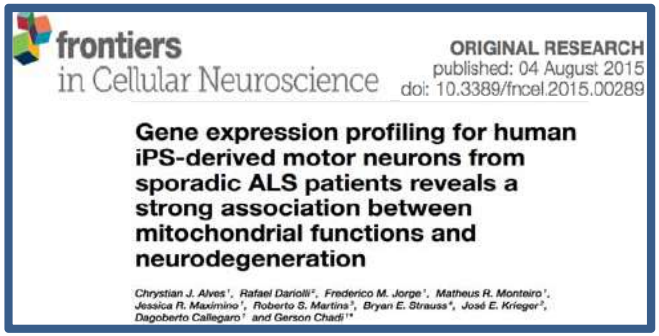
IPSC

Motor Neuron

Glial cells

ALS Patients Sporadic Form

N extensor hallucis brevis



frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 04 August 2015
doi: 10.3389/fncel.2015.00289

Gene expression profiling for human iPS-derived motor neurons from sporadic ALS patients reveals a strong association between mitochondrial functions and neurodegeneration

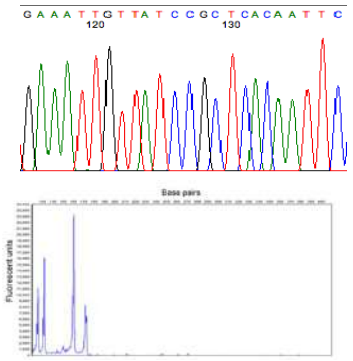
Chrystian J. Alves[1], Rafael Dariolli[2], Frederico M. Jorge[1], Matheus R. Monteiro[1], Jessica R. Maximino[1], Roberto S. Martins[3], Bryan E. Strauss[4], José E. Krieger[2], Dagoberto Callegaro[1] and Gerson Chadi[1]*

ALS - Search for Molecular Signals

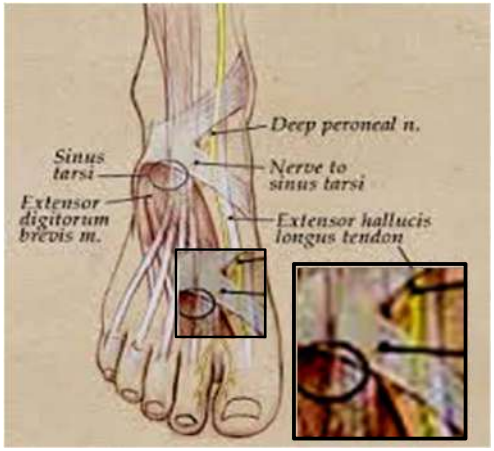
SPORADIC ALS

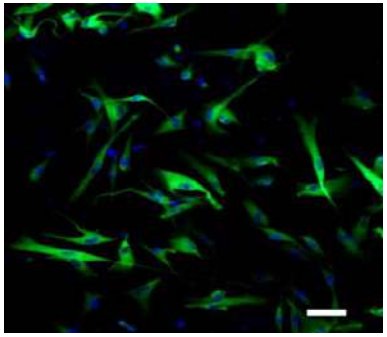
NO MUTATIONS FOUND

Sequencing



Nervus extensor hallucis brevis





Fibroblast

STEMCCA

SENDAY

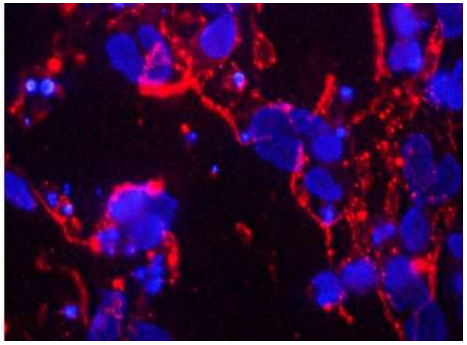
Yamanaka

IPSC

Differentiation

Hu and Zhang (2009)

Transformed Motor Neurons Sporadic ALS Patient



First time in Brazil

Sporadic ALS Patients

	ALS patients		Control patients	
Patient	Sporadic ALS	Sporadic ALS	non-ALS	non-ALS
Age	60	68	43	57
Gender	Male	Male	Female	Male
Biopsy date	August 2013	August 2013	August 2013	August 2013
Date of onset	March 2011	June 2011		
Site of onset	RLL	LUL		
Duration[1] (months)	29	26		
ALS-FR scale in 2012	34/40	34/40		
ALS-FR scale in 2014	30/40	/40		
Strength in the unilateral big toe	3/5	3/5		
Electroneuromyography	chronic disease with anterior tip cervico-thoraco-lumbar	chronic disease with anterior tip cervico-thoraco-lumbar and bulbar nucleus		
Medicines	Riluzole	Riluzole, B vitamins and venlafaxin		
Additional informations		Depressive symptoms		

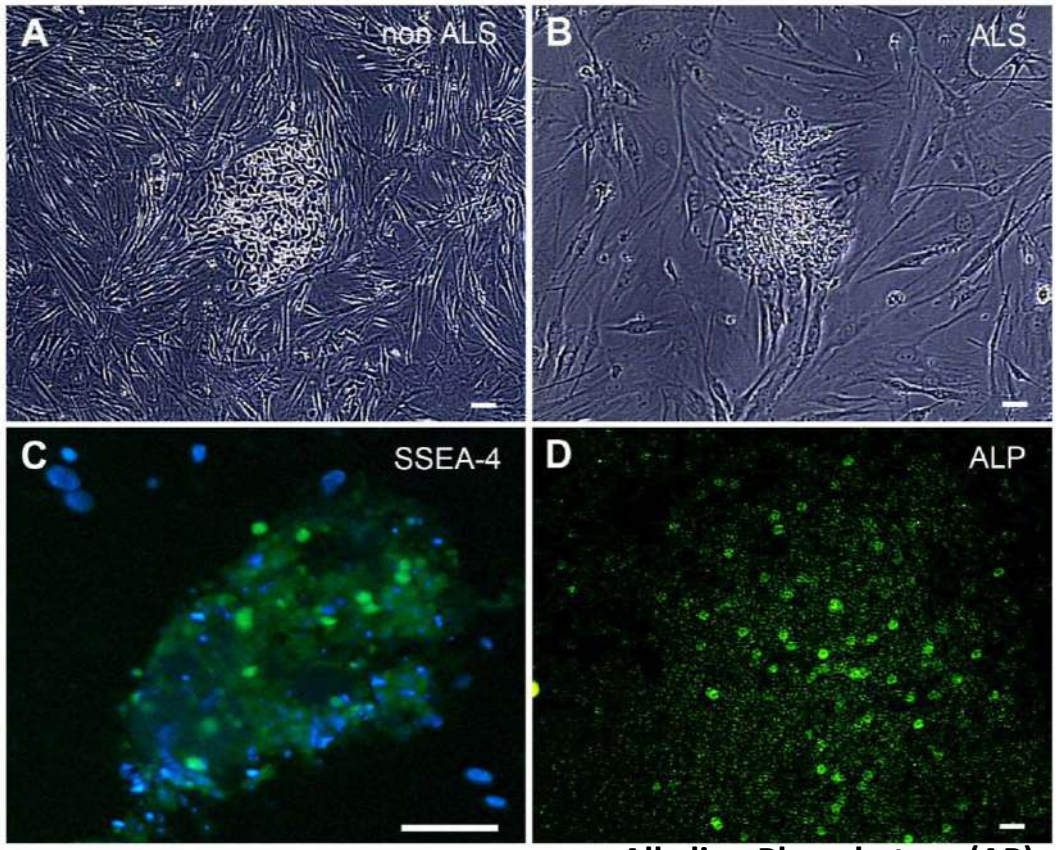
ALS Research Ambulatory Unit.
Hospital de Clínicas. HCFMUSP

