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A novel animal model for Amyotrophic Lateral Sclerosis: the SOD1^{G93A} transgenic swine.

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease that may occur in two clinically indistinguishable forms: sporadic (sALS) and familial (fALS), the latter linked to several gene mutations, mostly inheritable in a dominant fashion [*Rosen et al., 1993*]. The disease is characterized by selective and progressive degeneration of upper and lower motor neurons, leading to muscle weakness, atrophy and evolving to complete paralysis that results in patient death in 2 to 5 years after symptoms onset.

Research on ALS has mainly relied so far on experimental rodent models carrying a variety of Cu/Zn Superoxide Dismutase 1 (SOD1) mutations. Currently, the most widely employed model is a transgenic mouse with a glycine to alanine conversion at the 93rd codon (G93A) of the SOD1 gene. These mice reliably reproduce the ALS patients phenotype progression, developing a rapidly progressive motor neuron degeneration. Death occurs about four months after symptoms onset [*Turner & Talbot, 2008*], not reflecting the disease course in human patients. Although the use of these murine models is currently widespread both in clinical trials and in basic research, aimed at a resolution of the pathogenic mechanisms underlying the disease, doubts have been recently raised, from numerous reliable sources [*Schnabel, 2008; Benatar, 2007; Van Den Bosch, 2011; Gordon et al., 2007*] about rodents suitability to faithfully reproduce the human disease. Since human and rodent species differ in life-span, physiology, anatomy and biochemical aspects, data extrapolation has proved to be difficult. As a matter of fact, encouraging results of drug tests in rodents have never been so far successfully translated to humans, and, in some cases, molecules delaying disease progression in transgenic mice, such as minocycline, have resulted even detrimental in ALS patients [*Scott et al., 2008*] also because of the heterogeneity of mouse genetic background [*Schnabel, 2008*].

The scientific community has already accepted swine as an attractive model, alternative to non-human primates, for pharmacological and surgical testing as well as for biomedical research on the basis of its anatomical, physiological and biochemical features that are more closely related to human species than the rodent ones. Furthermore, the prospect of obtaining genetically modified pigs further extended their biomedical potential especially to mimic inherited human diseases [*Bendixen et al., 2010*].

In particular, regarding Central Nervous System (CNS) anatomy, pig brain cortical surface resembles human gyrencephalic neocortex and similarities with the human brain have also been demonstrated for the hippocampus, subcortical and diencephalic nuclei and brainstem structures. Furthermore, pig brain size permits an easy identification of cortical and subcortical structures by conventional imaging techniques and offers invaluable opportunities for microsurgical techniques and intrathecal drugs administration.

Consistently, the swine large size and long lifespan allow to perform numerous and repetitive samplings from the same animal, thus enabling to get a higher amount of data to characterize in detail preclinical and clinical phases. The longer lifespan makes swine also a suitable animal model for long-term evaluation of safety and efficacy of innovative therapies. Moreover cloning techniques are well established in this species, allowing thus to solve problems related to the variability of genetic background.

On this basis, our group has produced by *in vitro* transfection of cultured somatic cells combined with Somatic Cell Nuclear Transfer (SCNT) the first swine ALS model.

To achieve this goal, an ubiquitous $SOD1^{G93A}$ expression vector has been used, which is characterized by the ability to maintain high expression levels through the next generation of pigs [*Brunetti et al., 2008*], wherein the pCAGGS promoter is inserted between two insulators (5' MAR of chicken lysozyme gene) to prevent

silencing effects. This vector was used to transfect primary porcine adult male fibroblasts (PAFs), thus obtaining transgenic cell colonies to use as nuclei donor in SCNT procedures. After SCNT, $SOD1^{G93A}$ embryos were transferred in recipient sows, and four pregnancies developed to term.

Five piglets survived artificial hand raising, weaning, developed normally and reached adulthood. The remaining piglets died within 48–96 h after farrowing due to events commonly reported in commercial herds (i.e. neonatal diarrhoea or pneumonia, etc). Fibroblasts obtained from ear biopsy of living piglets were analyzed by immunocitochemistry (ICC) and revealed the transgenic protein expression. Furthermore Western Blot (WB) and immunohistochemistry (IHC) analysis were performed on dead piglets tissues, that proved to be all positive for the transgenic protein presence. Immunohistochemistry revealed granular mutant protein aggregates in the CNS.

Unlike rodent models that show an extremely high expression transgene level and a rapid disease course [*Turner & Talbot, 2008*], our swine model presents an expression level comparable to that of human patients, where a single allele mutation results in a toxic gain of function. In rodents, the mutant SOD1 expression level for a given mutation determines disease severity, higher levels yielding a more aggressive phenotype [*Bento-Abreu et al., 2010*]. However, since a $SOD1^{G93A}$ swine model has never been produced before, no data are available about the correlation between transgene expression level and disease onset timing and we can only make rough estimates as to when the first neurological symptoms may occur. Piglets are expected to show ALS symptoms in two or three years, while we cannot exclude a longer period. On one hand this could result in a longer pre-clinical phase and in an increase of animal maintaining costs, on the other hand our $SOD1^{G93A}$ swine could represent an invaluable opportunity to find early biomarkers and a closer and more faithful model to reproduce human pathology since ALS is typically an adult-onset disease.

Currently, an animal model recapitulating all the ALS crucial aspects has not yet been produced, although some transgenic mouse lines modulate a relatively faithful subset of disease features. However, since increasing difficulties are emerging in translating information gleaned from rodent models into therapeutic options for ALS patients, there is an urgent need for an intermediate research system.

I do believe that a swine model could provide this essential bridge between insights gained from rodent models and the reality of treating a human disease.

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I dedicate this thesis to all the people who believe in me and bring happiness in my life, and to a special person among them.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AHXR	Acute humoral xenograft rejection
ALS	Amyotrophic Lateral Sclerosis
ALS2	Alsin gene
ALT	Alanine Transaminase
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-l) propanoic acid
ANG	Angiogenin gene
APP	amyloid precursor protein gene
AST	Aspartate transaminase
βFGF	Fibroblast growth factor β
BSA	Bovine Serum Albumine
CDK5	cyclin-dependent kinase 5
CF	Cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
ChAT	Choline Acetyl Transferase
CMCT	Central motor conduction time
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor gene
Cra	"Cramping 1" mice
CSF	Cerebrospinal fluid
DCTN1	Dynactin gene
DMSO	Dimethyl sulfoxide
DTI	Diffusion Tensor Imaging
DWI	Diffusion Weighted Imaging
EAAT2	Excitatory Amino Acid Transporter
ECG	electrocardiography
eEF1A2	Translational Elongation Factor 2
EEG	electroencephalography
eGFP	Enhanced green fluorescent protein

EMG	Electromyography
eNOS	endothelial nitric oxide synthase
EP	evoked potential
ER	endoplasmic reticulum
ESCs	embryonic stem cells
FAD	familial, autosomal dominant AD
fALS	familiar Amyotrophic Lateral Sclerosis
FBS	Fetal Bovine Serum
FFPE	formalin-fixed/paraffin-embedded
FIG4	PI(3,5)P(2)5-phosphatase gene
fMRI	Functional Magnetic Resonance Imaging
FTDP	Frontotemporal Dementia with parkinsonism
FTLD	Frontotemporal Lobar Degeneration
FUS/TLS	fused in sarcoma/translocated in liposarcoma
GARP	Golgi-associated retrograde protein
GEFs	Guanine Exchange Factors
GFP	Green Fluorescent Protein
GGTA1	α1,3-galactosyltransferase
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
HAR	Hyperacute rejection
hCG	Human chorionic gonadotropin
hDAF	human-decay accelerating factor
HLA	human leukocyte antigen
HUVEC	Human Umbilical Vein Endothelial Cell
ICC	Immunocytochemistry
ICSI	Intra-cytoplasmatic sperm injection
IF	Immunofluorescence
IGHMBP2	immunoglobulin μ-binding protein 2
IHC	Immunohistochemistry
LC-MS/MS	liquid chromatography-tandem mass spectrometry

LDH	Lactic Acid Dehydrogenase
LMNs	Lower Motor Neurons
Loa	"Legs at odd angles" mice
MARs	Matrix Attachment Regions
MND	Motor Neuron Disease
MS	Mass Spectrometry
MRI	Magnetic Resonance Imaging
MUNE	Motor unit number estimation
NFH	gene coding the neurofilament heavy subunit
NFs	Neurofilaments
NLS	Nuclear Localization Signal
Nmd	"Neuromuscular degeneration" mouse
NMJ	Neuromuscular junction
NO	Nitric Oxide
NSC-34	Neuroblastoma hybrid cell line
NT	Nuclear Transfer
NTE	neuropathy target esterase protein
OP	Organophosphorous compounds
OPTN	Optineurin
PAFs	Porcine Adult Fibroblast
PB	Phosphate Buffer
PBS	Phosphate buffered saline
pCAGGS	CMV-IE enhancer + chicken beta actin hybrid promoter
PCR	Polymerase Chain Reaction
PERVs	porcine endogenous retroviruses
PET	Positron Emission Tomography
PFA	Paraformaldehyde
Pmn	Progressive motor neuronopathy mouse
PON1,2,3	Paraoxonase enzymes
PRPH	gene encoding for Peripherin
PVDF	polyvinylidene difluoride

RRM1, RRM2	RNA recognition motifs 1 and 2
RT	Room Temperature
sALS	sporadic Amyotrophic Lateral Sclerosis
SCNT	Somatic Cell Nuclear Transfer
SETX	Senataxin Gene
SMA	Spinal muscular atrophy
SMARD	spinal muscular atrophy with respiratory distress
SMN (1,2)	survival motor neuron genes.
SOD1	Cu/Zn superoxide dismutase 1
$SOD1^{G93A}$	glycine to alanine conversion at the 93^{rd} codon of the SOD1 gene
SPECT	Single-photon emission computed tomography
SPG11	Spatacsin
TARDBP	gene coding for TDP-43
TBST	Tris-Buffered Saline and Tween 20
TBPH	ortholog of Drosophila TARDBP
TDP-43	TAR-DNA-binding protein 43
TE buffet	Tris/EDTA buffer
TMS	Transcranial magnetic stimulation
UMNs	Upper Motor Neurons
VAMP	Vescicle-associated membrane protein B
VAPB	gene coding for VAMP protein
VEGF	Vascular Endothelial Growth Factor gene
VPS54	Gene mutated in Wobbler mouse encoding for GARP a subunit of the Golgi-associated retrograde protein complex
WB	Western Blot
WFN	World Federation of Neurology Research Group on Motor Neuron Diseases

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THE AMYOTROPHIC LATERAL SCLEROSIS

1.1 OVERALL INTRODUCTION

The existence of clinical conditions characterized by progressive muscular weakness and wasting became clearly recognized by the mid 19th century. In 1869 the famous French neurologist and physician Jean-Martin Charcot, studying the pathological features of this syndrome, described the characteristic alterations of the corticospinal tract and the loss of motor neurons and proposed the term amyotrophic lateral sclerosis [*Charcot & Joffroy, 1869*]. "Amyotrophic" refers to the muscle atrophy, weakness and fasciculation that derive from the degeneration of the motor neurons, whereas "lateral sclerosis" refers to the hardness to palpation of the lateral columns of the spinal cord in autopsy specimens, where gliosis follows degeneration of the corticospinal tracts. [*Rowland & Shneider, 2001*; *Silani et al., 2011*]. The term motor neuron disease (MND) is commonly used in the United Kingdom to indicate the ALS syndrome. In the United States, ALS is often known as Lou Gehrig's disease after the great baseball player who developed this disorder in the 1930s.

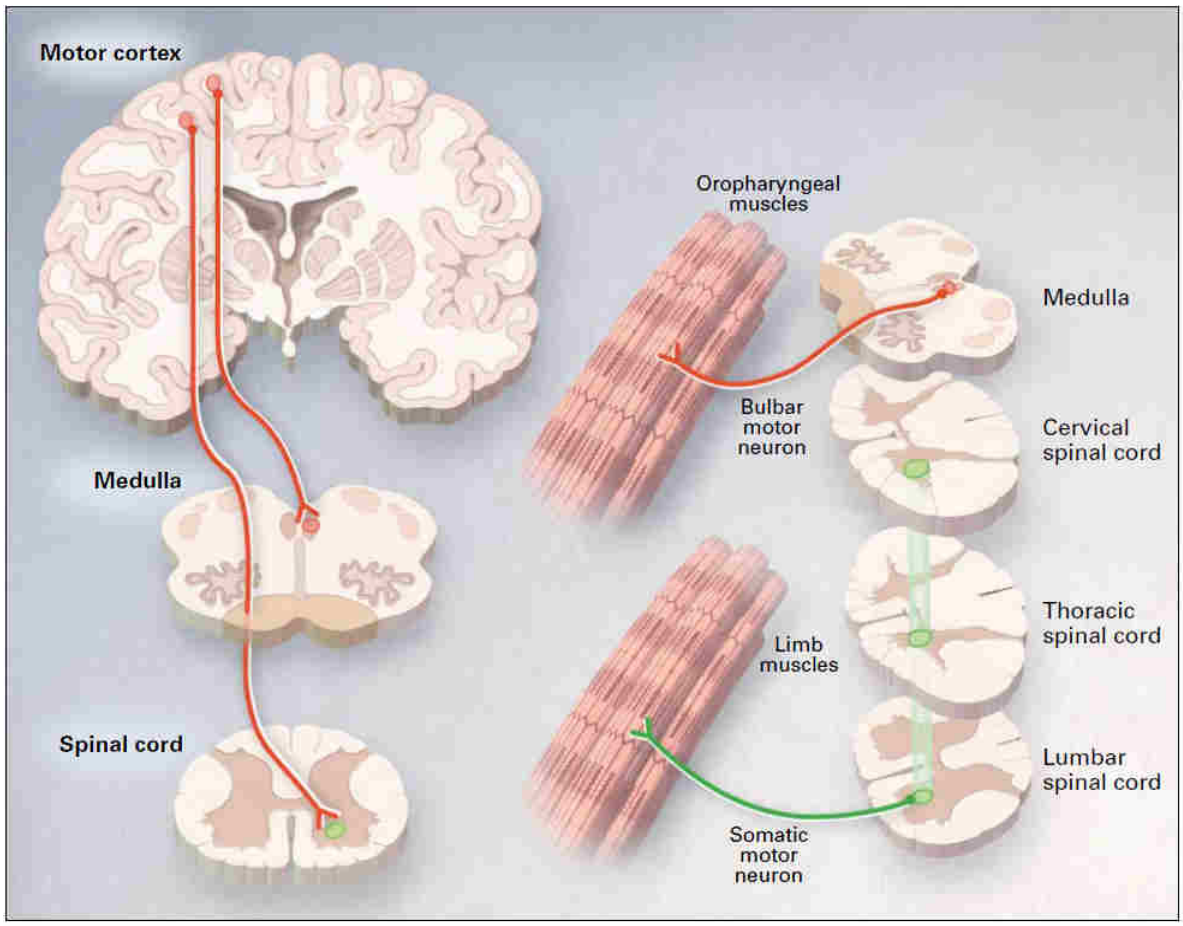
Amyotrophic Lateral Sclerosis is defined as a progressive and fatal neurodegenerative disease resulting from the selective degeneration of upper motor neurons (UMNs) in the motor cortex and lower motor neurons (LMNs) in the brainstem and in the spinal cord (**Figure 1.1**).

UMNs originate in the motor cortex and project to the spinal cord and LMNs connect the brain stem or spinal cord to muscle. When the motor neurons die, the nervous system becomes unable to initiate and control muscle movements. Because muscles no longer receive the input they need in order to function, they gradually weaken and deteriorate, producing deep atrophy.

ALS is the most frequent of the motor neuron disorders, representing 85-90% of cases of this kind of pathologies. The majority of ALS patients present a sporadic form (sporadic ALS, sALS) but in 5-10% of cases the disease is inherited (familial ALS, fALS) [*Mitchell & Borasio, 2007*].

Regrettably, at the moment, no primary therapy for this disorder is available and the only drug approved for its treatment (riluzole) only slightly promotes survival; therefore symptomatic measures are the mainstay of management of ALS.

Figure 1.1: The human Motor Neurons selectively affected in ALS.



Degeneration of motor neurons in the motor cortex leads to clinically apparent signs of upper motor neuron abnormalities: overactive tendon reflexes, Hoffmann signs, Babinski signs, and clonus. Degeneration of motor neurons in the brain stem and spinal cord causes muscle atrophy, weakness, and fasciculation. [Adapted from *Rowland & Shneider, 2001*]

1.2 CLINICAL FEATURES OF ALS

By definition, the features of ALS are signs and symptoms of lower motor neuron dysfunction associated with upper motor neuron dysfunction. LMN involvement determines weakness and fatigue, associated to progressive muscular atrophy, fasciculation (muscular twitching and shaking of contiguous groups of muscle fibers) and fibrillation (muscular twitching and shaking involving individual muscle fibers acting without coordination), reduced muscle tone and absence of tendon reflex. UMN involvement causes weakness, incoordination, stiffness and slowing of movement, with spasticity (persistent contraction of muscle), increased tendon reflexes, clonus (alternating contractions and relaxations) and extensor plantar responses. ALS probably begins a long time before its clinical manifestations, given that a substantial number of motor neurons can be lost before any clinical signs develop. In fact, several studies on animal models have shown that motor neuron dysfunction precedes the onset of symptoms and that compensatory reinnervation from nearby motor neurons permits a good maintenance of the motor function, although with an enlargement of motor units, until more than 50% of motor units have been lost; at this point symptoms appear and the number of motor units declines rapidly [*Côté et al., 1993*; *Kennel et al., 1996*].

No neuropathological, neurophysiological or biochemical markers are yet available to identify a patient as potentially susceptible for ALS prior to symptom onset. The different extent and localization of motor system involvement determines various early clinical features in different patients but ultimately, as the disorder progresses, the clinical expression of ALS is quite uniform, with extreme muscular wasting, spasticity and paralysis.

At the onset, ALS presents with lower motor neuron involvement, upper motor neuron involvement or bulbar involvement. Limb onset is the most frequent, and is found in 75-80% of cases, while bulbar onset is evidenced in only 20- 25%.

The most common initial presentation of ALS is focal asymmetric distal weakness and muscular atrophy. For example, the patient may present himself with a history of unexpected tripping, dragging of a foot and ultimately more diffuse weakness of the leg. Difficulty with buttoning clothes, turning keys in doors or simply poor coordination while performing fine movements are the symptoms of upper limbs involvement. Bulbar motor neuron degeneration leads to difficulty in swallowing (dysphagia) and speaking or forming words (dysarthria), associated to fasciculation of the tongue. Bulbar signs are often closely related to respiratory deficits, due to the involvement of diaphragmatic muscular weakness. This leads to a poor prognosis and to a shorter life expectancy.

With the progression of the illness, the disease spreads to contiguous muscle segments. The progressive loss of motor function results in increasing disability and paralysis, ultimately leading to a bed-bound state. Recent findings have revealed that the selectivity of ALS for the motor system is not absolute. In fact, some reports describe cognitive impairment in a subgroup of ALS patients. A battery of neuropsychological tests has shown a cognitive impairment in about 30% of ALS patients, ranging from mild impairment to frontotemporal lobar degeneration (FTLD).

The main symptoms are executive dysfunction with deficits in verbal and non-verbal fluency and concept formation [*Lomen-Hoerth et al., 2003*; *Kilani et al., 2004*; *Abrahams et al., 2005*; *Schreiber et al., 2005*; *Rippon et al., 2006*].

However, in the majority of the cases, the rate of cognitive decline is very slow as compared to the devastating motor deterioration. Respiratory deficits, due to the progressive atrophy of respiratory muscles, appear during the progression of the disease. When respiratory muscle activity is insufficient, patients need to use mechanised ventilatory support. Respiratory failure finally leads to the death of almost all ALS patients without mechanised assistance. In 50% of cases the death occurs within three years from diagnosis. **Table 1.1** includes all the main symptoms of the disorder.

Table 1.1: Symptoms attributable to ALS

Direct (owing to motor neuronal degeneration)	**Indirect** (as a results of primary symptoms)
• Weakness and atrophy	• Psychological disturbances
• Fasciculation and muscle cramps	• Sleep disturbances
• Spasticity	• Constipation
• Dysarthria	• Drooling
• Dysphagia	• Thick mucous secretions
• Dyspnoea	• Symptoms of chronic hypoventilation
• Emotional lability	• Pain

1.3 DIAGNOSTIC METHODS FOR ALS

Diagnosis of ALS is often critical, since many different clinical conditions may resemble this disease. To date, no test or specific marker can provide a definitive diagnosis of ALS; thus, it is primarily based on the symptoms and signs that the physician observes in the patient and on a series of tests to rule out other diseases.

Implication of UMN and LMN signs that cannot be explained by any other disease together with a neurodegenerative process progression is suggestive of ALS.

By The World Federation of Neurology (WFN) Research Group on Motor Neuron Diseases, criteria for clinical and pathological diagnosis have been defined during the ALS meeting held in El Escorial, Spain, in 1994 [*Brooks, 1994*] and updated at Airlie House, Virginia, four years later.

Based on these criteria patients can be classified into "Clinically definite", "Clinically probable", "Clinically probable - Laboratory supported" and "Clinically possible" categories. **Table 1.2** shows the essential features of the revised criteria. The El Escorial scale classifies the probability of a patient having ALS according to the degree of clinical certainty in relation to other pathologies. It includes four degrees of certainty for the diagnosis of ALS.

Physical and neurological examination could detect UMN and LMN clinical signs in four regions (brainstem, cervical, thoracic or lumbosacral spinal cord) of CNS. The time required to confirm with certainty an ALS diagnosis from first symptoms is about 1 year [*Chio & Silani, 2001*; *Borasio et al., 2001*].

Table 1.2: Summary of revisited El Escorial criteria

Definite
Lower motor neuron and upper motor neuron signs in three regions
Probable
Lower motor neuron and upper motor neuron signs in two regions
Probable with laboratory support
Lower motor neuron and upper motor neuron signs in one region or upper motor neuron signs in one or more regions with EMG evidence of acute denervation in two or more limbs
Possible
Lower motor neuron and upper motor neuron signs in one region
Suspected
Lower motor neuron signs only in one or more regions or upper motor neuron signs only in one or more regions
All categories need evidence of disease progression and absence of sensory signs not explicable on the basis of comorbidity.

1.3.1 Electrophysiological Studies

Electrophysiological studies primarily have the function of identifying LMN impairment and secondarily to exclude other pathologies. The revised El-Escorial criteria [*Brooks et al., 2000*] have proposed electrophysiological criteria for the diagnosis of ALS.

By nerve conduction studies it is possible to define and exclude other peripheral nerve, neuromuscular junction or muscle disorders that may mimic ALS. F-wave studies are particularly useful in assessing proximal conduction and abnormalities that have been reported in ALS. To support ALS diagnosis is essential to provides by Concentric needle electromyography (EMG) evidence of LMN dysfunction in

at least two of the four regions of CNS: brainstem (bulbar/cranial motor neurons), cervical, thoracic, or lumbosacral spinal cord. The revised El-Escorial criteria delineate EMG evidence required for ALS diagnosis: signs of active or ongoing denervation and chronic partial denervation are needed, although relative proportions vary from muscle to muscle [*Brooks et al., 2000*]. Transcranial magnetic stimulation (TMS) allows a non invasive evaluation of the corticospinal motor pathways, and allows detection of UMN lesions in patients who lack UMN signs. Central motor conduction time (CMCT) should be marginally prolonged to muscles of at least one extremity in ALS patients. Motor unit number estimation (MUNE) is a electrophysiological method that can provide an assessment of axons number innervating a muscle or group of muscles, that may have role in ALS progressive motor axon loss assessment [*Bromberg & Brownell, 2008*].

1.3.2 Neuroimaging Studies

In ALS diagnosis , the most important use of neuroimaging is to exclude treatable structural lesion that mimics ALS. Imaging studies are not required in cases of clinically definite disease with bulbar or pseudobulbar onset [*Brooks et al., 2000*]. Magnetic resonance imaging (MRI) can be used in revealing ALS lesions in the corticospinal tracts. The most characteristic finding in ALS is hyperintensity of the corticospinal tracts that is best visualised in the brain and brainstem [*Goodin et al., 1988; Thorpe et al., 1996; Abe et al., 1997; Waragai, 1997*]. Advanced neuroimaging modalities such as magnetic resonance spectroscopy, diffusion weighted imaging (DWI), diffusion tensor imaging (DTI), magnetic resonance voxel-based morphometry and functional imaging techniques (fMRI, PET and SPECT) do not have a use in routine diagnostics, but allowed to obtain promising results in the in vivo disease pathophysiology understanding, which may be realized in disease progression biomarkers identification and earlier diagnosis [*Ellis et al., 1998, 1999*; *Turner & Leigh, 2000*; *Kalra & Arnold, 2003*; *Turner et al., 2004, 2009*].

1.3.3 Muscle biopsy and other laboratory studies

For ALS diagnosis, skeletal muscle or other tissues biopsy is not required, but may be used to demonstrate LMN dysfunction when clinical or electrophysiological findings do not support this evidence. There are some laboratory tests that can be considered mandatory in the course of an ALS diagnosis, since they can be altered during this disease and include [*Brooks et al., 2000*]:

– muscle enzymes (serum creatine kinase [unusual above ten times upper limit of normal], AST (Aspartate transaminase), ALT (Alanine Transaminase), LDH (Lactic Acid Dehydrogenase);

– serum creatinine (related to loss of skeletal muscle mass);

– hypochloremia, increased bicarbonate (related to advanced respiratory compromise);

– elevated CSF protein (uncommonly more than 100 mg/dl)

1.4 EPIDEMIOLOGY OF ALS

ALS is considered as a rare disease: it occurs in about 1-3 people per 100,000 per year with a prevalence (number of surviving patients at any given time) of about 5-7 per 100,000. However, its personal and socioeconomic impact is greater and it has been calculated that its lifetime risk is approximately 1 in 1000 [*McGuire et al., 1996*]. The frequency of ALS appears to have been rising moderately over the past 50 years. Although this rise may indicate the increasing effect of some unidentified exogenous factors, more probably it simply reflects the greater life expectancy of the population, which allows longer survival of a subpopulation susceptible to ALS.

The average survival for sporadic ALS patients is approximately 3-5 years after the first symptoms. Onset of ALS in patients younger than 50 years of age is generally associated with a longer survival.

The incidence of ALS increases with age [*Kurtzke, 1991*], with a peak occurring between 55 and 75 years of age; therefore, aging is the most significant risk factor. ALS occurs predominantly in males, with a male to female ratio of 1.4 to 2.5; however, with increasing of age, this difference tends to diminish [*Kurtzke, 1991*]. Environmental risk factors are inconsistently reported in ALS; this may reflect a complex interaction between several environmental risk factors and specific genetic susceptibilities.

People of all races and ethnic backgrounds are affected by ALS and, with the exception of specific endemic areas in the Western Pacific, its worldwide frequency is uniform. Four geographic areas with a high prevalence (approximately 100-150 fold higher than the other regions) of ALS are described [*Kurland & Molgaard, 1982*; *Oyanagi & Wada, 1999*]:

- Guam and Rota islands
- 2 areas in the Kii peninsula
- Irian Jaya (Indonesia)
- Area of the Gulf of Carpentaria (North Australia)

These geographic areas with an apparently higher prevalence of ALS have long been a source of interest to epidemiologists. Although familial ALS is almost indistinguishable from the sporadic form in terms of clinical phenotype, some features differentiate it from an epidemiological point of view [*Mitsumoto et al., 1998*]. The average onset of fALS cases is approximately 47 years, a decade earlier than the sporadic type, and the mean survival is shorter; moreover, it occurs equally in males and females. Finally, in fALS, symptoms more frequently begin in the lower extremities compared to sALS.

1.5 NEUROPATHOLOGY OF ALS

Immunohistochemical and ultrastructural studies performed on post mortem tissues of ALS patients have helped to better describe the neuropathology of the disease. However, post-mortem examinations are conducted on tissues representing the final stage of the pathology; thus the alterations observed reflect a very advanced state of neuronal degeneration and give little information about the triggering events causing the cell death. It is also true that patients have different degrees of neuronal degeneration in various areas of their central nervous system; for example, many patients with aggressive form of bulbar onset ALS at the time of death often have relatively spared motor neurons in the spinal cord.

1.5.1 Motor system pathology

The major pathological features of ALS are:

I. Upper motor neuron abnormalities

II. Myelin pallor in the corticospinal tracts

III. Reduction in both size and number of LMN in the spinal ventral horns and in the bulbar nuclei.

I. UMN involvement

Upper motor neurons are defined as the neurons, localized in the motor cortex, that exert supranuclear control over LMN. The degree of degeneration observable at autopsy in the motor cortex of ALS patients is quite variable and may not be always evident, even in the presence of clear UMN signs. In the most severely affected cases, an evident loss of giant Betz cells in cortical layer 5, associated with an extensive astrogliosis and microgliosis, is reported. Since there are not good markers to distinguish UMN and other pyramidal cell types in the cortex, the identification of the Betz cell is often based on morphological and size criteria. Neuron cell bodies appear atrophied, with shorter fragmented dendrites [*Hammer*

et al., 1979]. Intracellular alterations are rarely identified in the spared Betz cells in classical ALS; occasionally, ubiquitinated neurofilament inclusions are reported. Pathological changes are rarely evident in somatosensory cortex, prefrontal cortex and premotor areas [*Kiernan & Hudson, 1991*].

II. Corticospinal tract alterations

Axonal degeneration of the descending corticospinal tract results in clear demyelination of the tract. As a consequence, the spinal cord of ALS cases shows pallor with myelin stain. Corticospinal fibers also show marked axonal swelling and spheroids [*Chou, 1992*]. An extensive gliosis is present, causing the typical sclerosis of lateral spinal cord tracts.

III. LMN involvement

Loss of large motor neurons localized in the lower brainstem and in the spinal cord is clearly observed at autopsy. Shrinkage and atrophy of the cell body precede neuronal death [*Kiernan & Hudson, 1991*]; the phenomenon is associated with alterations of axon and dendrite structures, which become thinner [*Nakano & Hirano, 1987*].

Certain motor neuron groups, such as those controlling eye movements and the Onuf's nucleus of the sacral spinal cord (that regulates the pelvic floor musculature) are spared by the disease. The remaining motor neurons present several abnormalities, listed below.

- <u>Ubiquitinated inclusions:</u>

Ubiquitin-positive inclusions are frequently found in the susceptible LMN groups of the spinal cord and brainstem of most cases and are defined as skein-like inclusions or Lewy body-like accumulations. The first are a specific hallmark of ALS whereas Lewy body-like inclusions are found also in other disorders. Both inclusions probably represent two different morphological stages of protein aggregation, from diffuse filamentous forms to dense and compact inclusions.

Besides phosphorylated neurofilaments and ubiquitin, Lewy body-like inclusions also contain cyclin-dependent kinase 5 (CDK5) [*Nakamura et al., 1997*]; dorfin, a RING finger-type E3 ubiquitin ligase, has also been found [*Hishikawa et al., 2003*]. Recently, the nuclear factor TDP-43 (TAR-DNA-binding protein 43) has been identified as a major component of ubiquitinated inclusions in sALS cases [*Neumann et al., 2006*]; TDP-43 is thought to function as a regulator of transcription and alternative splicing [*Buratti & Baralle, 2001*; *Buratti et al., 2004*; *Mercado et al., 2005*].

- Bunina bodies:

Bunina bodies are described as small, eosinophilic, irregularly shaped inclusions localized in the soma of motor neurons; probably they have a lysosomal derivation [*Sasaki & Maruyama, 1993*]. At the ultrastructural analysis, they appear like electron-dense, amorphous structures surrounded by vesicles, endoplasmic reticulum (ER) fragments, lipofuscin granules and are shown to contain cystatin C. Bunina bodies are reported to be present in 30-50% of cases; since they are not described in other disorders, they seem to be specific for ALS. Their pathogenesis and their relationship to neurodegeneration have not yet been unravelled.

- Hyaline conglomerate inclusions:

Hyaline conglomerate inclusions consist of large aggregates of phosphorylated and non phosphorylated neurofilaments associated with other "entrapped" cytoplasmic proteins and organelles [*Leigh et al., 1989*; *Sasaki & Maruyama, 1991*]. They have been identified in sporadic and familial ALS; however, they seem to be less specific for this pathology, since they have been found in other neurological disorders [*Sobue et al., 1990*].

- Globules and spheroids:

Phosphorylated neurofilaments are also found packed in axonal swellings in the anterior horns of ALS patients [*Corbo & Hays, 1992*; *Toyoshima et al., 1998*]. Spheroids are larger and tend to be localized in proximal axons and dendrites,

while globules are smaller and usually are more peripheral in the ventral horn. Both are presumed to represent focal abnormalities of axonal cytoskeletal regulation with a failure of axonal transport.

- Diffuse somatic phosphorylation of neurofilaments:

Diffuse accumulation of phosphorylated neurofilaments has been observed in the perikarya of motor neurons, especially in sALS [*Hirano et al., 1984*].

- Golgi fragmentation:

Both UMN and LMN of ALS cases show signs of fragmentation of the Golgi apparatus that is dispersed into numerous small isolated elements [*Mourelatos et al., 1994*; *Fujita & Okamoto, 2005*].

- Mitochondrial alterations:

In recent years, morphological alterations of mitochondria in the motor neurons of ALS patients have been observed. These include dense conglomerates of aggregates, dark mitochondria, swelling and vacuolization [*Hirano et al., 1984*; *Swerdlow et al., 1998*].

IV. Involvement of other cell types

Remarkable morphological and neurochemical evidence demonstrates the proliferation and activation of the microglial and astrocytic populations in the areas characterized by motor neuronal loss [*Kawamata et al., 1992*]. The concept of selective motor neuronal death has been challenged by studies reporting the loss of small neurons in the spinal cord of ALS cases [*Oyanagi et al., 1989*; *Raynor and Shefner, 1994*]. Stephens et al., 2006 report that the morphometric examination of the lumbar spinal cord of sALS patients revealed a substantial loss of ventral interneurons in addition to motor neurons. Therefore, the interneuronal population may degenerate to a similar extent and contemporary with the motor neuronal one.

1.5.2 Non-motor system pathology

Autopsy examination revealed in some patients alterations in extra-motor regions of the CNS, such as in the sensory system [*Hudson, 1981*], substantia nigra and hippocampus [*Wharton, 2003*].

1.5.3 Non-CNS pathology

Profound skeletal muscles atrophy is one of the earliest pathological changes in ALS patients [*Mitsumoto et al., 1998*]. Other alterations are reported in the skin, in which the collagen cross-linking results altered [*Kolde et al., 1996*].

1.6 GENETIC OF ALS

ALS is considered a multifactorial disease, with a complex interaction between genetic and environmental factors. As already explained, the majority of ALS cases are sporadic, while the 10% of patients have a positive familial anamnesis for motor neuron disease, generally with an autosomal-dominant inheritance pattern, although recessive pedigrees have been described [*Mulder et al., 1986*]. To date, 12 gene defects have been reported to cause ALS (**Table 1.3**). The protein products of these mutated genes are:

1.6.1 ALS1 - Cu/Zn Superoxide-Dismutase 1 (SOD1)

The linkage of ALS1 to chromosome 21q22.1 was described in 1991 by Siddique et al., and two years later, Rosen et al. (1993) described eleven disease-associated mutations in the SOD1 gene, spanning 9.3 kb, that is composed of five exons and encodes for a 153 amino acid forming the Cu/Zn superoxide dismutase 1 enzyme. This is the most common form of inherited ALS, accounting for about 20% of all familial ALS forms and corresponding to 2-3% of all ALS cases.