Generation and Characterization of SENDAI Transduced Pluripotent Stem Cell (hiPSC)

10^5 fibroblasts transduced with CytoTune iPS Reprogramming Kit containing Sendai virus vectors (Life Technologies; Cat. # A1378001). ***Macarthur et al. (2012)***

-individual delivery of 4 Yamanaka reprogramming factors, *OCT4, SOX2, KLF4, CMYC*

markers

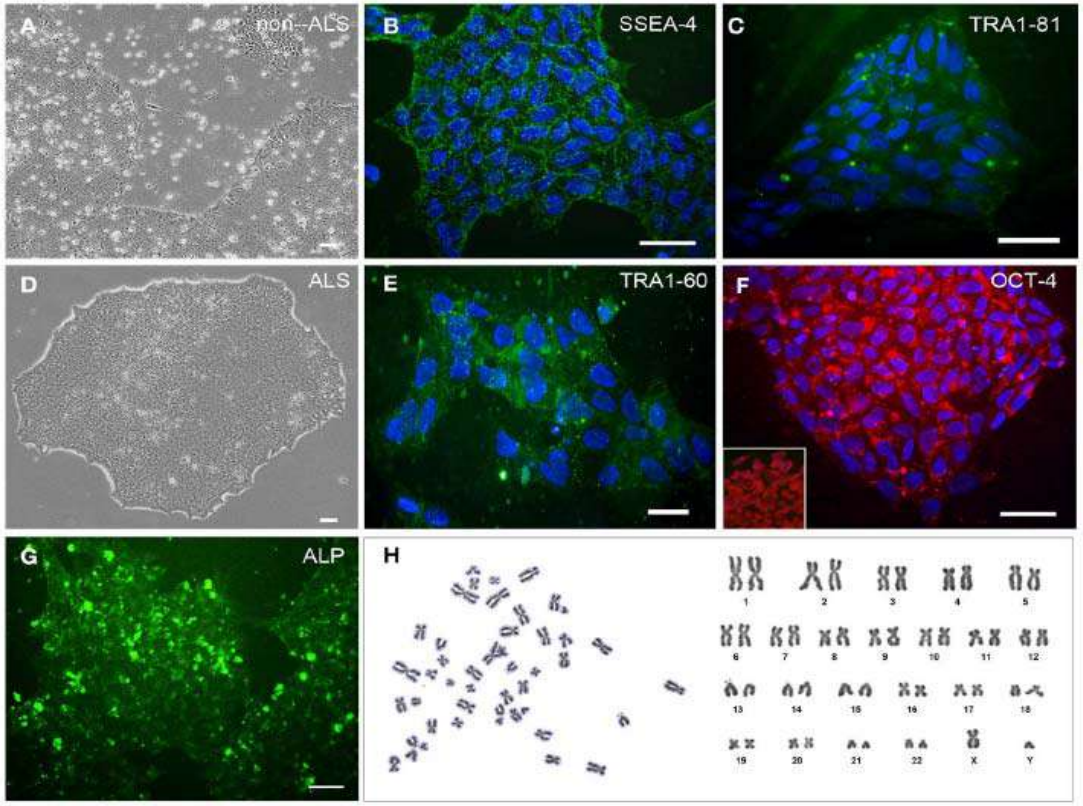


SSEA4
surface embryonic stem cell

Alkaline Phosphatase (AP)
pluripotency

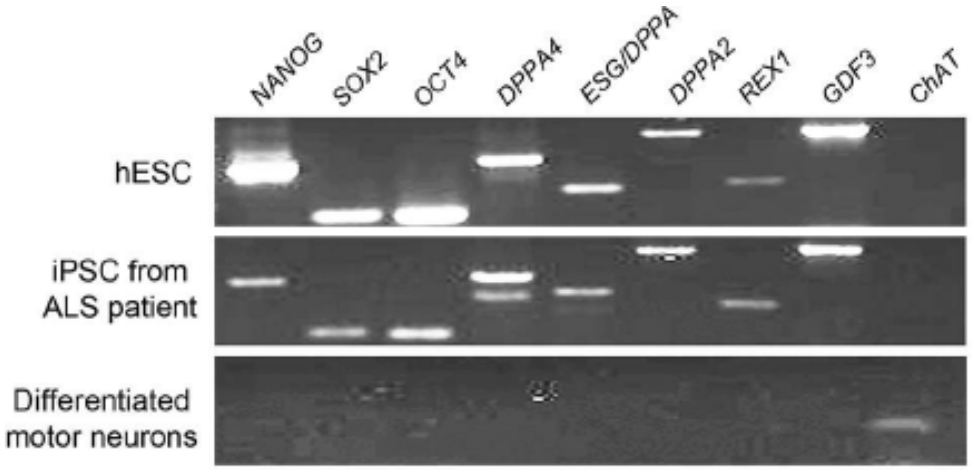
Generation and Characterization of STEMCCA Transduced Pluripotent Stem Cell (hiPSC)

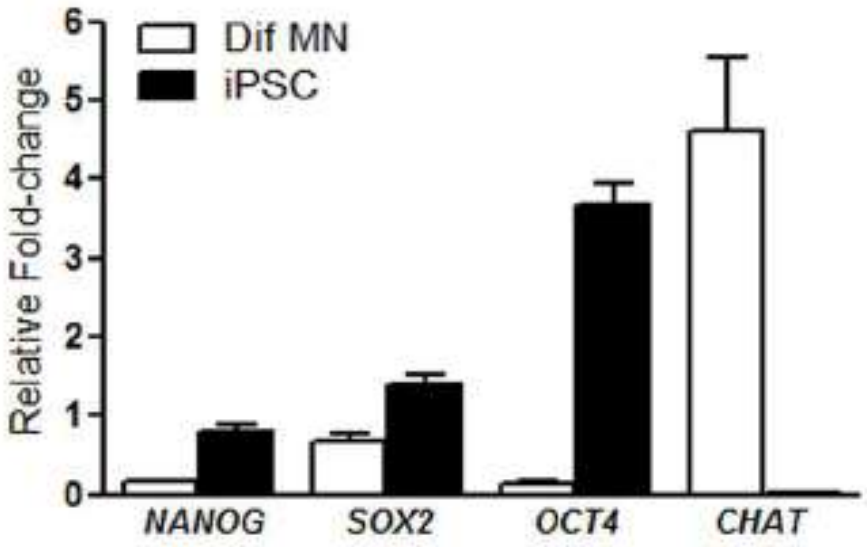
Human fibroblast (ALS patients, sporadic) reprogramming using hSTEMCCA-loxP lentivirus based on *Somers et al., 2010*



STEMCCA Cre-Excisable Constitutive Polycistronic Lentivirus expressing the embrionary genes *OCT4, SOX2, KLF4*, and *CMYC*

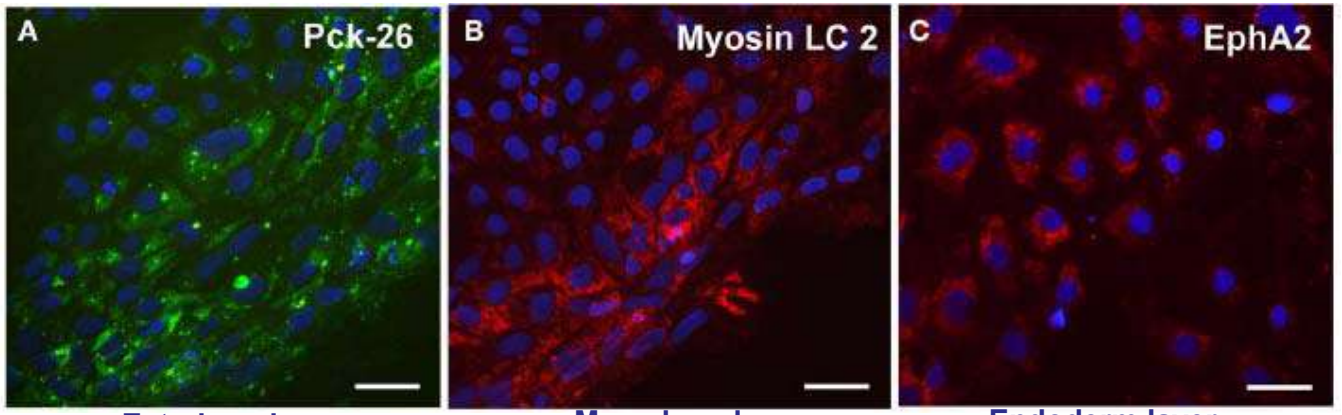
Pluripotency Markers of Generated hiPSC





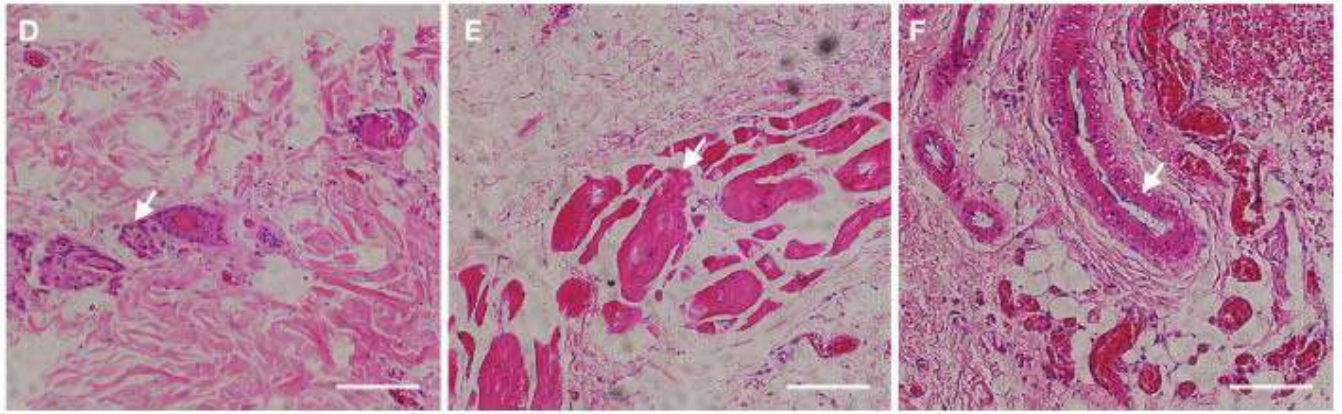
Spontaneous Differentiation of hiPS in 3 Germ Layers *in vitro and in vivo*

Immunocytochemical - in vitro hiPS derived from fibroblasts



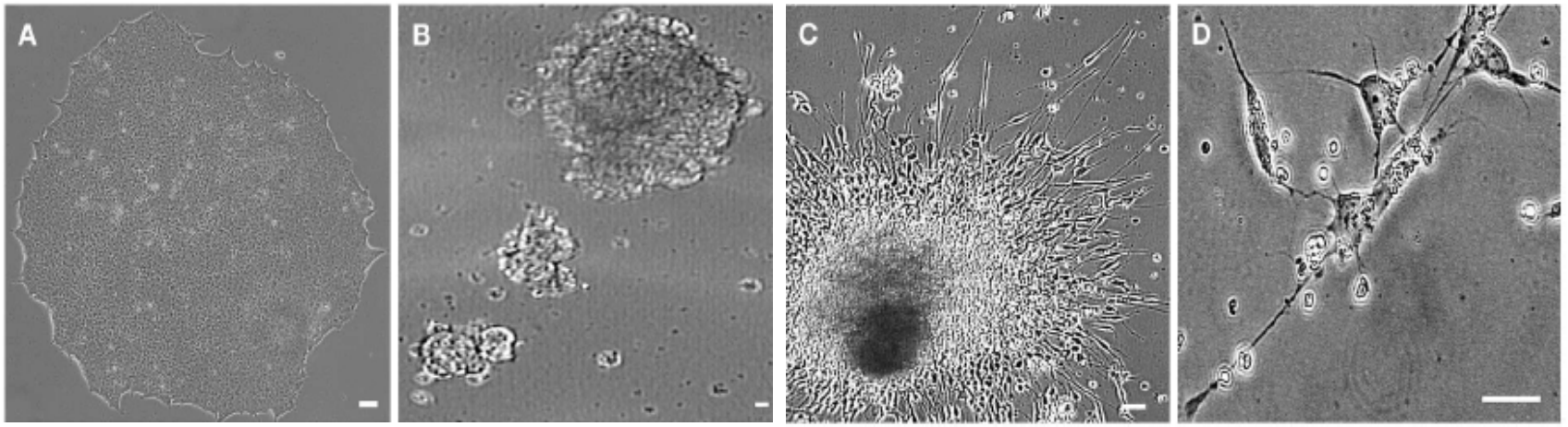
Ectoderm layer | Mesoderm layer | Endoderm layer

Hematoxylin and eosin staining of sections from a teratoma formed after a subcutaneous injection of hiPSC into nude rats