The SOD1 is a highly conserved, ubiquitously expressed, cytoplasmic proteins, that converts superoxide, produced as a by-product of

oxidative phosphorylation, to oxygen and hydrogen peroxide; it is very abundant, representing up to 2% of the soluble proteins of the brain [*McCord et al., 1969*].

SOD1 is an homodimeric metalloenzyme, composed by two 32 kDa monomer, where each consists of an eight-stranded beta-barrel and binds a copper and a zinc ion [*Getzoff et al., 1989*]. To date, more than 140 different SOD1 mutations have been identified in SOD1 gene and are localized in all the five exons, with no region of the polypeptide escaping from disease-causing mutations; 114 of them result in amino acid substitution and are known to cause the disease, whereas six silent mutations and five intronic variants do not. Although most mutations are missense, 12 are nonsense or deletion mutations that produce a truncated protein [*Andersen et al., 2003*].

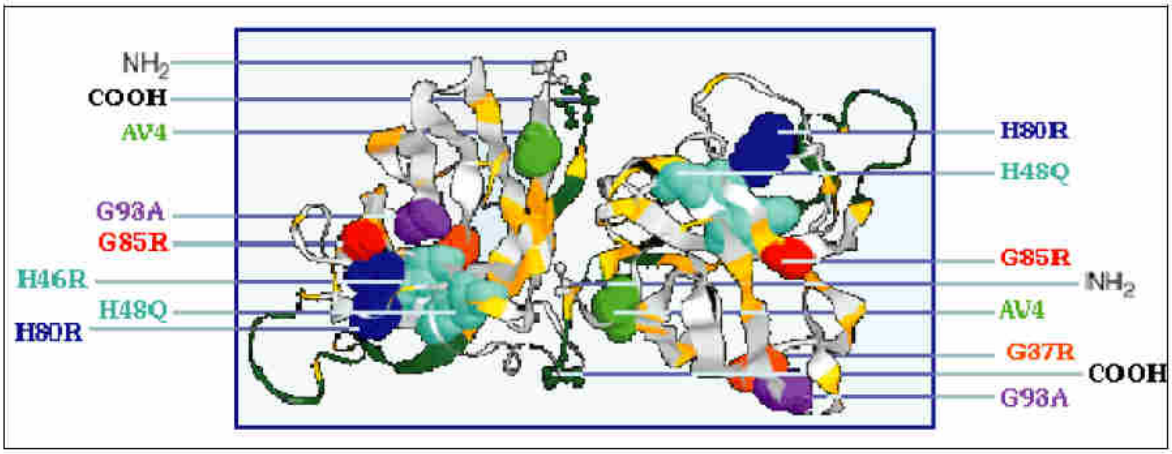
Table 1.3: Genes implicated in Familial ALS.

Type	Onset	Pattern	Linkage	Gene	Protein
ALS1	Adult	AD/AR*	21q22.1	*SOD1*	Cu/Zn superoxide dismutase
ALS2	Juvenile	AR	2q33-35	*ALS2*	Alsin
ALS3	Adult	AD	18q21	unknown	
ALS4	Juvenile	AD	9q34	*SETX*	Senataxin
ALS5	Juvenile	AD/AR*	15q15-21	*SPG11*	Spatacsin
ALS6	Adult	AD**	16p11.2-q21	*FUS/TLS*	Fused in sarcoma
ALS7	Adult	AD	20p13	unknown	
ALS8	Adult	AD	20q13.33	*VAPB*	VAMP-associatated protein B
ALS9	Adult	AD	14q11	*ANG*	Angiogenin
ALS10	Adult	AD	1q36	*TARDBP*	TAR DNA-binding protein
ALS11	Adult	AD	6q21	*FIG4*	PI(3,5)P(2)5-fosfatase
ALS12	Adult	AD/AR	10p15-p14	*OPTN*	Optineurin
ALS	Adult	AD/AR	7q21.3-q22.1	*PON1-2-3*	Paroxonase
ALS	Adult	AD	2p13	*DCTN1*	Dinactin
ALS	Adult	AD	9p13-p12	*VCP*	Valosin-containing protein

AD = autosomic-dominant; AR = autosomic-recessive. * Mutation p.D90A is transmitted AR in the main number of families. ****** H517Q mutation is trasmitted AR

Figure 1.2 shows human SOD1 three-dimensional structure and some mutations that cause ALS. All mutations are associated with autosomal dominant fALS, except for D90A and D96N, which can cause both dominant and recessive ALS [*Andersen et al., 1995*; *Robberecht et al., 1996*; *Orrell, 2000*]. The most frequent SOD1 mutation is A4V. Penetrance, clinical manifestations, age of onset, disease progression and survival vary greatly among specific mutations. For example, A4V and A4T are associated with an aggressive fALS type, [*Aksoy et al., 2003*], while slow progression over 10-15 years is reported for mutations such as G37R, D90A, G93C and G93V [*Arisato et al., 2003*].

Figure 1.2: Position of some mutations in the 3D structure of human SOD1.



To date, there is no certainty about the mechanisms by which SOD1 gene mutations cause ALS. It was initially proposed that the toxicity of mutated SOD1 was associated with the loss of superoxide dismutase activity, thus resulting in increased cellular levels of reactive oxygen species, oxidative stress, and neuronal death [*Deng et al., 1993*]. However, most of the mutated isoforms of the SOD1 gene retain their enzymatic activity; therefore it is impossible to establish a correlation between residual enzyme activity, clinical progression, and disease phenotype [*Radunović et al., 1997*].

The creation of transgenic mice expressing SOD1 with some of the mutations found in the human patients, which develop a motor syndrome similar to human ALS, showed that the catalytic activity is unchanged or elevated [*Gurney et al., 1994*; *Ripps et al., 1995*; *Wong, 1995a*; *Bruijn et al., 1997*). Furthermore, SOD1 knockout mice do not develop spontaneous motor neuron disease [*Reaume et al., 1996*]. Also, the expression of mutant SOD1 alleles in cell culture models induces apoptosis in neurons [*Pasinelli et al., 1998*]. In both in vitro and in vivo models, dismutase activity appears to be normal or elevated, suggesting the conclusion that SOD1 mutants acquire one or more toxic properties, irrespective of the amount of superoxide dismutase activity that each of them retains. The SOD1 propensity to misfolding to form cytoplasmic aggregates has been demonstrated by many studies. For their part, aggregates may result in cell death through sequestering other proteins essential for neuronal survival, by ubiquitin / proteasome system blockage, by chaperones depletion, by disrupting mitochondria and cytoskeleton and / or interrupting the axonal transport.

1.6.2 ALS2 - Alsin

A new gene linked to a rare, recessively inherited form of ALS characterized by juvenile onset and slow progression has been identified in 2001 [*Hadano et al., 2001*; *Yang et al., 2001*]. ALS2 variant is characterized by the predominance of facial and limb muscles spasticity.

The disease locus was mapped on chromosome 2q33-35 [*Hentati et al., 1994*], encodes a 184 KDa protein named alsin, spans 83 kb of genomic DNA and is composed of 34 exons. At least 13 different ALS2 mutations have been described so far, the majority of which are frameshift deletions resulting in a prematurely truncated protein, or nonsense mutations.

Alsin is a ubiquitously expressed protein, particularly abundant in neurons where it is localized to the cytosolic portion of endosomal membrane. The

aminoterminal region of alsin contains sequence motifs that are characteristic of guanine exchange factors (GEFs), involved in the recycling of a specific small G protein from its GDP-bound state to its GTP-state. The carboxyterminal half of alsin contains two further domains, which are similar to those of the Rho G-protein family that modulates dynamic actin assembly.

The function of alsin is not fully understood, but it is known that it acts as exchange factor for the small GTPase Rab5a in vitro [*Otomo et al., 2003*; *Topp et al., 2004*], suggesting a possible involvement in the organization of the cytoskeleton and in vesicle trafficking. In addition, alsin can interact with the small Rho GTPase Rac1 [*Topp et al., 2004*; *Tudor et al., 2005*].

Interestingly, alsin is also able to specifically bind to different mutant variants of SOD1 through the RhoGEF domain [*Kanekura et al., 2004*] and, in a cell culture paradigm, it has been shown to suppress mutant SOD1 toxicity. All the disease-causing alsin truncation mutations are highly unstable [*Hadano et al., 2001*]; this has led to the hypothesis that the disease is caused by loss of activity of the protein. However ALS2 knockout mice do not develop motor neuron disease [*Cai et al., 2005*].

1.6.3 ALS4 - Senataxin (SETX)

ALS4 is a rare slow progressive motor neuron disease, characterized from autosomal-recessive inheritance and juvenile onset.

ALS4 was initially mapped on chromosome 9q34, and was later discovered to be caused by mutations in SETX gene [*Chen et al., 2004*]. The gene is composed of 26 exons and encodes for the 303 kDa ubiquitously expressed protein senataxin.

Senataxin contains a DNA/RNA helicase domain with high homology to human RENT1 and IGHMBP2 (immunuglobulin-μ binding protein 2), two proteins known to have roles in RNA processing. Missense mutations in the SETX gene lead to a distal limb atrophy and to a motor neurons loss so slow that the life span is not reduced.

1.6.4 ALS5 - Spatacsin

ALS5 is an autosomal-recessive, juvenile-onset motor neuron disease characterized by distal muscle atrophy and weakness with bulbar muscles involvement. The disease has been mapped to a locus on chromosome 15q15- 21 [*Hentati et al., 1998*].

The identified variants are mostly frameshift mutations or nonsense substitutions. SPG11 gene, composed of 40 exons, encode for the protein spatacsin that contains four putative transmembrane domains, suggesting that spatacsin may be a receptor or a transporter. However, its physiological role is still unknown, although it is reputed to be involved in axonal transport [*Salinas et al., 2008*].

1.6.5 ALS6 - Fused in sarcoma / Traslocated in sarcoma

The ALS6 locus is mapped on chromosome 16p12.1-q21 on pedigrees with autosomal dominant ALS [*Abalkhail et al., 2003*; *Ruddy et al., 2003*; *Sapp et al., 2003*]. Recently novel variants in the FUS/TLS gene have been identified as the disease causing mutations [*Kwiatkowski et al., 2009*; *Vance et al., 2009*]. The overall mutational frequency is ~4% in fALS and ~1% in sALS [*Chio et al., 2009*; *Corrado et al., 2010*; *Bäumer et al., 2010*; *Hewitt et al., 2010*; *Millecamps et al., 2010*; *Rademakers et al., 2010*; *Robertson et al., 2011*; *Waibel et al., 2010*; *Yan et al., 2010*]. More than 30 different mutations have been hitherto described, which are mostly missense substitutions and the rest are frameshift or nonsense mutations.

The FUS gene, composed of 15 exons for a total of 9 kb, encodes a DNA / RNA binding protein which is normally found in the cell nucleus and is involved in cellular processes, such as genomic stability maintenance and transcription and splicing regulation [*Law et al., 2006*]. In CNS, upon the activation of glutamate receptors, FUS is involved in regulating mRNA transport towards the dendrites [*Fujii et al., 2005*]. The FUS protein is composed of an N-terminal transactivating domain, a central domain that contains both a RNA recognition

motif and a zinc finger motif, and a C-terminal region [*Morohoshi et al., 1998*], that contain the nuclear localization signal (NLS) [*Zakaryan et al., 2006*], where the majority of the identified mutations is clustered.

Neuropathological examinations of patient's tissues, harbouring FUS/TLS mutations, showed an increased cytoplasmic FUS staining, and FUS-immunoreactive cytoplasmic inclusions in LMN [*Kwiatkowski et al., 2009*; *Vance et al., 2009*]. Moreover, also data gained from in vitro experiments support the evidence that the FUS cytoplasmic redistribution is a key event in inclusion formation and motor neuron toxicity: cell lines transfection with GFP-FUS mutant fusion protein constructs resulted in increased cytoplasmic localization compared to transfection experiments with wild-type FUS vectors. Thus, it has been hypothesized that FUS mutations may contribute to ALS pathogenesis through the formation of cytoplasmic inclusions and/ or the loss of the physiological nuclear functions of the protein.

1.6.6 ALS8 - VAMP-associated protein B

A single P56S mutation in the VAPB gene, encoding for the VAMP (Vescicle-associated membrane protein B) causes ALS8. The VAPB gene is composed of six exons and spans 57.7 kb.

The VAMP is a ubiquitously expressed homodimer protein, belonging to the family of intracellular vesicle-associated/membrane-bound proteins that can associate with microtubules and, presumably, regulates vesicle transport. In particular, VAPB has been shown to act during the transport through the endoplasmic reticulum, Golgi apparatus and secretion. The P56S mutation dramatically disrupts the sub-cellular distribution of VAPB and induces the formation of intracellular protein aggregates [*Nishimura et al., 2004*]. The P56S-VAPB forms insoluble cytoplasmic inclusions in neural and non-neural cell lines, by sequestering wt-VAPB [*Suzuki et al., 2009*]. Supporting this hypothesis is the observation that the silencing of the VAPB homologous gene in Drosophila

melanogaster leads to larvae progressive paralysis by cytoplasmic inclusions formation and subsequent neurodegeneration. The phenotype is rescued by overexpressing the human VAPB gene [*Chai et al., 2008*].

1.6.7 ALS9 – Angiogenin

The hypothesis that some angiogenic factors may be involved in ALS pathogenesis is increasingly evident. Firstly is reported an association between two vascular endothelial growth factor gene (VEGF) promoter haplotypes and susceptibility to sALS. Moreover the down-regulation of VEGF expression leads to progressive motor neuron degeneration in mice [*Lambrechts et al., 2003*]. On the contrary, VEGF administration has neuroprotective effects and improves $hSOD^{G93A}$ transgenic mice disease phenotype [*Azzouz et al., 2004*]. In 2004, a single point mutation in the ANG gene, encoding for a VEGF effector named angiogenin has been associated to sALS susceptibility [*Greenway et al., 2004, 2006*]. The ANG gene is located on chromosome 14q11 and is composed of two exons, of which only one is encoding, for a total length of 5.4 kb.

Angiogenin protein is a member of the pancreatic ribonuclease superfamily and is mainly expressed in hepatocytes. After its secretion into the serum and the extracellular matrix, through uptaking by still unidentified endothelial receptors, angiogenin is translocated into the nucleolus where it stimulates tRNA transcription and protein translation [*Moroianu & Riordan, 1994*]. The majority of the 15 mutations hitherto described is clustered in the catalytic site or into the nuclear import signal and are consequently predicted to disrupt angiogenin secretion, ribonucleolytic activity and/or nuclear translocation, consequently producing angiogenesis alteration [*Wu et al., 2007*]. Thus, the loss of angiogenin functions may lead to motor neuron degeneration. Moreover, the mutant protein damages neurite growth and appears to be toxic on motor neurons in vitro [*Subramanian et al., 2007*]. Lastly, administration of human recombinant angiogenin prolongs $hSOD1^{G93A}$ mice lifespan [*Sebastia et al., 2009*].

1.6.8 ALS10 - TDP-43

In 2006 by Neumann et al. was identified the 43-kDa TAR-DNA binding protein as the main component of ubiquitinated cytoplasmic inclusions in ALS where is hyperphosphorylated and cleaved to generate abnormal C-terminal fragments. In unaffected neurons TDP-43 is localized in the cell nucleus and, instead, is absent from the nuclei of neurons with ubiquitinated inclusions; this phenomenon suggests a variation of the protein distribution between the nucleus and cytoplasm. TDP-43 is a 414 amino-acids, multifunctional DNA/RNA binding protein, ubiquitously expressed in the cells nucleus, and composed of two highly conserved RNA recognition motifs (RRM1 and RRM2), flanked by an N-terminal domain and a C-terminal tail, containing a glycine-rich region involved in others hnRNPs interactions [*Buratti et al., 2005*]. The TDP-43 specific functions in neurons is still unclair, although it seems to play a role in several biological processes, such gene transcription, splicing regulation, transport and stabilization of mRNA molecules [*Buratti & Baralle, 2008*].

To elucidate the TDP-43 pathogenic role in ALS are raised intense speculations: toxicity might be caused by TDP-43 aggregates that is sequestered away from its normal nuclear function or, conversely, that might have a independent toxic gain-of-function [*Lagier- Tourenne et al., 2010*; *Strong, 2010*; *Ticozzi et al., 2010*; *van Blitterswijk et al., 2010*].

In 2008, Gitcho et al. and Sreedharan et al. independently reported that pathogenic mutations in the encoding TDP-43 gene, named TARDBP and located in chromosome 1, cause several neurodegenerative diseases such as fALS, fALS, and FTLD. The proposed mutational frequency is ~5% for fALS and 0.5-2% for sALS. To date, more than 30 different missense substitutions in TARDBP gene, all of them clustered in the C-terminal glycine-rich region that is encoded by exon 6. So far was not possible to establish clear genotype-phenotype correlation, since most TARDBP mutations are private.

1.6.9 ALS11 - PI(3,5)P(2)5-phosphatase

In 2009, Chow et al. identified ten ALS-associated heterozygous mutations in the FIG4 gene, including substitutions and frameshift insertions and deletions. FIG4, composed of 23 exons, encodes for a 907-residue long phosphoinositide phosphatise that regulates the synthesis and turnover of phosphatidylinositol- 3,5-bisphosphate, a signalling lipid that mediates the retrograde transport of endosomal vescicles to the trans-Golgi network [*Rutherford et al., 2006*].