Neural rosettes | muscle tissue | gut-like epithelium

hiPSC-differentiated Motor Neurons

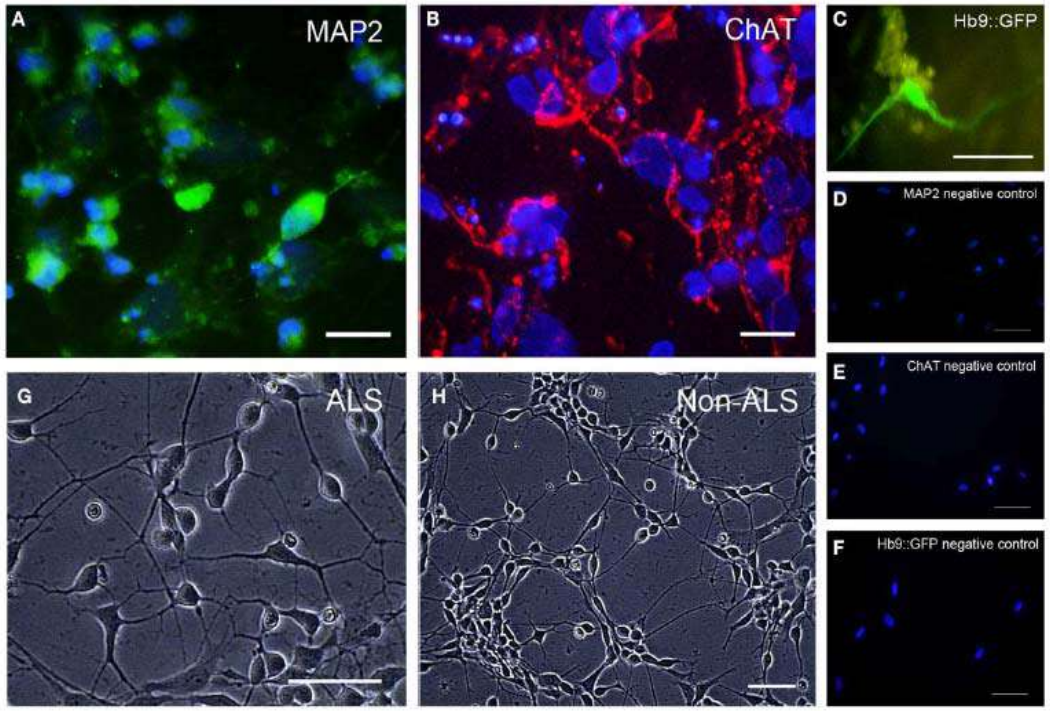


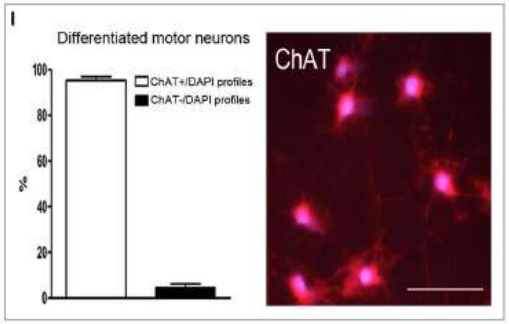
-After reaching confluence, hiPS colonies were cultured in suspension in the presence of embryoid body medium

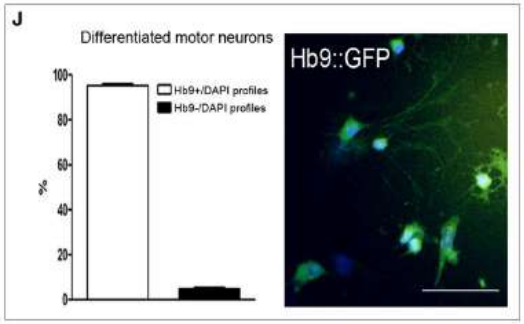
- The medium was replaced on day 4 by a neural differentiation medium containing DMEM/F12, N2-supplement, NEAA, antibiotic-antimycotic and heparin to induce the formation of the neural progenitor cells

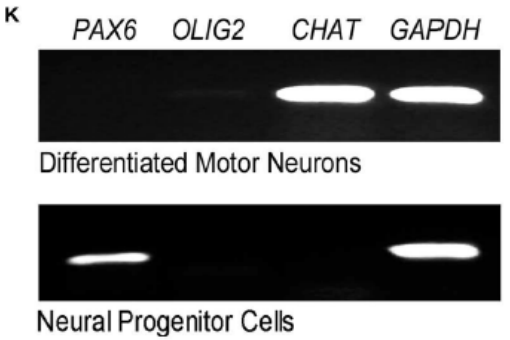
-Clusters attached to laminin-coated dishes after 1 week in suspension. Primitive neuroepithelial cells were posteriorized by addition of retinoic acid at day 10 and ventralizated by the addition of sonic hedgehog (Shh) and B27 supplement at day 14

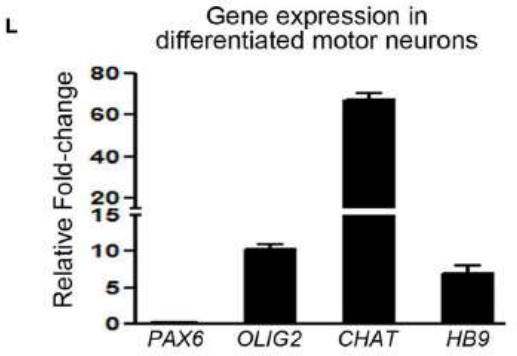
Characterization of hiPS-Derived Motor Neurons





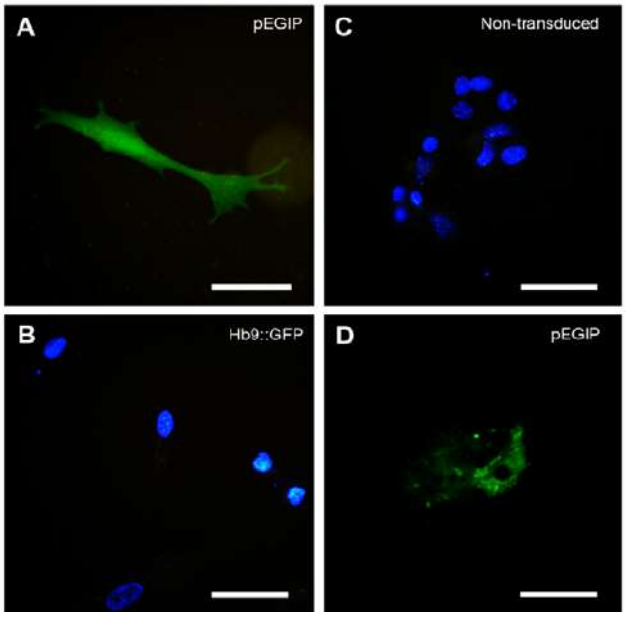


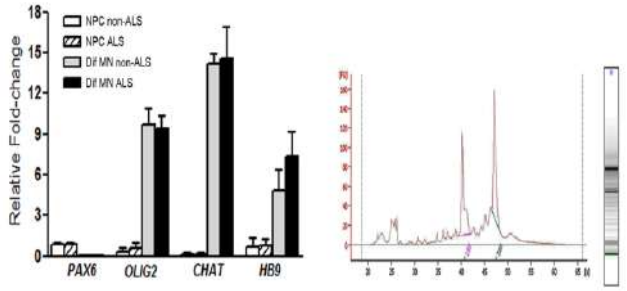




Quality Control

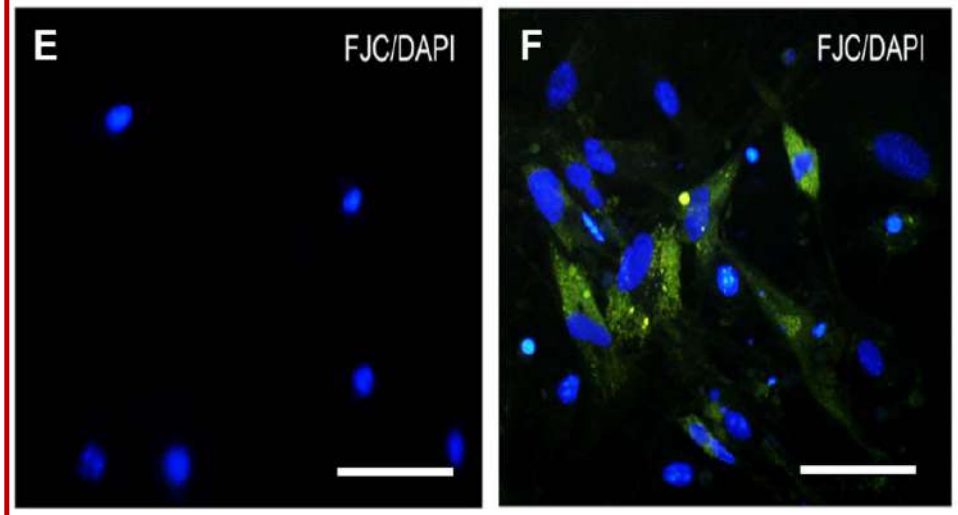
Control exper for MN markers





Vital state of motor neurons

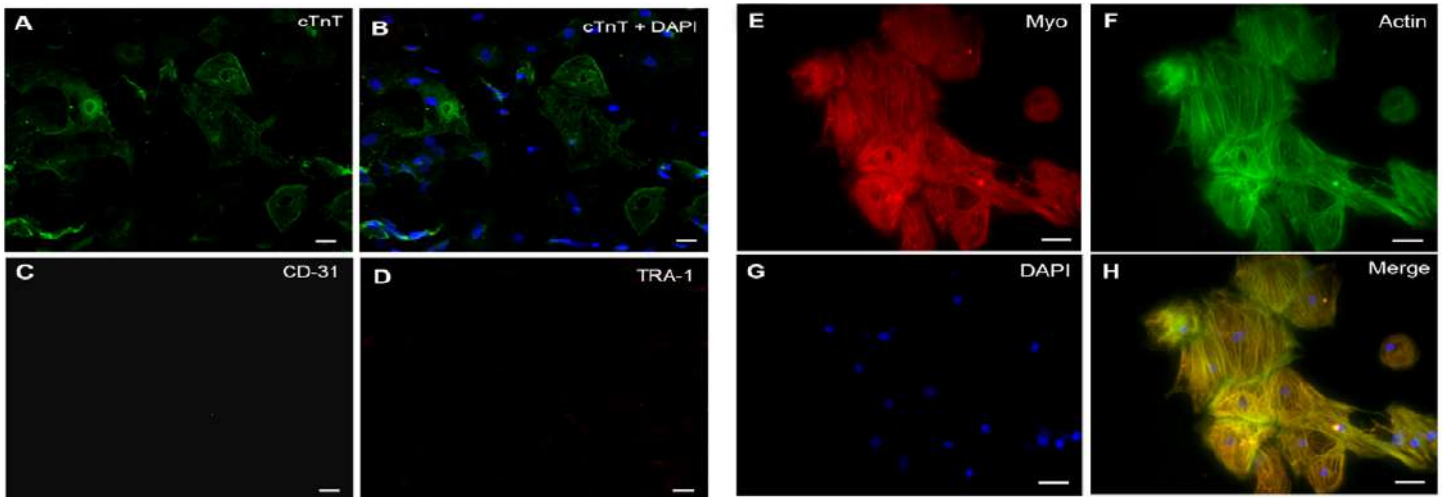
Vital state | **Pre-death state**



Human transf MN

SOD1 mouse MN Primary Culture

hiPS Differentiated Cardiomyocyte



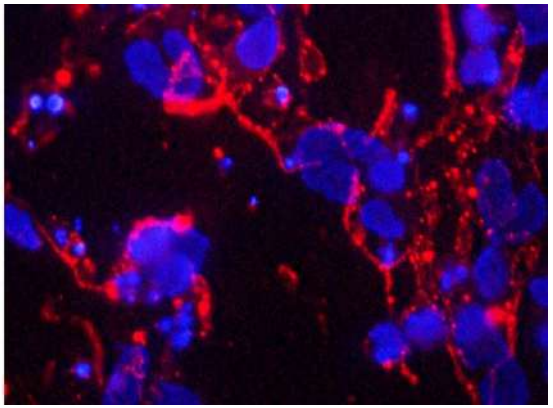
Video
Cardiomyocyte
Differentiation


40X

Transcriptome. Slide-based DNA microarrays

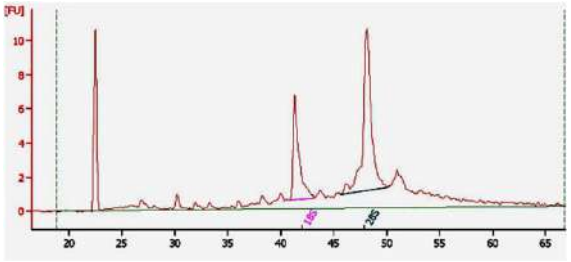
Transformed Motor Neuron - ALS Patient

Microarray Gene Profiling

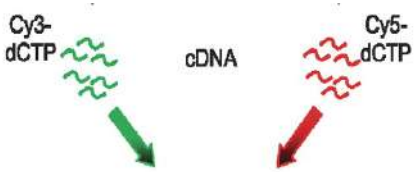


First time in Brazil

RNA Extraction



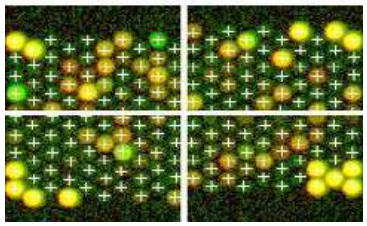
Reverse transcriptase labeling



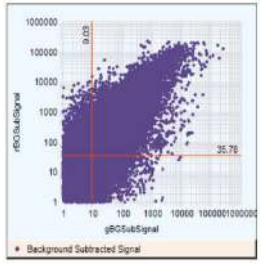
DATA ANALYSIS
Bioinformatics

Scanner and Image analysis

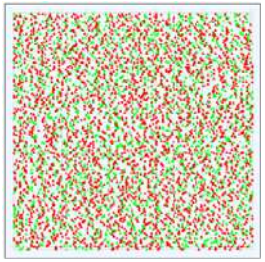
Feature Extraction Software



Grid Normal

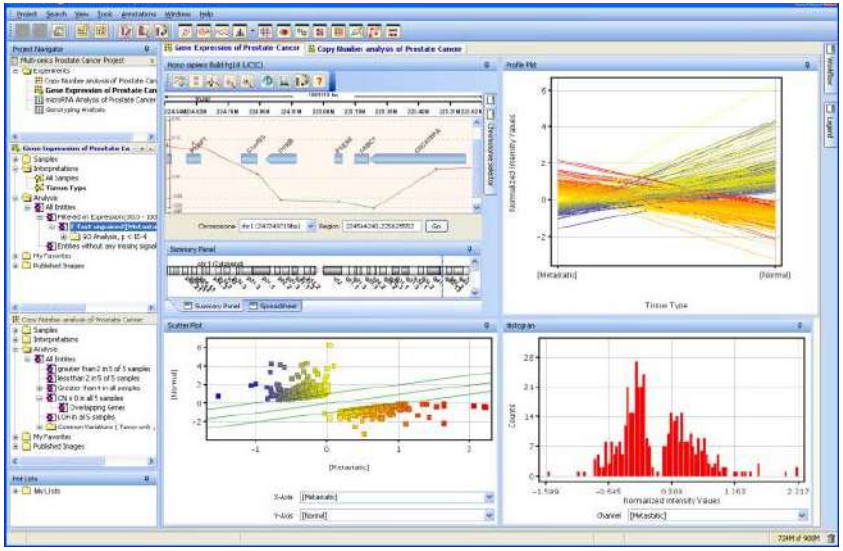


Background Correction



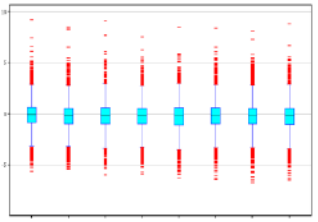
Up and Down-Regulated Genes

Transcriptome. Bioinformatic Analysis

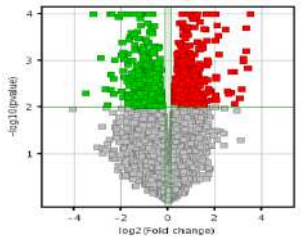


Statistical Analysis

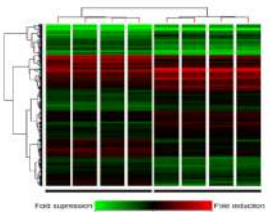
Normalization



Volcano Plot



Clustering



Transcriptome. Bioinformatic Analysis

Statistical Analysis

✓ Genes with *p < 0.05 were accepted as differentially expressed genes.*

✓ 1591 deregulated genes in MN SALS X MN CTR

✓526 up-regulated

✓1065 down-regulated

Functional Enrichment Analysis

KEGG

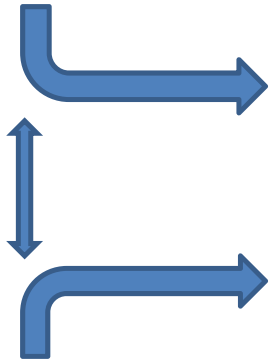
- **Kyoto Encyclopedia of Genes and Genomes**