1.6.10 Other fALS- associated genes

Mutations in several genes involved in cytoskeletal stability and axonal transport have been suggested to play a role in ALS pathogenesis.

Six deletions within the C-terminal domain of the NFH gene, encoding for the neurofilament heavy subunit, have been found in several sALS and fALS [*Figlewicz et al., 1994*; *Al-Chalabi et al., 1999*].

ALS related mutations was also found in PRPH gene [*Gros-Louis et al., 2004*; *Leung et al., 2004*], encoding for peripherin, a neuronal intermediate filament protein involved in axonal outgrowth, which frequently detected in ALS patients motor neurons ubiquitinated inclusions [*He et al., 2004*].

A single G59S mutation and three additional heterozygous missense mutations have been found in the p150 domain of DCTN1 gene, encoding for dynactin [*Münch et al., 2004*], a component of the dynein complex that is implicated in axonal transport. The mutation, which seems to affect the dynactin-dynein motor microtubules binding, is dominantly transmitted and provokes an atypical motor neuron disorder characterised by vocal cord paresis.

It is also been hypothesized that another group of candidate genes, involved in xenobiotics metabolism, may play a role in fALS pathogenesis. Organophosphorous compounds (OP), widely employed in agricultural and industrial fields, can disrupt the cholinergic transmission in the CNS and at

neuromuscular junctions. The covalent binding of OP to the neuropathy target esterase protein (NTE) leads to axonal degeneration in the spinal cord and peripheral nerves: mutations in the NTE gene have been associated with a progressive motor neuron disease [*Rainier et al., 2008*]. The paraoxonase enzymes (PON1, PON2 and PON3) represent the major detoxifying system for OP and are also involved in protecting cells against oxidative damage. Several studies have suggested an association between PON haplotypes and sALS susceptibility [*Saeed et al., 2006*; *Slowik et al., 2006*; *Morahan et al., 2007*; *Landers et al., 2008*]. A recent study identified eight mutations in the PON cluster in nine fALS and three sALS patients. However, to prove the segregation of these mutations with ALS, further studies will be needed to validate the PON mutations role in ALS pathogenesis.

Tau is a member of the microtubule-associated protein family, which has the principal functions of stabilizing microtubules and promoting their assembly by binding to tubulin. In addition, tau is likely to regulate motor protein-mediated transport of vesicles and organelles along the microtubules by modulating their stability [*Sato-Harada et al., 1996*; *Ebneth et al., 1998*]. In frontotemporal dementia with parkinsonism (FTDP), the mutation of tau gene affects the alternative splicing of exon 10, resulting in an excess of four repeat tau isoforms; this may cause a reduced binding of tau to microtubules in axons. No pure ALS case has been associated with tau mutations.

1.6.11 Susceptibility genes

Susceptibility genes are defined as genes that can potentially contribute to the development of ALS; in other words, mutations in these genes may lead to ALS interacting with other genetic or environmental risk factors.

Among the genetic alterations that may confer a higher predisposition to the development of ALS, have been described mitochondrial DNA micro deletions encoding for cytochrome-C oxidase [*Borthwick et al., 1999*], RNA processing errors in the glutamate transporter EAAT2 (Excitatory Amino

Acid Transporter) [*Lin et al., 1998*], an abnormal copy number of the survival motor neuron gene [*Corcia et al., 2002*] and gene deletions of the chromosome 5q13- linked neuronal apoptosis inhibitory protein gene [*Jackson et al., 1996*]. An increased frequency of the cytochrome P450 debrisoquine hydroxylase allele, encoding a cytochrome P450 monooxygenase involved in drug metabolism and associated with a "poor metabolizer" phenotype, has been also reported [*Siddons et al., 1996*]. Frequently, these alterations were described in only few cases or the results of different studies are conflicting.

Some evidence suggests that VEGF acts as a modifier of ALS in both human and mice. In a study conducted by Oosthuyse et al., ALS–like symptoms and neuropathology were observed in mice bearing a targeted deletion that eliminates the ability of VEGF gene to respond to tissue hypoxia [*Oosthuyse et al., 2001*]. These mice show a normal baseline expression of VEGF, but have a pronounced deficit in the ability to induce it in response to hypoxia. The motor deficits appear between 5 and 7 months of age and gradually progress; various classical features of ALS are observed, such as accumulation of neurofilaments in the motor neurons, degeneration of motor axons and muscle atrophy. As regards to the human pathology, in a large European study three single nucleotide polymorphisms in the promoter region of the VEGF gene have been associated with an overall 1.8–fold increased risk of developing ALS. The promoter variants in the VEGF gene in these patients coincided with reduced levels of plasma VEGF [*Lambrechts et al., 2003*].

Also neurofilament variants are probable modifying risk factors in sporadic ALS and may modulate disease expression. A set of small in-frame deletions or insertions in the repetitive tail domain of the large neurofilament subunit NFH has been identified in about 1% of 1,300 sporadic ALS patients examined [*Al-Chalabi et al., 1999*]. Although the known neurofilament sequence variants are not responsible by themselves of producing disease with high penetrance, it is likely that they are at least important risk factors for apparently

sporadic disease. The studies that link sALS to particular genetic variants so far known account only for a small number of the total cases; the causes could be a complex pattern of inheritance with very low penetrance, a high degree of heterogeneity and/or the existence of environmental factors predisposing to ALS.

In conclusion, high genetic heterogeneity and complex interactions between genetic and environmental factors are the main obstacles in the process of the finding of new ALS genetic determinants.

EXPERIMENTAL MODELS OF ALS

Successful therapies for almost all diseases can only come from the understanding of cellular and molecular mechanisms that underlie their pathophysiology. With the aim to obtain insights into such mechanisms, the use of experimental models is essential. This is particularly true in case of diseases whose etiopathology is still unknown, which precisely ALS is. In general, such studies are carried out in vitro and especially in vivo because animal models reflect more closely the human diseases progression [*Tovar y Romo et al., 2009*].

2.1 IN VITRO MODELS

2.1.1 Spinal cord cultures

To study motoneuron characteristics, spinal cord primary cultures have been widely used [*Ransom et al., 1977*]. In general, because of their large size, motoneurons are relatively easy to identify in plated dissociated tissues, but unfortunately, they are under-represented and this leads to the necessity of using the motor neuron enriched cultures. Motoneuron enrichment could be achieved with cellular densities centrifugation followed by biochemical analyses of acetylcholine production [*Schnaar & Schaffner, 1981*]. Further purification of the motoneuron population can be obtained by flow cytometry.

The disadvantages of modeling a complex disease such as ALS in such a limited system are obvious; in spite of this it was possible to draw some information such as, for example, the motoneurons vulnerability to glutamatergic excitotoxicity trough AMPA (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-l) propanoic acid) receptors [*Carriedo et al., 1996*]. Moreover, this kind of studies have helped to elucidate the role played in ALS pathogenesis by micro and astroglia and other non-neuronal cell types, since accumulating evidence indicates that ALS is a non cell-autonomous disease.

2.1.2 NSC-34 cells

Establishing an immortalized neuron cell line is a difficult task: to overcome this problem a hybrid neuroblastoma/ motoneurons cell line (NSC-34) was produced [*Cashman et al., 1992*]. Transgenic NSC-34^{SOD1} is considered a cellular model of ALS, since they exhibit some motoneuron alterations features as mitochondrial dysregulation [*Raimondi et al., 2006*] and Golgi fragmentation [*Gomes et al., 2008*].

2.1.3 Organotypic Cultures

On plated cell cultures the majority of cellular interactions modulating the ALS processes, such motoneuron signalling as well as glia trophic support, are lost.

To maintain intact tissue structures and its cellular interactions is possible to cultivate an entire spinal cord slice where neurons retain their metabolic characteristics [*Delfs et al., 1989*]. By organotypic cultures it has been verified that some neurotrophic factors protect motoneurons from excitotoxic death [*Corse et al., 1999*; *Tolosa et al., 2008*]. As in cell culture, a major organotypic systems limitation is that they do not always carefully mimic what would be happening in vivo.

2.2 IN VIVO MODELS

One of the major breakthroughs in the field of ALS research derives from the development of animal models of disease that proved useful both for the study of pathogenetic mechanisms and to test potential pharmacological approaches. The study of experimental models of ALS is useful to investigate the triggering events occurring earlier in the pathology. Nowadays, many models of motor neuron degeneration have been discovered or developed. However, even if some of them are associated with gene mutations found in human ALS or considered risk factors for the disease, they recapitulate only some of the features

of the human disease. Nevertheless, these models may provide useful insights in deciphering the mechanisms of selective motor neuron degeneration as potential therapy targets.

2.2.1 Spontaneous or Induced Models of ALS

Mice carrying naturally occurring mutations on identified or unidentified genes provide animal models of motor system impairment, as recapitulated in **Table 2.1**.

2.2.1.1 Wobbler Mouse

The Wobbler mouse arose as the result of a spontaneous mutation at the "Institute of Animal Genetics" in Edinburgh [*Mitsumoto & Bradley, 1982*]. It shows a progressive forelimb weakness and atrophy, accompanied by a marked decrease of muscular strength and motor ability; the symptoms are associated with proximal axonal degeneration and vacuolar changes within the motor neurons of the cervical spinal cord with little involvement of the brain.

The gene mutated responsible for this autosomal recessive this syndrome is VPS54, encoding a subunit of the Golgi-associated retrograde protein (GARP) complex involved in the transport of vesicles from endosomes to Golgi apparatus [*Schmitt-John et al., 2005*].

2.2.1.1.1 Nmd Mouse

Mice showing neuromuscular degeneration, with autosomal recessive mutation localized on the gene coding for the ATPase/DNA helicase have been described [*Cook et al., 1995*; *Cox et al., 1998*]. Called Nmd (neuromuscular degeneration) mice, they present rapidly progressive hind limb weakness and motor neuron cell body degeneration and have a life span that range from 2 to 20 weeks.

Table 2.1: Overview of spontaneous or induced mouse models showing motor neuron degeneration.

Name	Mutated gene	Gene product	Inheritance	Human disease	Reference
Wobbler	*VPS54*	Subunit of the GARP complex	recessive	NA	Duchen & Strich, 1968
Nmd	*IGHMBP2*	Immunoglobulin μ-binding protein 2	recessive	SMARD1	Cook et al., 1995
MND	Cln8	neuronal ceroid lipofuscinose	dominant	NA	Ranta et al., 1999
Pmn	*TBCE*	tubulin-specific chaperone E	recessive	motor neuropathy HRD/SSS	Schmalbruch et al., 1991
Wasted	*eEF1A2*	translational elongation factor	recessive	NA	Chambers et al., 1998
Loa	*DYNC1H1*	dynactin	dominant	Sensory neuropathy	Rogers et al., 2001
Cra	*DYNC1H1*	dynactin	dominant	sensory neuropathy	Hrabé de Angelis et al., 2000

SMARD: spinal muscular atrophy with respiratory distress, HRD: hypoparathyroidism-retardation dysmorphism syndrome, SSS: Sanjad-Sakati syndrome, and NA: not available

2.2.1.1.2 MND mouse

MND (motor neuron degeneration) is a spontaneous, dominant mutation localized to chromosome 8 in the coding region of the gene Cln8, belonging to the family of neuronal ceroid lipofuscinose-related genes [*Ranta et al., 1999*]. MND mice exhibit an adult-onset, progressive deterioration of motor function with spastic paralysis moving from caudal to cranial spinal cord levels. They undergo a premature death at 10-12 months [*Messer et al., 1987*]. The number of choline acetyl transferase (ChAT) immuno-positive lumbar motor neurons is not different from normal mice [*Mennini et al., 2002*]. Neuropathological hallmarks are inclusion bodies containing ubiquitin, mitochondrial alterations, lipofuscin accumulation and neurofilament abnormalities. However, the presence of abnormal autofluorescent cytoplasmic inclusions rich in lipofuscin found in neurons, as well as in many other somatic organs, makes these animals a useful model for human neuronal ceroid lipofuscinosis rather than for ALS [*Bronson et al., 1993*].

2.2.1.1.3 Pmn Mouse

The "Progressive motor neuronopathy" also known as "paralyse natural mutant" mouse carry a recessive point mutation on chromosome 13 [*Brunialti et al., 1995*].

Two groups identified the Pmn mutation as a Trp to Gly substitution at the last residue of the tubulin-specific chaperone E protein [*Bommel et al., 2002*; *Martin et al., 2002*], that is essential for the proper tubulin assembly and for the maintenance of microtubules in motor axons. Distal axonopathy with paralysis of the limbs and muscular atrophy are the most relevant clinical signs, while motor neuron cell bodies and proximal axons are relatively preserved [*Schmalbruch et al., 1991*]. Symptomatic phase begins at 2 weeks of age and evolves rapidly to death.

2.2.1.1.4 Wasted Mouse

It carries a recessive mutation on chromosome 2, in the gene coding for the translational elongation factor eEF1A2 [*Chambers et al., 1998*]. The symptom onset is around 2 weeks of age and the progression is very fast, leading to death within a month. Spinal and brainstem motor neurons are lost, while UMN are not affected [*Doble & Kennel, 2000*].

2.2.1.1.5 Loa and Cra Mice

Two independent *N*-ethyl-*N*nitrosourea-(ENU) induced mutagenesis experiments generated the mutant mouse lines: "Legs at odd angles" (Loa) [*Rogers et al., 2001*] and "Cramping 1" (Cra) [*Hrabé de Angelis et al., 2000*]. These animals show motor neuron disorders with substantial similarities to human pathology progression and features, such as ubiquitinated inclusions containing among other proteins mutated SOD1 and neurofilaments (NFs).

Hafezparast and colleagues relate the Loa and Cra phenotypes two different dynein encoding gene point mutations [*Hafezparast et al., 2003*], thus supporting the hypothesis that disturbances in axonal transport lead to motor neuron loss.

2.2.2 Genetic Models of ALS

The discovery of human mutations linked with ALS has made possible the development of etiological models in a wide variety of organisms, including nematodes, fruit fly, zebrafish and rodents. These transgenic systems had an important role in the understanding of the ALS pathophysiological basis.

2.2.2.1 ALS1: Superoxide Dismutase 1 models

SOD1 is ubiquitously expressed and protects from oxidative stress by converting superoxide to hydrogen peroxide. ALS-causative mutations in SOD1 were initially thought to affect SOD1 superoxide dismutase activity, thus inhibiting its protective role against oxidative stress. However, SOD1 knockout mice do not develop ALS features, thus excluding the loss-of-function hypothesis; instead mutations in SOD1 cause a toxic gain of function [*Ho et al. 1998*; *Reaume et al. 1996*]. **Table 2.2** recapitulated all the SOD1 models that have been hitherto produced.

SOD1 Mice models

Several transgenic mouse strains were created by the introduction of the sequence coding for human mutant SOD1 under the control of a promoter that enables ubiquitous expression of the transgene [*Shibata, 2001*]. The first ALS animal model has been created in the murine species: the fALS related SOD1 mutation, where at residue 93 a glycine is converted into an alanine (G93A), was expressed under the control of the human SOD1 promoter [*Gurney et al. 1994*]. In the following years, investigators have generated different lines over-expressing human SOD1 with G37R or G85R mutations [*Dal Canto & Gurney, 1994*; *Wong et al., 1995a*], or mouse SOD1 with G86R mutation [*Ripps et al., 1995*].

These animals develop a phenotype resembling ALS, with an adult onset progressive motor paralysis, muscle wasting and reduced lifespan.