Gene Ontology (GO) terms

- **Biological Process**
- **Molecular Functions**
- **Cellular components**

KEGG - Kyoto Encyclopedia of Genes and Genomes

Term		Genes
hsa05012	Parkinson's disease	20
hsa00190	Oxidative phosphorylation	18



Highly Involvement of Mitochondrial related genes

Gene Ontology (GO) terms

- Biological Process
- Molecular Functions
- Cellular components

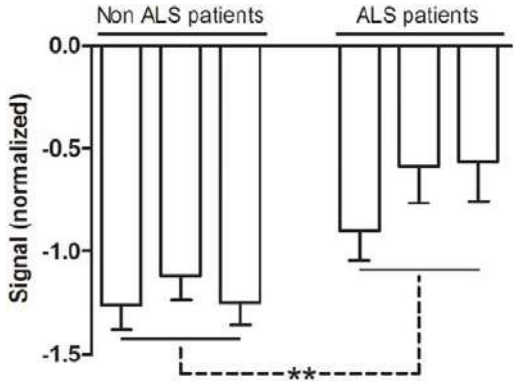
Molecular Signals

Cellular Component

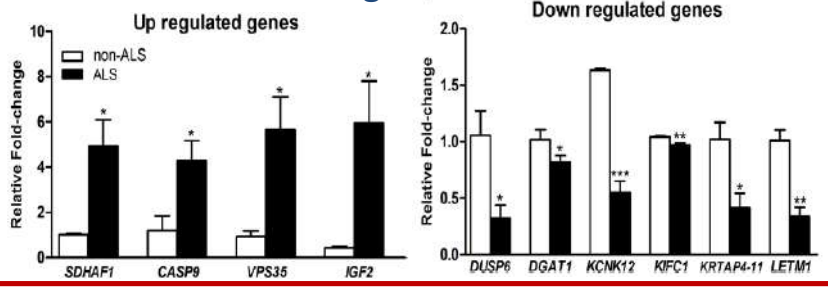
four GO term related with mitochondrion

- ✓ mitochondrion (105 genes)
- ✓ mitochondrion part (65 genes)
- ✓ mitochondrion matrix (30 genes)
- ✓ mitochondrion lumen (30 genes)

Mitochondrion gene expression in hiPS-derived MN



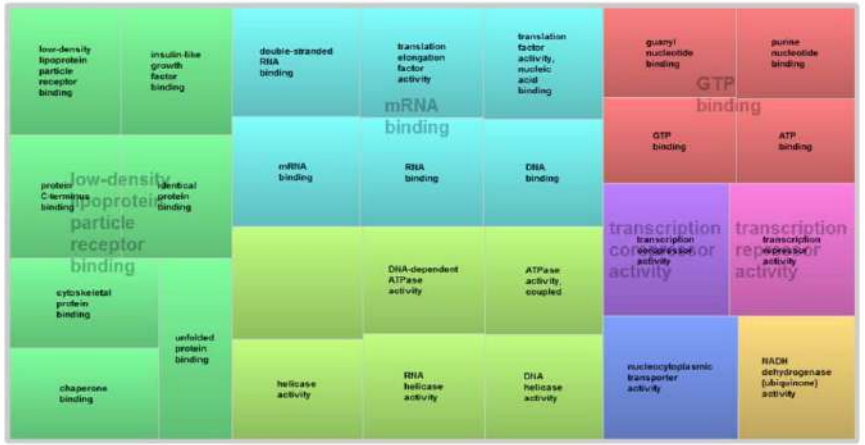
qPCR verification of dereg genes related to mitochodrion signaling and dysfunction



Gene Ontology terms grouped by REVIGO

Reduce + Visualize Gene Ontology

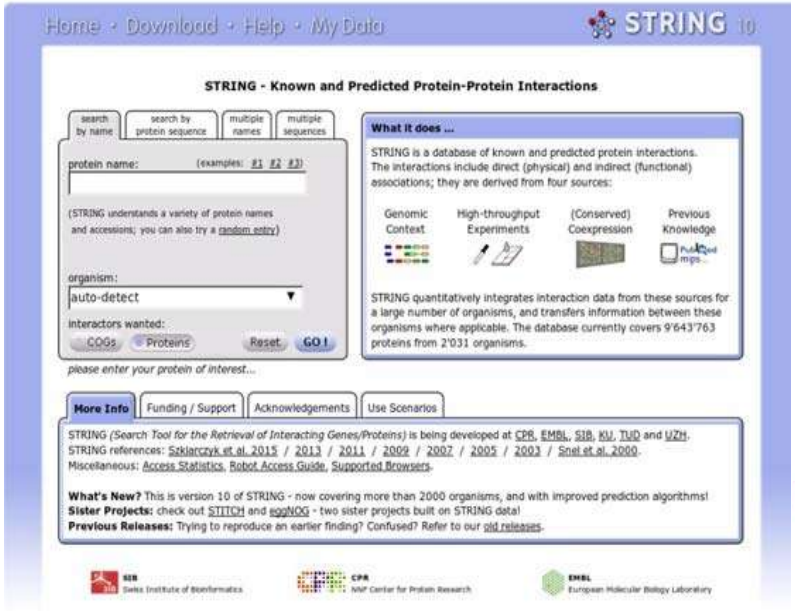
Molecular Function

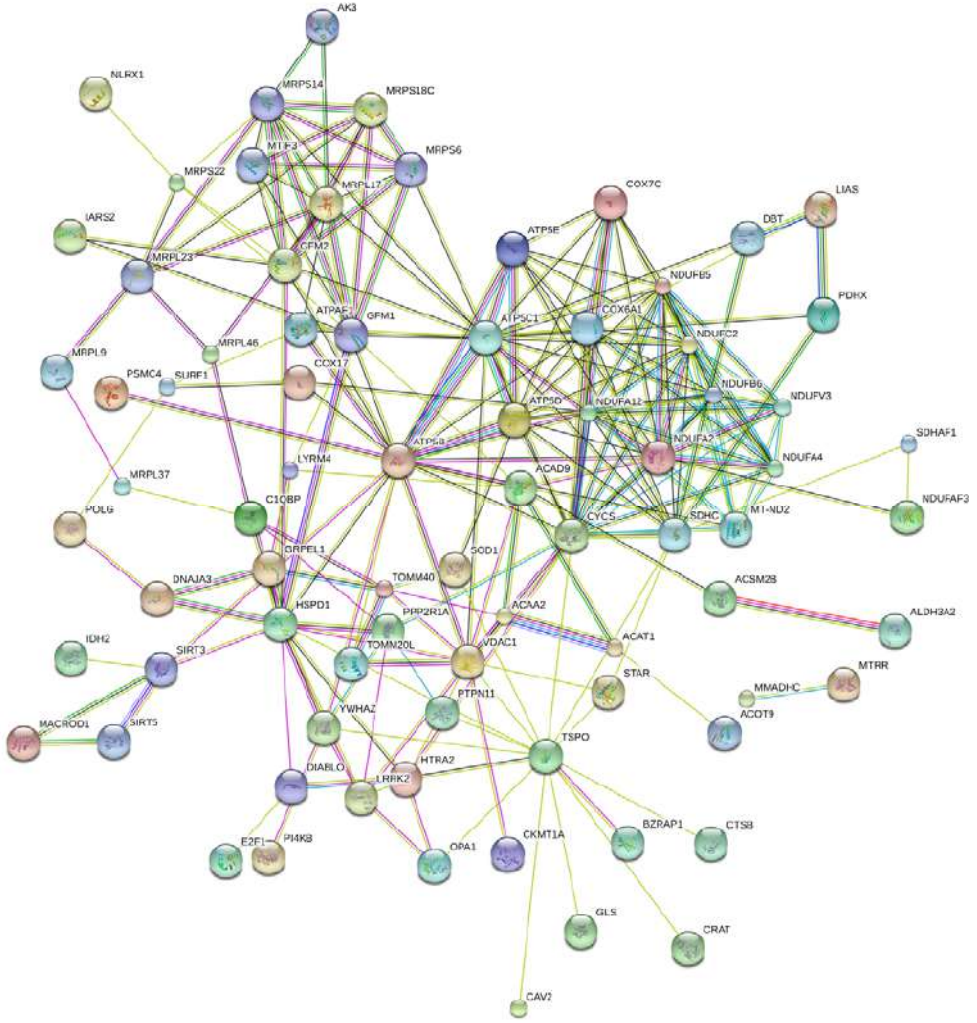


Biological Process

Network Analysis

STRING **Functional protein association networks**



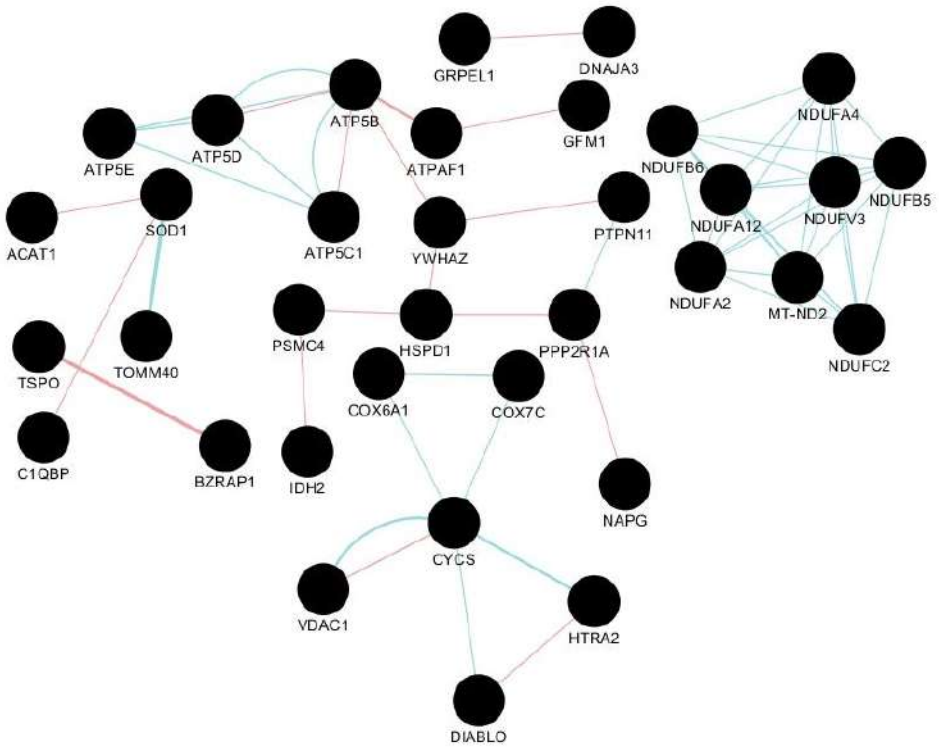


Network Analysis

Cytoscape

Pathway

Physical interactions

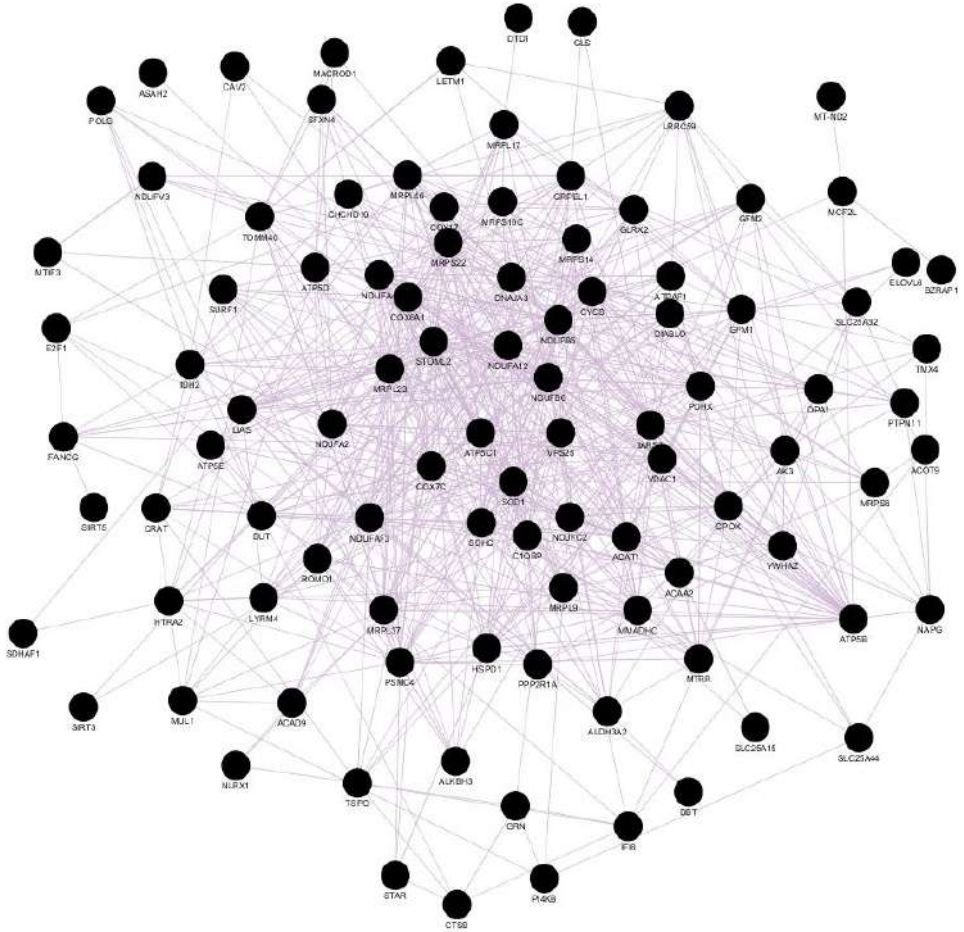


Top 20 Degree	
Gene	**Degree**
ATP5B	7.0
NDUFC2	7.0
NDUFA4	7.0
NDUFB5	7.0
NDUFA2	7.0
NDUFA12	7.0
NDUFB6	7.0
NDUFV3	7.0
MT-ND2	7.0
CYCS	6.0
ATP5D	4.0
ATP5C1	4.0
YWHAZ	3.0
HSPD1	3.0
PPP2R1A	3.0
SOD1	3.0
ATP5E	3.0
ATPAF1	2.0
PSMC4	2.0
PTPN11	2.0