Table 2.2: Summary of SOD1 animal models

Specie	Mutation	Promoter/ tissue expression	Protein expr. (fold)	Activity (fold)	Symptom Onset (weeks)	Survival (weeks)	Phenotype	Reference
Mouse	A4V [c]	Human SOD1	nd	nd	35	48	ALS-like phenotypes: tremor, progressive motor abnormalities and paralysis; gliosis, ubiquitinated SOD1 inclusions, mitochondrial vacuolation, axonal and MN degeneration.	Deng et al. (2006)
	G37R	Human SOD1	nd	14	15–17	25–29		Wang et al. (1995a)
	H46R	Human SOD1	nd	nd	20	Nd		Chang-Hong et al. (2005)
	H46R/H48Q	Human SOD1	nd	0	17–26	Nd		Wang et al. (2002)
	H46R/H48Q/H63G/H120G	Human SOD1	nd	0	36–52	24–30		Wang et al. (2003)
	L84V	Human SOD1	nd	nd	21–26	37–45		Tobisawa et al. (2003)
	G85R	Human SOD1	1	0	35–43	46–54		Bruijn et al. (1997)
	G86R	Human SOD1	1.5	nd	39.5–48	17		Wang et al. (2005a)
	G86R [b]	Mouse Sod1	nd	0	13–17	61		Ripps et al. (1995)
	D90A	Human SOD1	20	6–8	52	17–26		Jonsson et al. (2004)
	G93A	Human SOD1	17	13	13–17	40–60		Gurney et al. (1994)
	G93A [d!]	Human SOD1	8	nd	24–26	60		Gurney (1997)
	I113T	Human SOD1	nd	nd	52	43		Kikugawa et al. (2000)
	T116X	Human SOD1	nd	nd	41	Nd		Deng et al. (2006)
	L126X	Human SOD1	0–0.5 [c]	nd	28–56	47		Wang et al. (2005)
	L126X	Human SOD1	0–1 [c]	nd	44	18		Deng et al. (2006)
	L126delTT	Human SOD1	2	0	17	36		Wakisaka et al. (2005)
	G127X	Human SOD1	0.5–1	0	35	24		Jonsson et al. (2004)
Rat	H46R	Human SOD1	6	0.2	20	24	ALS-like phenotypes	Nagai et al. (2001)
	G93A	Human SOD1	2.5	3	14	17		Nagai et al. (2001)
	G93A	Human SOD1	8–16 [d]	Nd	16	17		Howland et al. (2002)
Dog	E40K/K80K [e]	Endogenous	1	1	25 years [f]	6–19 month [f]	Degenerative myelopathy, axonal lesions, no neuronal cell body loss, functional deficits in UMN followed with LMN	Awano et al. (2009); Coates & Wininger (2010)
Zebrafish	G93R [g]	Zebrafish Sod1	3	Nd	48	72–108	Motor defects, muscle atrophy, MN loss, reduced survival	Ramesh et al. (2010)
Drosophila	WT	Motor neurons	3–7 [h]	nd	3	Normal	Motor deficits, SOD1 aggregation, glial cell stress activation, no MN loss	Watson et al. (2008)
	A4V	Motor neurons	3–5 [h]	nd	4	Normal		
	G85R	Motor neurons	1–2 [h]	nd	2	Normal		
C. elegans	G85R,M46Y/H48Q	Pan neuronal	nd	nd	Movement defects at 4 days	G85R: reduced H46R/H48Q: nd	G85R: Locomotor defect at larval stage 4, SOD1 aggregation, abnormal synaptic processes, no MN loss H46R/H48Q: Less severe locomotor defect	Wang et al. (2009b)
	G85R, G93A, G127X	Muscle	≤1	nd	nd	nd	SOD1 aggregation, 25–30% reduced motility in day 6 adults	Gidalevitz et al. (2009)

nd not described, [all] G1 low copy, WT wild-type, [a] Double transgenic with SOD1 WT [b] Mouse transgene [c] mRNA expression is high, protein is low [d] Expression increases with age: 8-fold at presymptomatic, 16-fold at end stage [e] Spontaneous mutation in endogenous gene [f] Varies according to dog breed/size [g] Zebrafish transgene [h] Relative to levels of transgene GFP expression, expression increases with age

Pathological changes mainly consist of depletion of motor neurons in the spinal cord, atrophy, gliosis, axonal swelling and presence of ubiquitin-positive inclusions. By contrast, mice overexpressing wild-type SOD1, generated by targeted gene deletion, remain clinically normal and do not develop motor phenotypes, consequently the SOD1 disruption alone seem to be insufficient to cause spontaneous motor neuron degeneration in mice. The mutant SOD1 transgenic models are summarised in **Table 2.2** and have been recently reviewed by Turner and Talbot (2008). In brief, ALS-like phenotypes of mutant SOD1 mouse models can vary according to mutation and transgene expression level. For instance, mice that express high or low amounts of the $SOD1^{G93A}$ transgene develop ALS-like phenotypes earlier or later, respectively, suggesting a mutant SOD1 dosage effect.

Transgenic mice carrying 23 copies of human $SOD1^{G93A}$ are considered the standard model of ALS in therapeutic studies [*Bendotti & Carri, 2004*]. The model develops a motor system disease prevalently affecting lower motor neurons. Ultrastructural and microscopical analysis reveals that the earliest pathological sign in these mice is the vacuolization of large neurons in the anterior horns of the spinal cord [*Bendotti et al., 2001*]; it has been hypothesized that these vacuoles originate from the dilation of rough endoplasmic reticulum and from degenerating mitochondria. At the end stage, motor neuronal depletion is evident and hyaline, filamentous inclusions immuno-positive for ubiquitin and neurofilaments are present in some of the surviving neurons [*Gurney et al., 1994*; *Migheli et al., 1999*]. Transgenic mice expressing low levels of $SOD1^{G37R}$ mutant show a motor disease restricted to lower motor neurons, whereas higher copy number causes more severe abnormalities and affects a variety of other neuronal populations. The most obvious cellular abnormality is the presence in axons and dendrites of membrane-bounded vacuoles, which appear to be derived from degenerating mitochondria [*Wong et al., 1995b*]. Transgene expression of mutant

human SOD1^{G85R} or its murine counterpart (SOD1^{G86R}) develops a very aggressive pathology with a rapid progression to paralysis and death within two weeks from the first symptoms. Differences on disease progression and survival in various SOD1 mutant mice depend on the mutation and the copy number of the transgene. The age of onset, the duration and several pathological features also vary in dependence of the mouse strain in which the mutation is expressed; this background effect suggests the existence of strong modifying genetic factors.

SOD1 Rat models

Two human SOD1 mutations have been modelled in rats: SOD1^{H46R} and SOD1^{G93A} (**Table 2.2**) [*Howland et al., 2002*; *Nagai et al., 2001*]. In these models, the onset of the disease occurs early and the progression is very rapid, directly proportional to mutant SOD1 expression. The SOD1^{G93A} mutation causes a more aggressive disease in rats than the SOD1^{H46R} mutation [*Nagai et al., 2001*]. Pathological abnormalities are similar to those observed in the mouse model with evidence of gliosis and vacuolization in both spinal cord and brainstem prior to clinical onset and motor neuron death [*Howland et al., 2002*]. These rat models offer the advantage of increased size, facilitating preclinical trials using intrathecal catheters.

SOD1 Zebrafish models

Expression of SOD1^{G93R} transgene in zebrafish causes slow-progressing ALS phenotype with muscle atrophy, motor neuron loss, and reduced survival [*Ramesh et al., 2010*]. Age-dependent neuromuscular junction (NMJ) defects are also seen. However, denervation was not observed, which could be due to the regenerative capacity of zebrafish motor neurons [*Reimer et al., 2008*]. Further, the hSOD1^{WT}, SOD1^{G93A}, SOD1^{G37R}, and SOD1^{A4V} isoforms were used to produce zebrafish transgenic embryos: mutant but not wild-type SOD1, causes abnormalities in motor neuron axon [*Lemmens et al., 2007*]. Similar to rodent, these defects are dose-dependent.

***Drosophila* and *C. elegans* models**

The expression of either human wild-type SOD1, or $SOD1^{A4V}$ and $SOD1^{G85R}$ mutations in *drosophila* models causes progressive deficits in climbing ability of transgenic flies; thus, human wild-type and mutants SOD1 forms are toxic to flies although motor neuron survival and life span are unaffected [*Watson et al., 2008*]. Motor neuron abnormalities might not translate to motor neuron loss due to the short life span of the fly.

Several human SOD1 transgenic *C. elegans* strains have been created. Neuronal expression of human $SOD1^{G85R}$ causes locomotor defects and provokes mutant SOD1 aggregates formation, morphological defects in the ventral nerve cord processes, and dorsal cord synapses reduction. These animals do not experience motor neuron loss or reduced life span [*Wang et al., 2009b*].

To analyse SOD1 aggregation, mutant human $SOD1^{G85R}$, $SOD1^{G93A}$, and $SOD1^{G127X}$ were expressed in *C. elegans* muscle where forms aggregates vary according to mutation [*Gidalevitz et al., 2009*].

2.2.2.2 ALS2: Alsin models

Alsin knockout mice are generated by several research groups, [*Cai et al., 2005*; *Hadano et al., 2006*; *Devon et al., 2006*; *Yamanaka et al., 2006*] (**Table 2.4**) but these models do not present a well defined pathological motor phenotype, suggesting that, in mouse, alsin knock out is unable to provoke important motor defects.

2.2.2.3 ALS6: Fused in Sarcoma models

It is estimated that FUS/TLS mutations causes fALS in 4%-5% of cases. FUS/TLS deletion in mice causes postnatal mortality [*Hicks et al., 2000*] and until now mutant and/or wild type FUS/TLS transgenic models are no available. It is hypothesised that mutant FUS/TLS induces the disease by a "gain-of-function" mechanism.

2.2.2.4 ALS8: VAMP-associated protein B models

Recently transgenic mice expressing in the nervous system either wild-type or mutant (P56S) VAPB gene using a modified prion promoter were created [*Tudor et al., 2010*]. These mice do not develop motor phenotype and show no alterations in survival.

2.2.2.5 ALS10: TDP-43 models

Mutations in the TARDBP gene are rare and account for less than 5% of fALS. Various TDP-43 models have been hitherto created (**Table 2.3**) [*Gitcho et al., 2008*; *Kabashi et al., 2008*; *Sreedharan et al., 2008*; *Van Deerlin et al., 2008*; *Yokoseki et al. 2008*].

Mouse models

To date, three research groups have created wild-type TDP-43 transgenic mice, expressing the TDP-43 gene under the control of Thy-1.2, CaMKII and mouse prion promoter (mPrp). Two groups express human TARDBP^{A315T} from the mPrp while a third group expresses human TARDBP^{M337V} from the mPrp; a fourth group expresses TARDBP without a NLS from the CaMKII promoter [*Igaz et al., 2011*; *Shan et al., 2010*; *Stallings et al., 2010*; *Tsai et al., 2010*; *Wegorzewska et al., 2009*; *Wils et al., 2010*; *Xu et al., 2010*]. The majority of these models were reviewed recently by Wegorzewska and Baloh (2011) that highlight the variation in phenotypes between models according to promoter and expression levels.

Overexpression of TARDBP^{WT} is toxic in almost all mouse models created. Both human and mouse TARDBP^{WT} overexpression results in dose-dependent gait dysfunctions, tremors, paralysis, and eventually in premature death [*Shan et al., 2010*; *Wils et al., 2010*; *Xu et al., 2010*].

The CaMKII promoter mediated TARDBP expression provokes defects in learning and memory, as well as hippocampus and neocortex degeneration [*Igaz et al., 2011*; *Tsai et al., 2010*].

These findings have never been reported in patients where altered TDP-43 expression has not been revealed. TDP-43 mislocalisation from the nucleus to the cytoplasm and subsequent aggregation is seen in ALS patients and might prevent normal nuclear TDP-43 function [*Neumann et al., 2006*].

To investigate this possibility, Igaz et al. (2011) generated mice expressing human TDP-43 without NLS. TARDBP$^{\Delta NLS}$ mice have more aggressive neurodegeneration compared to TARDBP^{WT} lines.

Wegorzewska et al. (2009) generated mice expressing TDP-43^{A315T} threefold higher than mouse endogenous one. These animals show gait alterations at about 13 weeks, complete paralysis and die at ~22 weeks of age. Since in their experiment Wegorzewska et al. (2009) did not produce TARDBP^{WT} mice, it was not possible to make a direct comparison between mutant and wild-type protein pathogenicity [*Wegorzewska & Baloh, 2011*].

Stallings et al. (2010) generate mice expressing both TARDBP^{WT} and TARDBP^{A315T}: the TARDBP^{A315T} animals present a more aggressive phenotype compared to that shown by Wegorzewska et al. (2009) mice. Even the TARDBP^{WT} mice die prematurely although mutant TDP-43 appears more toxic than the wild-type form [*Stallings et al., 2010*]. Interestingly, high expression of hTDP-43 in rodents can lead to a down regulation of mouse endogenous one, suggesting that the protein auto-regulates its expression. Thus, one hypothesis might be the loss on its self-regulating ability to lead to the TDP-43-mediated neuronal death [*Igaz et al., 2011*; *Xu et al., 2010*].

Kraemer et al. (2010), Sephton et al. (2010) and Wu et al. (2010) failed to generate Tardbp knockout mice, suggesting that TDP-43 is essential for embryonic development. Interestingly, Kraemer et al. (2010) reported muscle weakness in heterozygote Tardbp$^{+/-}$ mice. All of this data highlight two key points: the vital function of TDP-43 in development, and the effect of mutated TDP-43 levels on neuronal survival.

Table 2.3: Summary of TDP-43 animal models

Specie	Mutation	Promoter/ tissue expression	Protein expr. (fold)	Symptom Onset (weeks)	Survival (weeks)	Phenotype	Reference
Mouse	WT	mPrp	3–4	None	Normal	Mild gliosis and diffuse neuronal ubiquitin staining	Shelkovnikova et al. (2013)
	WT	mPrp	2.5	2	4.5–4.5	Motor deficits, gliosis, rare cytoplasmic phospho-TDP-43 aggregates and CTFs, mitochondrial aggregates. Axonal degeneration but no MN loss	Xu et al. (2010)
	A315T	mPrp	3	13	22	UMN degeneration and 20% LMN loss, gliosis, rare cytoplasmic phospho-TDP-43 aggregates and CTFs	Wegorzewska et al. (2009)
	A315T	mPrp	4	4	37.5	Gliosis, muscle atrophy, rare phospho-TDP- 43 inclusions, increased TDP-43 CTFs with age	Shelkovnikova et al. (2013)
	WT	Thy1.2	3.8–6.1 [a]	2–8.5 [a]	4–29 [a]	30% loss of layer V UMNs, 25% loss LMNs, gliosis, increased TDP-43 CTFs with age, rare phospho-TDP-43 aggregates	Wils et al. (2010)
	WT	Thy1.2	M: 3.5 F: 1.3	2–2.5 13	nd	Motor abnormalities without MN loss. Mitochondrial aggregation, no cytoplasmic TDP-43/phospho-TDP-43 inclusions, intranuclear TDP-43, and FUS inclusions	Shan et al. (2010)
	WT [b]	CaMKII	2	8.5	71	Impaired learning, progressive motor deficits, hippocampal atrophy, 15–20% cortical neurons have TDP-43-positive cytoplasmic inclusions	Tsai et al. (2010)
	WT	CaMKII TRE	0.8 [c]	8–49	nd	Mosaic expression, cerebral atrophy, gliosis, motor spasticity,\13% cytoplasmic phospho-TDP-43 aggregates	Igaz et al. (2011)
	ΔNLS	CaMKII TRE	7.9 [c]	6	nd	More pronounced cerebral atrophy than WT, gliosis, motor spasticity, very rare phospho-TDP-43 aggregates	Igaz et al. (2011)
Rat	WT	Endogenous	nd	None	Normal	No MN pathology	Zhou et al. (2010a)
	M337V	TRE	nd	4.5–4.8 [d]	6.5–7 [d]	17% LMN loss, gliosis, rare cortical phospho-TDP-43 aggregates	Zhou et al. (2010a)
Drosophila	WT [e]	Sensory neurons	nd	nd	nd	Increase in larval sensory neuron dendritic branching	Lu et al. (2009)
	WT, Q331K, M337V and CTF	Sensory neurons	nd	nd	nd	Increase in larval sensory neuron dendritic branching. Q331K and M337V promote loss dendritic branching. CTF has no effect on branching	Lu et al. (2009)
	WT	Eye	nd	nd	nd	Retinal degeneration	Li et al. (2010b)
	WT	Mushroom bodies	nd	nd	Normal	Age-dependent axonal loss and neuron death	Li et al. (2010b)
	WT	Motor neurons	nd	nd	nd	Reduced larval locomotor activity, reduced axonal branches at NMJs, cytoplasmic TDP-43 aggregate formation, axon swelling, and MN death	Li et al. (2010b)
	WT	Eye	nd	nd	Normal	Dose- and age-dependent retinal degeneration	Hanson et al. (2010)
	WT	Motor neurons	nd	2–3	2.4–3.4	Progressive motor dysfunction leading to paralysis, no cytoplasmic TDP-43 aggregation	Hanson et al. (2010)
	WT	Eye	nd	nd	nd	Age-dependent retinal degeneration	Elden et al. (2010)
	WT and Q331K	Motor neurons	nd	nd	nd	Both display motor deficits, Q331K motor deficits are greater	
	WT	Pan neuronal	nd	nd	Lethal	Reduced survival	
	WT, A315T, 2675, 346C, A382T, N390D, 147V/ F149I, ΔNLS, CTF	Pan neuronal	nd	N.d	Lethal: WT, ΔNLS, G287S, A315T, G348C, A382T, N390D Non-lethal: F147I,F149I,CTF	Transgene severity from most severe to least severe: WT, A315T, G348C, A382T, ΔNLS, G287S, N390D, F147I/F149I, CTF	Vogel et al. (2010)
	WT, A315T, G287S, G348C, A382T, 390D, F147I / F149I, ΔNLS	Motor neurons	N.d	nd	nd	Progressive motor deficits and paralysis, WT being the worse and CTF and 147I / F149I showing only mild deficits	
	WT, ΔNLS, ΔNES	Eye	nd	N.d	nd	Mild retinal degeneration with WT or ΔNES, more severe degeneration with ΔNLS	Miguel et al. (2011)
	WT, ΔNLS, ΔNES	Neuronal	nd	nd	WT: L1 larval lethality ΔNLS and ΔNES: pupal lethality	Conditional expression in cebellneuri: WT survival 16 days, ΔNLS survival 21 days, ΔNES survival 26 days, control survival 30 days. Misaggregates in nucleus, inclusions not required for degeneration	
	WT, ΔNLS, ΔNES	Glia	nd	nd	WT and ΔNLS: larval/pupal lethality ΔNES: nonlethal	nd	
	WT, ΔNLS, ΔNES	Muscle	nd	nd	WT and DNLS: larval/pupal lethality ΔNES: nonlethal	nd	Miguel et al. (2011)
C. elegans	WT, ΔRRM1, ΔRRM2, ΔNLS, ΔCTD	Pan neuronal	nd	Larval stage	nd	WT TDP-43: uncoordinated movement, nuclear accumulation of TDP-43, NMJ defects ΔRRM1, ΔRRM2, ΔCTD, ΔNLS: No uncoordinated phenotype. Granular staining pattern for ΔRRM1, ΔRRM2, ΔCTD	Ash et al. (2010)
	WT, G290A, A315T, M337V, S409A/S410A	Pan neuronal	nd	nd	nd	G290A, A315T, M337V severe locomotor defects and paralysis, WT moderate locomotor defects. Nuclear, phospho-TDP-43 insoluble aggregates, MN degeneration. S409A/S410A mutations reduce paralysis of TDP-43 mutants	Liachko et al. (2010)

nd not described, WT wild-type, mPrp mouse prion promoter, CaMKII calcium/calmodulin-dependent kinase II; CTF C-terminal fragment, FUS fused in sarcoma, TRE tetracycline response element, NMJ neuromuscular junction, ΔNLS removal/disruption of nuclear localization signal, ΔNES removal/disruption of nuclear export signal; ΔCTD removal of C-terminal domain, ΔRRM1/2 removal of RNA recognition motif 1/2 [a] Variation in phenotype proportional to TDP-43 expression [b] Mouse transgene [c] TRE induction 28 days after birth [d] TRE induction 4 days after birth [e] Drosophila homegene

Rat models

Both TARDBP^{WT} and TARDBP^{M337V} have been expressed in rats [*Zhou et al., 2010a*] under the control of the human TARDBP promoter. TARDBP^{M337V} expression causes early mobility problems, paralysis, and death before pubertal age while rats expressing the TARDBP^{WT} isoforms at comparable levels not develop motor phenotype thus suggesting that the mutant TDP-43 isoforms is more toxic than the wild-type one.

Zebrafish models

Two groups created zebrafish embryos expressing both human wild-type and mutant TARDBP mRNA. Laird et al. (2010) showed that mutant and wild-type TARDBP mRNA decreased axon length and increased aberrant branching. These defects were exaggerated with TARDBP^{A315T}, and axonopathy severity was dependent on mRNA dose. Interestingly, mutant and wild-type TDP-43 localised to the nucleus, suggesting that cytoplasmic TDP-43 mislocalisation is not required for axonal defects [*Laird et al., 2010*]. Intriguingly, Tardbp knockout fishes present similar motor deficits and axonopathy that could partly be rescued by human wild type TARDBP mRNA expression, but not by mutant TARDBP, suggesting that pathogenic TARDBP mutations cause a loss of function. Finally, the TDP-43 C-terminal domain was shown to be required for pathogenicity in zebrafish [*Kabashi et al., 2010*]. Development of adult TDP-43 zebrafish should provide insight into how these motor defects manifest in adulthood.

***Drosophila* and *C. elegans* models**

Knocking down endogenous TBPH, the *Drosophila* TARDBP ortholog, has proved deleterious to animal development by preventing the hatching from the pupal stage of the fruit flies majority. Animals reaching adulthood display dramatic motor defects such as an inability to fly/walk and reduced life span [*Feiguin et al., 2009*].

Expression of either ortholog TBPH or human TDP-43 in *Drosophila* has had varied outcomes; however, a recurring theme is of wild-type and mutant TDP-43 overexpression being pathogenic with dendritic branching increasing. Whilst aberrant dendritic branching is not seen in ALS, these studies further emphasise the neuronal specific toxicity caused by expression of wild-type and mutant TDP-43, providing insight into its pathogenetic role. TDP-43 expression in eyes causes retinal degeneration, in mushroom bodies an age-dependent axonal loss and aggregate formation, whilst in motor neurons provokes axon swelling and motor neuron death [*Li et al., 2010*]. Thus, the action of TDP-43 toxicity might vary from cell to cell [*Miguel et al., 2011*].

Wild-type or mutant TDP-43 expression in *C. elegans* is also toxic. In the two transgenic *C. elegans* models created, the human TARDBP sequence is expressed under the snb-1 promoter control, causing animals to become uncoordinated. In one model, the number of GABAergic motor neuron synapses is dramatically diminished, and there is occasional axonal defasiculation [*Ash et al., 2010*].

In the other model expression of pathogenically mutated G290A, A315T, or M337V TDP-43, in respect to the wild type isoforms, causes a more severe progressive locomotor defect and decreased life span [*Liachko et al., 2010*]. In mutant TARDBP animals there are early axonal abnormalities and GABAergic neurodegeneration. Interestingly, prevention of aberrant phosphorylation of mutant TDP-43 results in an amelioration of motor deficits, suggesting that TDP-43 hyperphosphorylation is important in *C. elegans* pathogenesis. Knockout of tdp-1, the TARDBP C. elegans ortholog, in contrast to what is seen in mice and Drosophila, does not cause obvious defects [*Liachko et al., 2010*], suggesting a slightly divergent role between TDP-43 and tdp-1 in development. This difference might relate to the absence of a glycine-rich C-terminal domain in tdp-1 of *C. elegans*. Transgenic studies in *C. elegans*, however, might point toward a greater conservation in neuronal function.

2.2.2.6 Models for rare fALS and candidate genes

Dynactin

The p150-glued is the largest isoform of the dynactin complex, whose physiological function is expressed by microtubules binding. Mutated isoforms of p150glued show a lower binding efficiency causing a "loss-of-function" [*Puls et al., 2003*]. Moreover, it has been demonstrated, by in vitro studies on cell culture, that mutant p150glued^{G59S} causes protein aggregation [*Levy et al., 2006*]. It has been found that motor neuron loss in transgenic mice is caused by a dominant negative mechanism [*Laird et al., 2008*, *Chevalier-Larsen et al., 2008*]. Lai et al. (2007) generated a mutant p150Glued^{G59S} knock-in mouse. Homozygous mice present embryonic lethality, while heterozygous ones develop motor neuron phenotype at 10 months of age together with excessive accumulation of cytoskeletal and synaptic vesicle proteins at the neuromuscular junctions, loss of spinal motor neurons, increase of astrogliosis, and shortening of gait [*Lai et al., 2007*]. Mice overexpressing p150Glued^{G59S} under the control of the neuron-specific Thy-1 promoter was developed by Laird et al. (2008) and by Chevalier-Larsen et al. (2008) that observed a motor phenotype resulting in muscle weakness, paralysis, and eventually death (**Table 2.4**).

Neurofilaments and Peripherin

NFs consist of three subunits (NF-L, NF-M, and NF-H) and, attempting to determine their role in the pathology of motor neurons, over the years, a large number of transgenic mice with modifications related to NFs were made. Knocking out one of the NF subunits as well as simultaneously two of them does not provoke an obvious phenotype. Moreover, even the NFs subunit overexpression results in motor neuron death, though NF accumulations in neuronal cell bodies were found. However, the same NF-L^{L394P} mutation causing the Charcot-Marie-Tooth disease, type 2E [*Mersiyanova et al., 2000*] in mice induce selective motor neuron death [*Lee et al., 1994*], although no effect on mouse life span was reported.

Peripherin is another intermediate filament, mostly expressed in the peripheral nervous system and upregulated after injury and by inflammatory cytokines.

A frameshift deletion in the peripherin gene was discovered in ALS patients [*Leung et al., 2004*; *Gros-Louis et al., 2004*]. Transgenic mice overexpressing wild-type peripherin develop a 2 years of age motor dysfunctions associated with motor axons loss and peripherin inclusion bodies appearance.

The onset of this motor phenotype is accelerated by the absence of NF-L as shown by peripherin overexpressing/ NFL knockout crossbreed animals that also present a dramatic loss of motor neurons [*Beaulieu et al., 1999*].

Growth Factors

Motor neurons survival depends on growth factors whose absence can cause neuron death. This is well illustrated by CNTF knockout mice, in which the ciliary neurotrophic factor gene is deleted and who develop with aging atrophy and motor neurons loss [*Masu et al., 1993*].

Another example of this phenomenon is represented by VEGF knockout mice, that exhibit an adult-onset, slowly progressive motor neuron loss leading to muscle atrophy and a motor phenotype [*Oosthuyse et al., 2001*], despite the fact that these animals do not become paralyzed and have a normal life span.

Tau

Different transgenic mouse models over-expressing various human tau isoforms have been generated showing a clear motor phenotype [*Spittaels et al., 1999*; *Zhang et al., 2004*; *Tatebayashi et al., 2002*; *Ramsden et al., 2005*; *Schindowski et al., 2006*; *Tanemura et al., 2002*; *Yoshiyama et al., 2007*].

These mice acquire an age-dependent CNS pathology, similar to FTDP, and show axonal degeneration in brain and spinal cord, progressive motor disturbance and behavioural impairment. In spite of these findings is not yet clear how tau is involved in ALS pathogenesis [*Taes et al., 2010*].

Table 2.4: Overview of mouse model for atypical or rare fALS and candidate genes.

Disease	Gene product	Inheritance	Animal	Genetic modification	Reference
ALS2	Alsin	Recessive	Mouse	KO(exon 3)	[Cai et al., 2005]
				KO (stop codon in exon 3)	[Hadano et al., 2006]
				KO (exon 3 and 4)	[Devon et al., 2006]
				KO (exon 4)	[Yamanaka et al.,2006]
ALS8	VAPB	Dominant	Mouse	PrP; VAPB P56S	[Tudor et al., 2010]
ALS	Dynactin	Dominant	Mouse	Knock-in G59S p150Glued	[Lai et al., 2007]
				Thy-1; G59 p150Glued	[Laird et al., 2008, Chevalier-Larsenet al, 2008
CMT2E/1F	Neurofilament-L	Dominant	Mouse	NF-L L394P	[Lee et al., 1994]
NA	Peripherin	NA	Mouse	overexpression	[Beaulieu et al., 1999]
NA	VEGF	NA	Mouse	$VEGF^{\delta/\delta}$	[Oosthuyse et al., 2001]
FTDP-tau	tau	Dominant	Mouse	4R human tau	[Spittaels et al., 1999]
				R406W human tau	[Zhang et al., 2004, Tatebayashi et al., 2002]
				P301L human tau	[Ramsden et al., 2005]
				G272V, P301S human tau	[Schindowski et al., 2006]
				V337M human tau	[Tanemura et al., 2002]
				P301S human tau	[Yoshiyama et al., 2007]

VEGF: Vascular endothelial growth factor, PrP: prion promoter, CMT: Charcot-Marie-Tooth, FTDP: frontotempora dementia with parkinsonism, and NA: not available.

2.3 PATHOGENETIC HYPOTHESES IN ALS

The current knowledge on disease mechanisms in ALS comes from the study of the effect of SOD1 mutations that provoke motor neuron disease through the acquisition of one or more toxic properties.

Studies performed on human ALS autopsy samples or on SOD1 mutant mice have suggested the involvement of various processes as possible triggers or secondary events in ALS pathology.

Thus, despite extensive research, the mechanisms underlining the motor neuron death still remain unknown, and, to date, most researchers consider ALS as multifactorial disease in which a complex interplay between multiple mechanisms including genetic factors, oxidative stress, excitotoxicity, protein aggregation, damage to mitochondria and axonal transport determines the motor neuronal death (**Figure 2.1**).

2.3.1 Oxidative damage

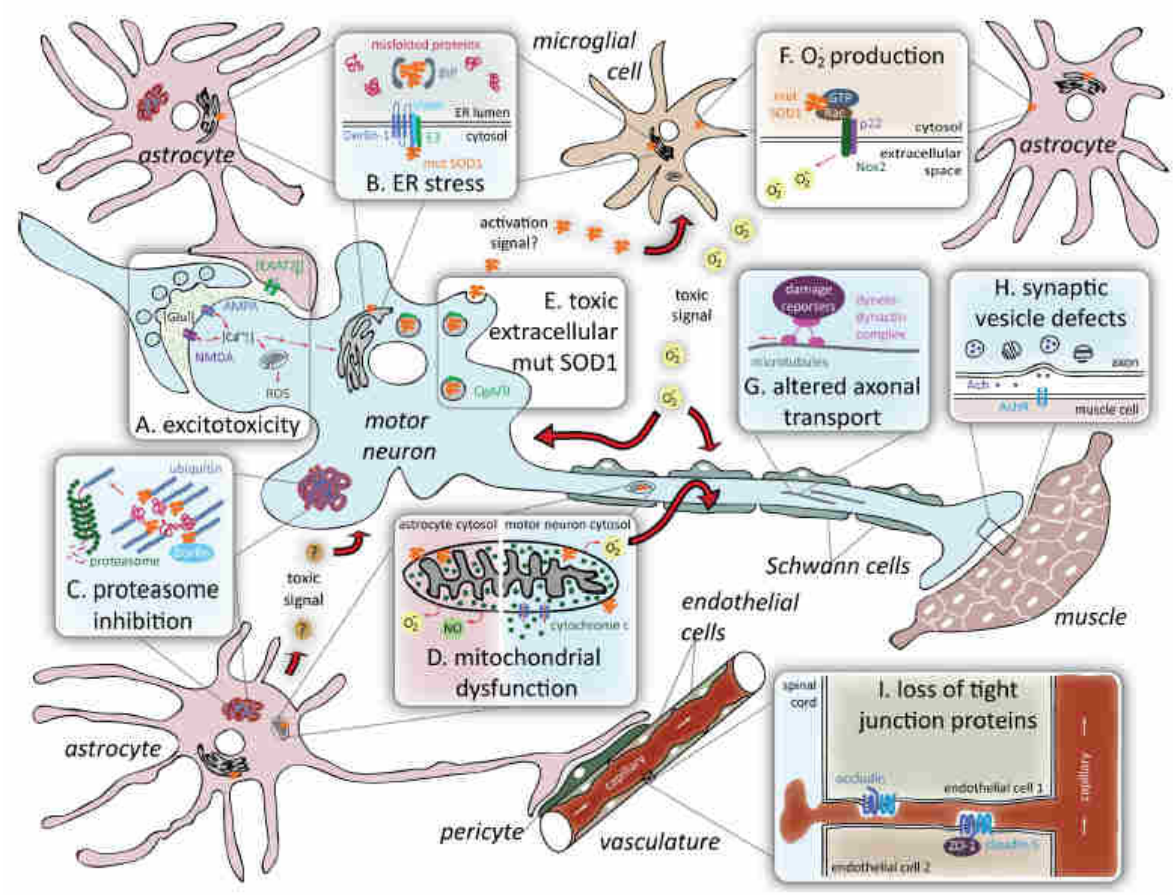
The role of oxidative stress as primary or secondary event in the pathogenesis of ALS still remains controversial.

Increase in markers of oxidative damage has been reported in human patients affected by both sporadic and inherited forms of ALS [*Beal et al., 1997*; *Ferrante et al., 1997*; *Liu et al., 1999*] and in transgenic mouse models of the disease [*Ferrante et al., 1997*; *Andrus et al., 1998*].

However, in other studies, no significant differences in oxidative damage markers were found [*Shaw et al., 1995*; *Bruijn et al., 1997*]. Some studies suggested that a possible source of oxidative insult may be represented by the gain of toxic function of mutated SOD1 [*Cleveland & Rothstein, 2001*] that alters the enzyme activity through aberrant copper catalysis or improper metal binding.

Figure 2.1: Proposed mechanisms of toxicity in SOD1-mediated ALS.

(**A**) Excitotoxicity is the hyperactivation of motor neurons resulting from failure to rapidly remove neurotransmitter glutamate from synapses due to deficiency in the glutamate transporter EAAT2 in the neighboring astrocytes. (**B**) ER stress is induced by abnormal interactions of mutant SOD1 with ER proteins. (**C**) Proteasome inhibition due to "overload" of the proteasome degradation pathway with ubiquitinated misfolded protein aggregates may damage astrocytes and motor neurons. (**D**) Mitochondrial dysfunction mediated by mutant SOD1 deposition on the mitochondrial membrane provokes release of cytochrome c in motor neurons, whereas in astrocytes it leads to nitroxidative stress. (**E**) Toxic extracellular mutant SOD1 is secreted from motor neurons and astrocytes after interaction with components of neurosecretory vesicles. (**F**) Superoxide production from microglia or astrocytes can damage neighboring motor neurons. (**G**) Altered axonal transport including an increase in retrogradely transported stress-related proteins was reported in mutant SOD1-expressing motor neurons. (**H**) Synaptic vesicle defects such as stalling and loss from distal synapse in vulnerable motor neurons is an early event in ALS. (**I**) Loss of tight junction proteins within capillary endothelial cells results in the disruption of the blood–spinal cord barrier and the occurrence of microhemorrhages within the spinal cord well before disease onset.



[Adapted from *Ilieva et al., 2009*]

2.3.2 Mitochondrial dysfunction and apoptosis

The mitochondrial involvement hypothesis comes from observations of morphological abnormalities such as mitochondrial swelling and vacuolation, in parallel with mitochondrial respiratory dysfunction in rodent models and ALS patients [*Jung et al. 2002*]. Since apoptosis is intimately linked with mitochondria, recent work into mitochondrial involvement in ALS [*Sheridan & Martin 2010*] has centred on mitochondrial abnormalities, mutant SOD1 association with mitochondria, and activation of apoptosis.

Abnormal clustering and aggregation of mitochondria and membranous structures are observed in SOD1 and TDP-43 mouse models [*Guo et al. 2010*; *Sotelo-Silveira et al. 2009*]. Wild-type and mutant TDP-43^{A315T} mice display vacuolated mitochondria that cluster in dendrites and motor neuron axons. Therefore, abnormal mitochondrial morphology could affect mitochondrial function through cell-autonomous and non cell-autonomous mechanisms. Regarding the second hypothesis, embryonic astrocytes as well as embryonic motor neurons [*Bilsland et al., 2008*] expressing SOD1^{G93A} display mitochondrial membrane potential reduction and intramitochondrial [Ca^{2+}] increase. Zhou et al. (2010b) speculate that increased mitochondrial Ca^{2+} release in muscle could increase mutant SOD1 aggregation causing a pathological feedback loop, resulting in defective Ca^{2+} signalling and muscle atrophy. Thus, dysfunctional mitochondria exist in motor neurons, glia, and muscle, all of which might influence motor neuron survival.

2.3.3 Alterations of cytoskeleton and axonal transport

The idea that cytoskeleton abnormalities may play a role in ALS pathology arises from early reports of neurofilament accumulations in the cell bodies and proximal axons of motor neurons of both sporadic and familial ALS [*Hirano et al., 1984*]. Subsequently, it has been shown that in mice the over-expression of NF-H or NF-L subunits causes selective degeneration and motor neurons death [*Côté et al., 1993*; *Xu et al., 1993*], though it has not yet been clarified

whether neurofilament disorganization represents a secondary product of pathological processes or whether it directly contributes to the death of the motor neurons.