Top 20 Betweenness	
Gene	**Betweenness**
ATP5B	82.0
YWHAZ	75.0
HSPD1	54.0
PPP2R1A	25.0
ATPAF1	22.0
PSMC4	22.0
CYCS	16.0
PTPN11	14.0
SOD1	6.0
NDUFC2	0.0
NDUFA4	0.0
NDUFB5	0.0
NDUFA2	0.0
NDUFA12	0.0
NDUFB6	0.0
NDUFV3	0.0
MT-ND2	0.0
ATP5D	0.0
ATP5C1	0.0
ATP5E	0.0

Network Analysis

Co-expression



Top 20 Degreee	
Gene	**Degree**
ATP5B	43.0
STOML2	42.0
NDUFB5	42.0
NDUFA12	38.0
IARS2	37.0
DNAJA3	36.0
SOD1	34.0
NDUFAF3	32.0
PSMC4	31.0
COX7C	30.0
C1QBP	30.0
SDHC	29.0
ATP5C1	29.0
MRPL23	29.0
NDUFA4	29.0
NDUFB6	27.0
VDAC1	27.0
PDHX	26.0
COX6A1	25.0
GFM1	25.0

Top 20 Betweenness	
Gene	**Betweenness**
STOML2	742.57
IARS2	626.61
DNAJA3	616.75
ATP5B	600.89
NDUFB5	548.26
VPS25	427.44
MCF2L	386.00
NDUFAF3	304.15
PSMC4	301.72
NDUFA12	291.77
MRPL23	280.68
C1QBP	257.33
MRPL17	253.24
MRPL9	237.65
GFM1	226.95
SDHC	188.58
NDUFB6	183.65
GRPEL1	183.33
PDHX	173.26
MRPL46	171.12

Translational Neurology based on Personalized Medicine

Autologous *in vitro* screening for therapeutic targets

Molecular Analyses – transformed Motor Neurons

- Molecular modeling,
- Gene expression networks,
- Protein Interactomes, etc

- Algorithm to reinforce individual patient data in a defined context

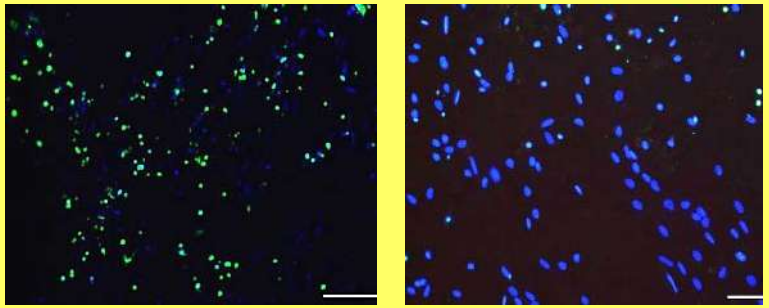
Selected Molecules

Molecular Analyses - Schwann Cells

Therapeutic tests

DEATH (TUNEL, green)

Transf MN from ALS (spor) fibroblasts



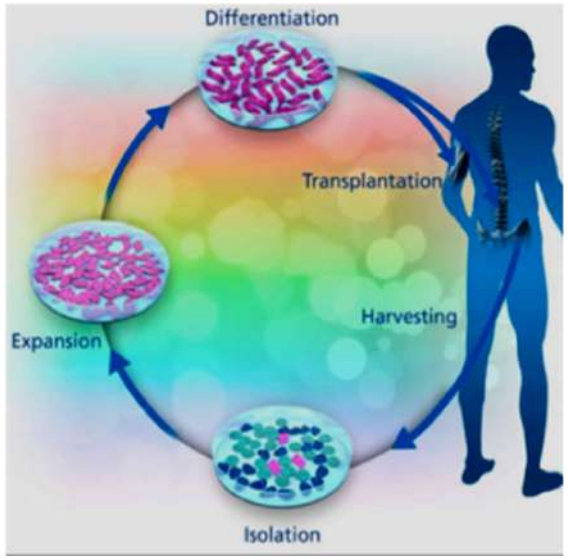
Factor (-) Factor (+)

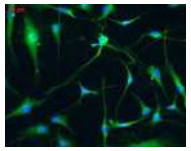
Clinical Trial

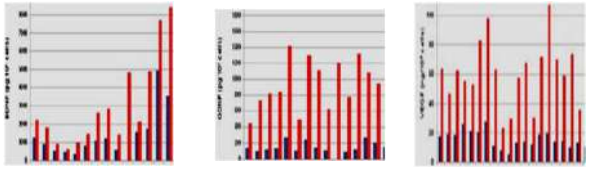
Phase 1/2 CLINICAL TRIAL WITH MESENCHYMAL STEM CELL TO BRAZILIAN ALS PATIENTS

-sponsored by Ministry of Health

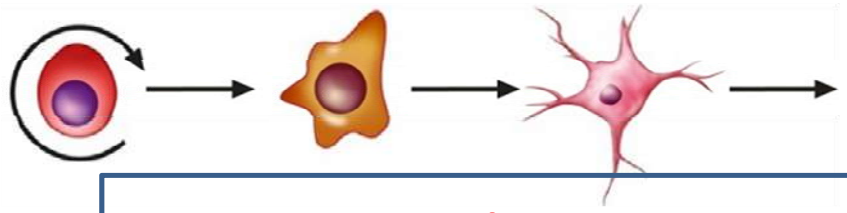
-FMUSP, UNIFESP, PUC-PR







GDNF BDNF VEGF

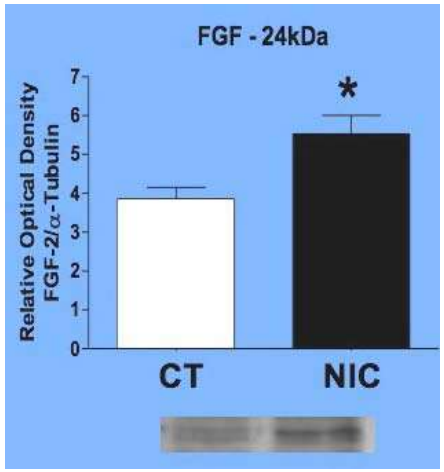


ALS

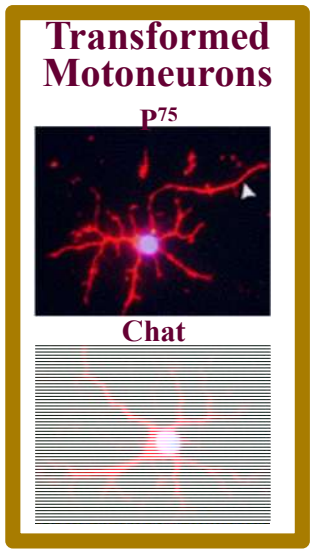
Stimulus *In vitro*

Clinical

Pre Clinical



FGF-2



University of São Paulo

USP Medical School – Clinics Hospital /Institutes








ALS Brazil Project

www.projetoelabrasil.com.br

Neuroregeneration Center

Department of Neurology. FMUSP

Gerson Chadi
Dagoberto Callegaro
Frederico M de Haidar Jorge
Rafael Carra
Jessica Ruivo Maximino
Samantha Nakamura dos Santos
Welinson Paiva
Joyce Meire Gilio
Gabriela N S Rebelo
Vinícius Iamonti
Erich Fonoff
Amanda Mendes
Maiara Gedo
Juliana Pereira
Edmar Zanoteli
Mirian Akiko Furutani de Oliveira

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Generation of MSC for ALS clinical trial

Collaborators

Prof. José E. Krieger
Prof. Brian E Strauss
Prof. Roberto S Martins
Dr. Rafael Dariolli

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