In neurons, the transport of molecules and organelles is dictated by the highly polarized anatomy of these cells: axonal proteins are synthesized in the cell body and must be transported in an anterograde manner along the axons and dendrites to reach synapses, whereas substances such as peripherally located trophic factors must be transported centrally from the synaptic regions by retrograde transport. The molecular motors for anterograde and retrograde transport are kinesin and the dynein-dynactin complex, respectively. Several factors indicate that defects in axonal transport might contribute to the degeneration of motor neurons in ALS [*Zhang et al., 1997*; *Borchelt et al., 1998*; *Williamson et al., 1999; Murakami et al., 2001*] such as the demonstration that misfolded SOD1 inhibits fast kinesin-based anterograde transport [*Bosco et al., 2010*]. Moreover the identification of misfolded SOD1 in sALS patients without SOD1 mutations suggests that axonal transport damage could be a unifying ALS disease mechanism.

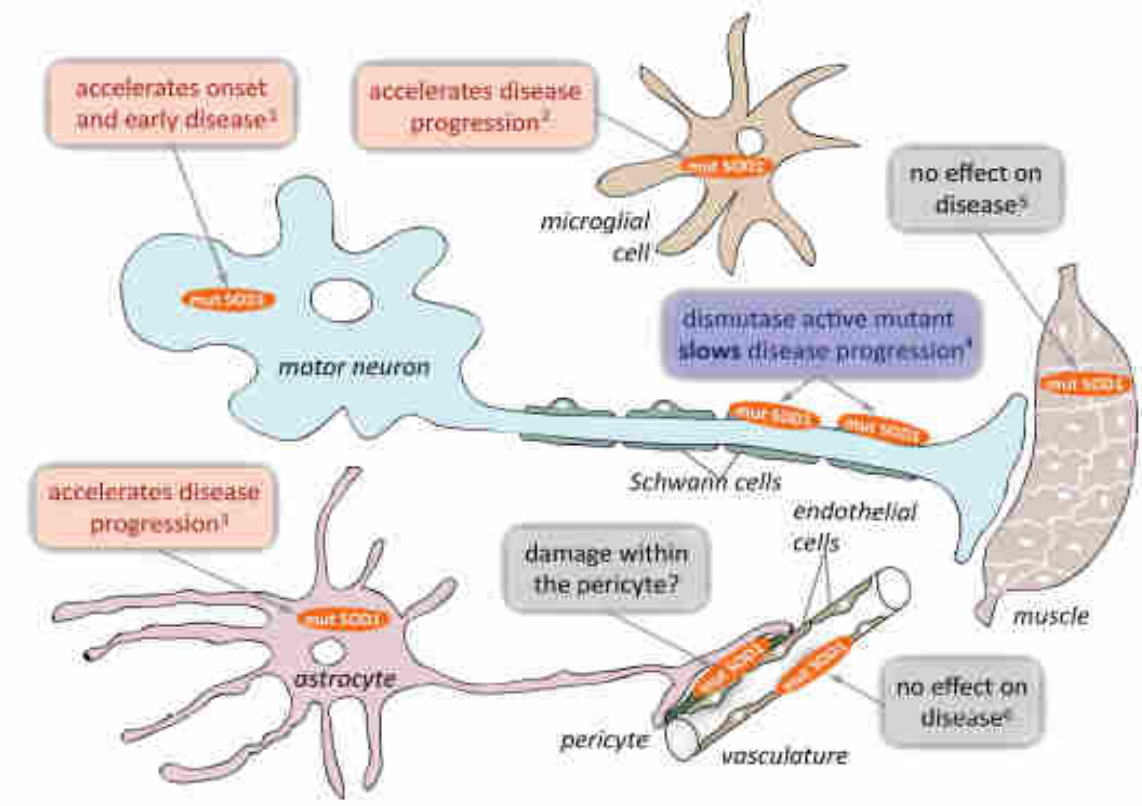
2.3.4 Excitotoxicity

Glutamate-induced excitotoxicity is considered another major mechanism that may contribute to the aetiology of ALS. An over-stimulation of neuronal glutamate receptors can cause neuron death by increasing the cytosolic free calcium thus overriding the storage abilities of mitochondria and ER and eventually activating the death cascades (**Figure 2.1 A).**

The first indication of an involvement of glutamate-mediated excitotoxicity in ALS arose from studies showing increased levels of glutamate in the plasma [*Plaitakis & Caroscio, 1987*] and in the cerebrospinal fluid of a subset of ALS patients [*Rothstein et al., 1990*; *Shaw et al., 1995*]. It is likely that the regulation of AMPA receptors expression is related to motor neuron degeneration in ALS patients [*Fray et al., 1998*] and in SOD1 mutant mice [*Bendotti et al., 2001*].

Figure 2.2: Contribution of mutant SOD1 within different cell types in ALS.

Despite the apparent selectivity for motor neurons, multiple lines of evidence indicate that non-neuronal cell types contribute to pathogenesis and disease progression in SOD1-mediated neurodegeneration. Mutant SOD1 expression in motor neurons directs the onset and development of early disease, but does not influence its progression. In contrast, mutant SOD1 expression in microglia or astrocytes accelerates disease progression without affecting its onset. Expression of a dismutase-active mutant SOD1 specifically in Schwann cells was found to slow disease progression, but the role of a dismutase-inactive mutant in these cells has not been tested. Mutant SOD1 expression within muscle or endothelial cells does not affect ALS onset or progression, although some reports suggest that muscle might be a direct target of mutant SOD1 toxicity. Lastly, the vasculature is damaged very early in disease, leading to loss of tight junctions between endothelial cells and microhemorrhages, but whether any of this is from mutant SOD1 within pericytes, the terminal astrocyte, or coming from cells outside the vasculature is not established. **1** [*Ralph et al., 2005*; *Boillée et al., 2006 b*; *Jaarsma et al., 2008*], **2** [*Beers et al., 2006*; *Boillée et al., 2006b*; *Wang et al., 2009c*], **3** [*Yamanaka et al., 2008*], **4** [*Lobsiger et al., 2009*], **5** [*Holzbaur et al., 2006*; *Miller et al., 2006*; *Dobrowolny et al., 2008*; *Towne et al., 2008*], **6** [*Zhong et al., 2009*].



[Adapted from *Ilieva et al., 2009*]

2.3.5 Non cell-autonomous effects

Recently, various evidence highlight the crucial role played by the cross-talk between neurons and non-neuronal cells, including microglia, astrocytes, skeletal muscle cells and immune system peripheral cells such as macrophages and T lymphocytes, in motor neuronal death induction (**Figure 2.2**).

Firstly, the activation of either astrocytes or microglia a phenomenon named gliosis, is common to all ALS models including rodents and Drosophila SOD1 models and TDP-43 rodents [*Turner & Talbot 2008*; *Watson et al., 2008*; *Wegorzewska & Baloh, 2011*].

Secondly, motor neurons expressing mutant SOD1 did not degenerate if they were surrounded by wild type glia; reciprocally, wild type motor neurons surrounded by SOD1 mutated glia showed ubiquitin-positive inclusions [*Clement et al., 2003*].

The importance of microglia for the pathological processes has been unravelled by experiments with deletable transgenes demonstrating that the lack of mutant SOD1 only in microglia and peripheral macrophages does not change the onset of the disease, but increases the survival [*Boillée et al., 2006a*].

The implication of astrocytes and microglia in ALS has led to attempts to inhibit gliosis in order to delay disease progression. Replacing astrocytes, transplanting mesenchymal stem cells, and performing bone marrow transplants have all proved somewhat successful in either reducing gliosis and/or extending survival of $SOD1^{G93A}$ mice [*Lepore et al., 2008*; *Ohnishi et al., 2009*; *Vercelli et al., 2008*].

Treatment with anti-inflammatory drugs such as minocycline reduces neuroinflammation and extends survival of $SOD1^{G93A}$ mice [*Neymotin et al., 2009*; *Yang et al., 2010*]. Following this work, a number of ALS patients sought a prescription for minocycline but unfortunately, with detrimental effect [*Gordon et al., 2007*].

2.3.6 Protein misfolding and aggregation

Protein misfolding and aggregate formation are key features in ALS patients and other proteinopathies. Convergence of a possible common disease mechanism between sALS and SOD1-linked fALS was established recently when an aberrant wild-type SOD1 conformation was identified in sALS and fALS tissue not carrying SOD1 mutations [*Bosco et al., 2010*].

It is unclear how protein misfolding or aggregate formation affects disease onset and progression or, indeed, what the mechanisms of SOD1 aggregation are. Mutations in SOD1 (and potentially TARDBP) can affect protein folding, creating an alternate ''misfolded'' conformation that can associate and form aggregates, potentially through cysteine- mediated disulphide crosslinking [*Karch & Borchelt, 2010*]. However, it is uncertain which protein species are toxic: the misfolded monomers, misfolded oligomers, or insoluble aggregates.

It is interesting to note that spinal cord homogenates derived from $SOD1^{G93A}$ mouse may promote aggregates formation in a prion-like fashion that, in turn, can penetrate cells and provoke aggregate formation [*Chia et al., 2010*; *Münch et al., 2011*]). If this phenomenon, observed in vitro, occurs also in vivo, it could explain the rapid ALS progression and the disease prion-like spread. In fact, at the beginning, the in vitro aggregates formation is slow, as the in vivo disease incubation period is, but later aggregates proliferate rapidly which, if translated in vivo, would result in widespread cellular toxicity and cell death [*Chia et al., 2010*; *Münch et al., 2011*; *Ravits et al., 2007*].

Finally, it has also been hypothesized that proteasome activity could be inhibited by SOD1 aggregates, leading to accumulation of aberrantly folded forms of SOD1 and other proteins [*Cleveland and Rothstein, 2001*] (**Figure 2.1 C**). Cheroni et al. (2009) reported decreased proteasome activity in lumbar spinal cords of SOD1 mice: this finding suggests a vicious cycle in which protein aggregation increases misfolded SOD1 levels [*Hoffman et al., 1996*] and

simultaneously sequesters essential cellular components such $SOD1^{WT}$ within the aggregates, causing further cell damage [*Bruijn et al., 1998*].

On the other hand, despite the TDP-43 aggregates prevalence in ALS patients, they are rare in TDP-43 models [*Wegorzewska & Baloh, 2011*], that invariably exhibit motor neuron degeneration or neuronal defects; thus, aggregate formation may not be required for TDP-43-induced neurodegeneration.

2.3.7 RNA processing

The discovery of the RNA binding protein TDP-43 in cytoplasmic aggregates of ALS patients and pathogenic TARDBP mutations in ALS suggests a role for RNA processing in ALS [*Gitcho et al., 2008*; *Kabashi et al., 2008*; *Neumann et al. 2006*; *Sreedharan et al., 2008*; *Van Deerlin et al., 2008*].

Further highlighting the emerging role of RNA processing in motor neuron degeneration is the identification of causative FUS mutations in ALS, another gene encoding a RNA-binding protein [*Kwiatkowski et al., 2009*; *Vance et al., 2009*]. Taken together, these observations suggest that a loss or alteration in normal RNA processing deleteriously affects motor neuron survival; however, it remains unclear which areas of RNA metabolism directly affect motor neuron survival. TDP-43 overexpression in mice causes a dramatic disruption of gene expression, suggesting an important role in transcriptional regulation [*Igaz et al., 2011*]. Further, in times of stress, TDP-43 and FUS associate with stress granules, that likely regulate the stability of selected mRNAs [*Anderson & Kedersha, 2006*; *Bosco et al., 2010*]. If this process is altered by TDP-43 mislocalisation, aggregation, or loss of function, TDP-43 may be unable to dissociate from these granules, translation of key housekeeping proteins may be inhibited, and motor neuron function/viability may be affected. Combined with this is the defect in RNA metabolism seen in SOD1 mouse models in which protein translation is disrupted by increased levels of oxidised mRNA, presumably through increased oxidative stress [*Chang et al., 2008*; *Volkening et al., 2009*].

However, these studies only begin to identify the mechanisms potentially involved in ineffective RNA processing in ALS. The analysis and creation of further TDP-43 animal models, the expected arrival of new FUS animal models, and the creation of SOD1 animal models alternative to standard mice will allow the ALS research community to investigate this mechanism further.

THE SWINE AS AN ANIMAL MODEL FOR NEURODEGENERATIVE DISEASES

Animal models of human diseases have always played a central role in biomedical research for the exploration and development of new therapies. However, the evolutionary gap between humans and many of the applied animal models (eg rodents) has always prevented a direct applicability of the acquired knowledge to human therapy. In this regard, the pig model offers many advantages. Being a domesticated eutherian mammal, the pig has evolved in the same way as humans and represents a taxon with different selected phenotypes [*Rothschild & Ruvinsky, 1998*]. On the other hand, the pig also represents an evolutionary clade distinct enough from primates and rodents to provide considerable power in the understanding of genetic complexity, that is, how genetic variation contributes to diverse phenotypes and diseases.

As outlined in **Table 3.1** the swine has been used as a model for various aspects of human biology, thanks especially to its physiology, organ development and disease progression but also to the possibility of studying its organs using standard human technologies and to perform repetitive sampling of tissues. Detailed drug susceptibility testing is facilitated by large cell lines availability. In addition, the swine genome sequencing shows a high homology with the human one, also as regarding the chromosomes structure, thus allowing improvements of genetic and proteomic tools for pigs. On the basis of these numerous propitious aspects the swine allows a great number of biomedical researches and, in order to facilitate their handling for experimental purposes, several miniature strains have been created.

2.3 TRANSGENESIS IN PIGS

Transgenic animals can serve as models for many human diseases. In mice, the technology of homologous recombination in embryonic stem cells (ESCs) provides an ideal tool for modelling genetically based disorders. Nevertheless, the relatively short lifespan of mice and the genetic and physiological differences

between mice and humans makes the comparison and application of data between the two species quite difficult.

Transgenic pigs can serve as an ideal biomedical model bridging this gap since they are physiologically closer to humans, have a longer lifespan than mice, and are easily bred in controlled conditions. The first genetic modification in pigs was accomplished through microinjection of hundreds copies of a foreign DNA into the pig zygotes pronuclei that resulted in the exogenous DNA and in the generation of the first transgenic pigs [*Hammer et al., 1985*].

Table 3.1: Genetically Modified Pigs for Use in Biomedicine

Use/modification	Issue addressed/ transgene activity	References
Xenotransplantation		
Membrane cofactor protein, MCP (CD46)$^{+}$	Hyperacute rejection: complement modifier	Diamond et al. (2001)
Human-decay accelerating factor, hDAF (CD55)$^{+}$	Hyperacute rejection: complement modifier	Langford et al. (1994)
CD59^{+}(MIRL)	Hyperacute rejection: complement modifier	Fodor et al. (1994)
α-1,2-Fucosyltransferase (FUT2)$^{+}$	Hyperacute rejection: carbohydrate remodeling	Koike et al. (1996)
β-1,4-Acetylglucosaminyltransferase (MGAT2)$^{+α}$	Hyperacute rejection: carbohydrate remodeling	Miyagawa et al. (2001)
α -1,3-Galactosyltransferase (GGTA1)$^{-/-}$	Hyperacute rejection	Lai et al. (2002a)
Heme oxygenase I (HMOX1)$^{+}$	Post-hyperacute rejection	Petersen et al. (2008)
Ecto-ATPase (CD39)$^{+}$	Post-hyperacute rejection	Dwyer et al. (2007)
Thrombomodulin (THBD) $^{+}$	Post-hyperacute rejection	Petersen et al. (2007)
Human leukocyte antigen, DP (HLA-DP)$^{+}$	Cell mediated rejection	Tu et al. (1999)
Human leukocyte antigen, DR (HLA-DR)$^{+}$	Cell mediated rejection	Huang et al. (2006)
Human leukocyte antigen, E (HLA-E)$^{+}$/B2M^{+}	Cell mediated rejection	Weiss et al. (2009)
Tumor necrosis factor ligand superfamily, member 10 (TNFSF10)$^{+}$	Cell mediated rejection	Klose et al. (2005)
Tumor necrosis factor-alpha-induced protein 3 (TNFAIP3)$^{+}$	Cell mediated rejection	Oropeza et al. (2008)
Fas ligand (FASLG)$^{+}$	Cell mediated rejection	Choi et al. (2010)
Cytotoxic T lymphocyte-associated 4 (CTLA4)$^{+}$	Nonvascular rejection	Martin et al. (2005)
Belatacept (LEA29Y)	Nonvascular rejection	Klymiuk et al. (2009)
Porcine endogenous retroviruses (PERV) knock-down shRNA	Porcine endogenous retrovirus	Dieckhoff et al. (2008)
Disease model		
Rhodopsin, mutant P347L (RHO P347L)$^{+}$	Retinitis pigmentosa	Petters et al. (1997)
Cystic fibrosis transmembrane conductance regulator (CFTR)$^{+/-}$ and CFTR$^{ΔF508/+}$	Cystic fibrosis	Rogers et al. (2008)
Huntington (HTT) $^{+}$	Huntington's disease	Uchida et al. (2001)
Omega 3 fatty acid desaturase^{+}	Cardiovascular disease	Lai et al. (2006)
Catalase (CAT)$^{+}$	Cardiovascular disease	Whyte et al. (2011)
Endothelial nitric oxide synthase 3 (NOS3)$^{+}$	Cardiovascular disease	Whyte & Laughlin (2010)
Proprotein convertase, subtilisin/kexin-type, 9 (PCSK9)$^{+}$	Familial hypercholesterolemia	Bolund et al. (2010)
Apolipoprotein E (APOE) knock-down shRNA^{+}	Familial hyperlipidemia	El-Beirouthi et al. (2009)
Gastric inhibitory polypeptide receptor, dominant negative (GIPR(dn))$^{+}$	Diabetes	Renner et al. (2010)
Hepatocyte nuclear factor-1 homeobox A, dominant negative (HNF1A(dn))$^{+}$	Diabetes	Umeyama et al. (2009)
Insulin 2, mutant C93S (INS2 C93S)$^{+}$	Diabetes	Renner et al. (2010)
Amyloid precursor protein (APP) K670N^{+} /M671L^{+}	Alzheimer's disease	Kragh et al. (2009)
Mouse mammary tumor virus (MMTV)/v-Ha-ras^{+}	Mammary tumors	Yamakawa et al. (1999)
Survival motor neuron (SMN)$^{+/-}$	Spinal muscle atrophy	Lorson et al. (2011)

Adapted from Whyte and Prather 2011

This ability to insert new genetic material and/or subsequently delete or replace genes opened new possibilities for the use of pigs as a research animal. The medical community had already accepted pigs as an excellent model for surgical testing based on their respective organs similarity to human heart, coronary vasculature, liver, kidney, lungs, and brain [*Swindle, 2007*].

The prospect of obtaining genetically modified pigs further extended their biomedical potential, especially to mimic inherited human diseases and to establish experimental DNA-altering treatments (i.e., gene therapy). Pigs are ideal for this type of genetic research as the size and composition of the porcine genome is similar to that of humans [*Bendixen et al., 2010*].

However, for a long time, the only technique for creating transgenic pigs has been pronuclear injection of a gene construct [*Uchida et al., 2001*] (**Figure 3.1 A**). DNA is microinjected into the pronuclei of zygotes collected from a super-ovulated female, and then transferred to a recipient pig by embryo transfer [*Hammer et al., 1985*]. The technique is reliable and widely used, but only about 1% of injected eggs produce transgenic pigs [*Niemann, 2004; Prather et al., 2008*]. Moreover this method suffers from several drawbacks, the most serious being the random integration sites of the gene construct in the host genome, the lack of control over transgene expression levels, and differences in transgene expression in the offspring.

Some alternative approaches addressing the above problems in transgenic pig production include sperm-mediated DNA transfer [*Lavitrano et al., 2002*] (**Figure 3.1 B**) a technique which not been confirmed in later studies.

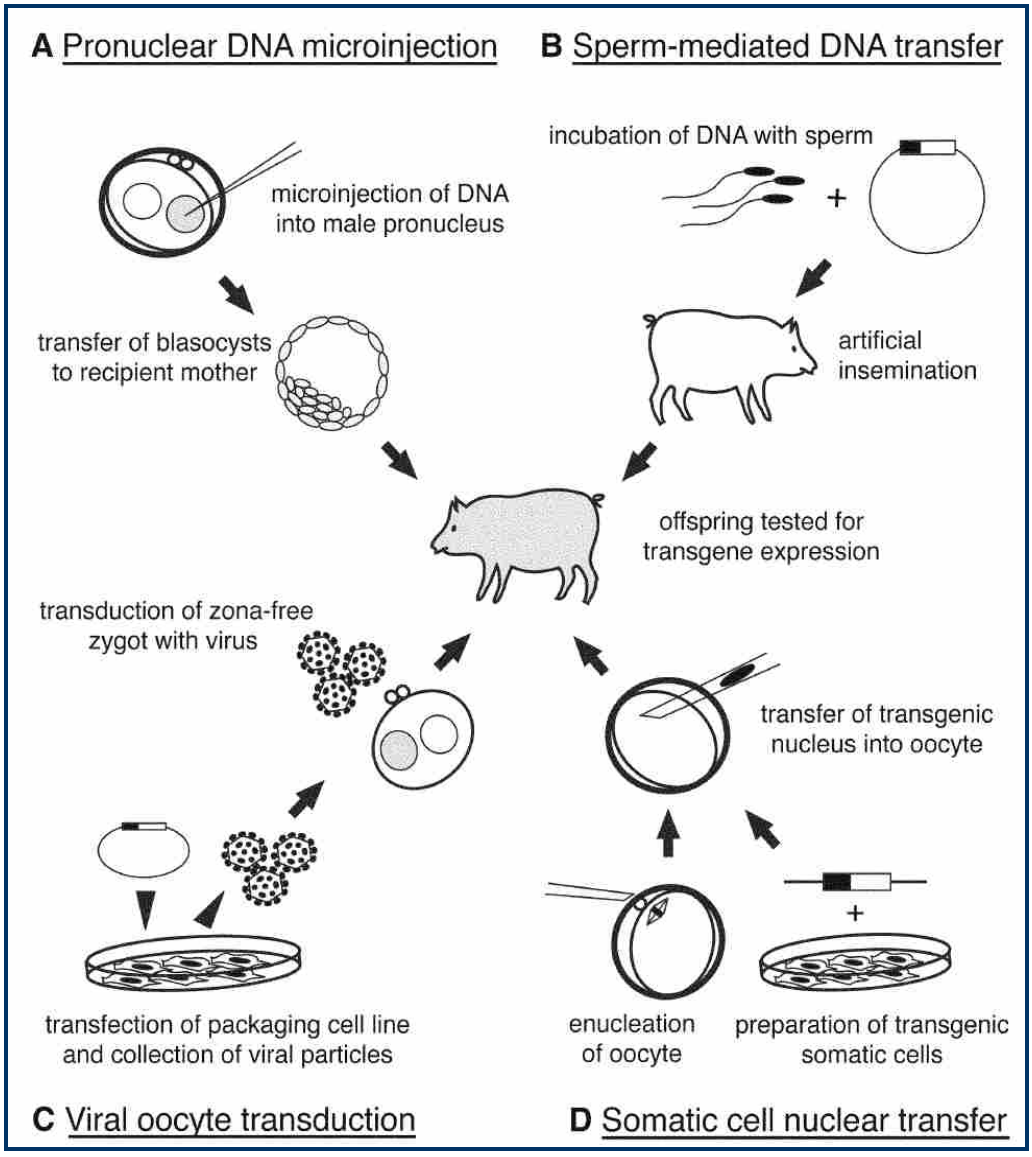
An alternative method, oocyte transduction, uses a replication deficient virus to deliver the transgene to the porcine oocytes and can increase the percentage of transgenic offspring with seventy percent of piglets born carry the transgene DNA [*Cabot et al., 2001*]. By this technique a recombinant lentiviral vector is injected into the perivitelline space of one-cell porcine embryos (**Figure 3.1 C**).

Lentiviral gene transfer results in a high efficiency of transgenesis and, importantly, the transgene is transmitted through the germ line [*Hofmann et al., 2003*]. However, retroviruses undergo epigenetic modification, and retroviral expression is shut off either during embryogenesis [*Jaenisch, 1976*] or shortly after birth [*Chan et al., 1998*]. Moreover the use of lentivirus (HIV-1; EIAV) poses many safety and ethical concerns, due to the proven ability to activate oncogenes as well as the possibility of re-acquiring their pathogenic characteristics. These methods suffer of some limitations including the inability to pre-screen embryos for transgene integration prior to embryo transfer, the lack of expression specificity arising from random integration of foreign DNA, and the fact that only transgene addition is permitted, not deletion (i.e., gene knockout). Targeted introduction of transgenes and loss-of-function mutations via homologous recombination in ESCs has been used for genetic manipulation of mice for decades [*Capecchi, 2000*], but this method is not established in pig because of lacking of ESCs stable source.

The most popular method of producing genetically modified pigs to date is through genomic modification of somatic cells followed by nuclear transfer (NT), first reported by Park et al. (2001) (**Figure 3.1 D**). In this process, the nuclei of somatic cells are transferred into enucleated metaphase II oocytes, and then this complex is activated by electrofusion. Reconstructed embryos are then cultured and transferred to synchronized recipients sows for gestation. The advantages of NT for gene transfer in pigs were described by Robl & First (1985), and nuclei from porcine blastomeres were used to produce the first cloned pigs from embryonic donor cells [*Prather et al., 1989*]. Soon after the demonstration that the nuclei of adult somatic cells could undergo proper reprogramming to produce viable mammalian offspring [*Wilmut et al., 1997*], the first cloned pig derived from differentiated cells (porcine fetal fibroblasts) was reported [*Onishi et al., 2000*] and was soon followed by the cloning of pigs from cultured adult granulosa cells [*Polejaeva et al., 2000*].

Figure 3.1: Methods available for the production of transgenic animals.

(**A**) Pronuclear DNA microinjection: Recombinant DNA is injected into the male pronucleus of a one-cell porcine embryo. Injected embryos are cultured *in vitro* up to the blastocyst stage or directly transferred into surrogate mothers. (**B**) Sperm-mediated DNA transfer: Linearized plasmid DNA is incubated with sperm. Sperm will internalize surface-bound DNA and can then be used for artificial insemination. (**C**) Viral oocyte transduction: The transgene transfected into a packaging cell line, and viral particles are collected. The virus is either injected into the perivitelline space between the zona pellucida and the zygote, or the zona pellucida is removed enzymatically, and the zona-free zygote is co-cultured with viral particles. The transduced embryos are transferred to surrogate mothers. (**D**) Somatic cell nuclear transfer: Somatic cells are transfected with the transgene and selected for transgene integration. The nuclei of transgenic cells are transferred into enucleated metaphase II oocytes. The activation of embryonic development is induced chemically or by electric current. The embryos are cultured up to the blastocyst stage *in vitro* and then transferred to surrogate mothers.



[Adapted from Vodička et al. 2005]

Despite these achievements, NT is an inefficient process in pigs, as it does in all mammals without stably cultured ESCs. Many reconstructed pig embryos fail early in pregnancy, requiring large numbers of embryo transfers per recipient. The prevailing view is that incomplete epigenetic reprogramming of donor cell nuclei results in aberrant gene expression during development [reviewed in *Zhao et al., 2010*]. Recently, some advances have improved the ability to genetically modify porcine donor fibroblasts to produce cloned transgenic pigs and by NT technology many useful disease models have been hitherto produced and are poised to have significant impacts in biomedical research.

2.4 BIOMEDICAL APPLICATIONS

2.4.1 Cell Tracking

One of the first transgenic swine models is cloned animals expressing enhanced green fluorescent protein (eGFP) [*Park et al., 2001; Lai et al., 2002b*]. Since that time, pigs expressing multi-gene fluorescent proteins (red, blue, green, and yellow) have been produced [*Matsunari et al., 2008; Webster et al., 2005*]. Tissues obtained from such transgenic pig are employed in studies that require marked cells or organs. For example, fetal eGFP-porcine cells were used to evaluate the retinal progenitor cells survival after an allograft in wild-type recipient pigs with damaged retinas [*Klassen et al., 2008*].

2.4.2 Pig-to-Human Xenotransplantation

The first landmark swine model derived specifically for human health was in the field of xenotransplantation. The urgent need for organs replacement is unmet by human organ donations, with over 110, 460 recipient candidates awaiting organ transplants in the United States [*United Network for Organ Sharing, 2011*]. The pig stands out as the most suitable donor for animal-to-human xenotransplantation because of the similar size and physiological capacity

of its organs. Practically, however, the use of xenografted tissues of non-human origin faces many immunologic barriers. This has spurred a large scientific effort to make pig organs transplantable to humans.

Rejection of pig organs by human recipients is caused by humoral and cell-mediated responses [reviewed in *Klymiuk et al., 2010*]. Hyperacute rejection (HAR) occurs within 24 hr of transplantation and results from antibody recognition of α1,3-galactose (α-Gal) epitopes synthesized by the enzyme α1,3-galactosyltransferase (GGTA1). Acute humoral xenograft rejection (AHXR), usually begins after the first week of transplantation, and is caused by mismatched human leukocyte antigen (HLA) complex antigens that are present on all cells.

To circumvent HAR, swine fibroblasts were produced with a knockout of the gene GGTA1. Production of cloned swine from these fibroblasts was a major victory in the search for human organ replacements [*Lai et al., 2002a*]. Antibody-initiated complement activation is one dominant mechanism through which AHXR occurs.

Transgenic cloned swine that overexpress the complement regulatory protein human-decay accelerating factor (hDAF) and an enzyme that modifies α-Gal,N-acetylglucosaminyltransferase III have been developed to protect against AHXR and HAR, respectively [*Fujimura et al., 2004*]. Swine with multiple genetic modifications (e.g., GGTA1 knockout combined with hDAF overexpression) appear to be the most promising route to widespread availability of organs for human transplant.

2.4.3 Cardiovascular Disease

Swine are historically considered an excellent model for the human cardiovascular system [*Turk & Laughlin, 2004*].

As a potential dietary source of essential and beneficial fatty acids, genetically modified pigs have been developed to express D12 fatty acid desaturase from spinach (Spinacia oleracea) [*Saeki et al., 2004*] to increase linoleic acid, as well as pigs expressing a humanized Caenorhabditis elegans gene, fat-1, encoding an n-3

fatty acid desaturase to increase the n-3/n-6 fatty acid ratio in meat [*Lai et al., 2006*]. These pig models can also be used to examine the cardiovascular effects of an altered n-3/n-6 fatty acid ratio in the swine themselves.

Another important regulator of vascular health is the signalling molecule, nitric oxide (NO). Transgenic swine that overexpress endothelial nitric oxide synthase (eNOS) [*Hao et al., 2006; Whyte & Laughlin, 2010*], responsible for producing NO in the inner lining of blood vessels, will increase our understanding of the complex regulation of vasodilation and potential therapies for diseases related to endothelial dysfunction.

These large-animal cardiovascular models will enable real-time measurement of functional parameters including blood flow, temperature, tissue oxygenation, perfusion and diffusion that are difficult or impossible to monitor in similar genetically modified rodent models.

2.4.4 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease [reviewed in *Messick, 2010*] caused by mutations in the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel. Symptoms of CF arise from faulty water and ion transport across epithelial tissues, leading to inadequate hydration of mucous secretions. Over 1,600 mutations related to CF have been described, and although several CFTR knockout mouse models have been developed, none of them develop the obstructive lung disease symptoms seen in humans [*Bragonzi, 2010*].

In an effort to produce an accurate CF model, pigs were cloned from fetal fibroblasts in which the CFTR gene was mutated to produce null and CF^{DF508} alleles [*Rogers et al., 2008; Welsh et al., 2009*]. These CF piglets display comparable defects, such pancreatic destruction and early focal biliary cirrhosis, to those occurring in newborn humans with CF [*Meyerholz et al., 2010*].

Recently, the CF pigs phenotype resolved a long-misunderstood question about the order of events in the inflammation/infection pathogenic cycle. The initial event displayed in CF pigs is impaired bacterial elimination from the lungs, followed by a cascade of inflammation and pathology [*Stoltz et al., 2010*]. This key finding about CF pathogenesis provides new options for CF therapy and prevention.

2.4.5 Alzheimer's disease

Alzheimer's disease (AD) is a progressive manifestation of dementia that typically begins with subtle failure of memory, gradually becoming more severe, and eventually, incapacitating the patient [reviewed in *Avramopoulos, 2009*]. The familial, autosomal dominant AD (FAD) is correlate to Presenilins and APP (amyloid precursor protein) genes mutations, that are associated with increased production of proteolytic fragment Aβ which aggregates into fibrils and toxic oligomeric forms, initiating synaptic damage and neurodegeneration [*Walsh et al., 2005*].

A genetically modified pig model for FAD was developed by Kragh et al., 2009. Porcine fibroblasts hemizygous (carrying a single copy) for a neuron-specific splice variant of human APP were generated via transfection with a cDNA construct, followed by NT cloning. This splice-variant carries an AD-causing dominant mutation known as the Swedish mutation [*Kragh et al., 2009*]. The transgene was expressed in the brain, but the authors speculate that it may take until the age of 1–2 years before Aβ accumulates to symptomatic levels in the porcine brain [*Götz & Götz, 2009*] thus reflecting the typical human onset, that for the majority (95%) of AD cases occurs in the adulthood.

Pig models that address the genetic foundations of other forms of AD could provide translational data for early detection and new treatment in patients.

2.4.6 Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is an autosomal-recessive neurodegenerative disease [reviewed in *Prior, 2010*]. Clinical manifestations of SMA include motor neuron loss and skeletal muscle wasting, which are consequence of deletion/mutation of the survival motor neuron (SMN1) gene.

A highly conserved gene (SMN2) is also present exclusively in humans. Mutations in SMN2 have no clinical consequence if SMN1 is retained. In the case where SMN1 is mutated, the disease severity correlates inversely with SMN2 gene copy number, although SMN2 alone cannot prevent the disease.

A recent porcine model for SMA was developed specifically to evaluate the efficacy, pharmacology, and toxicology of SMA therapeutics. This SMA porcine model is being produced in three stages: first, a knockout of the SMN allele produced $SMN^{+/-}$ pigs that, like their human $SMN^{+/-}$ counterparts, are phenotypically normal. Next, efforts are underway to introduce the human SMN2 transgene into swine fetal fibroblasts to eventually generate $SMN^{-/-}$/SMN2 pigs through breeding and a second round of nuclear transfer that will lead to completion of the pig model of SMA [*Lorson et al., 2011*].

Effective SMA therapeutics do not currently exist, highlighting the value of a genetically modified pig model for this disease.

2.5 THE USE OF SWINE IN NEUROSCIENCE

Rodents and primates have been the preferred animal species studied within neuroscience. However the use of pig experimental models has increased dramatically, reflecting the considerable resemblance of pigs to human anatomy and physiology. Despite considerable progress in understanding neurobiological disorders, there are enormous efforts for refinement of knowledge about the neurobiological processes underlying normal and the abnormal human behaviour.

In this context, the use of swine as an alternative animal species could be appropriate for obtaining advanced understanding of general neuronal and behavioural processes.

The potential for using pigs in paediatric brain research was recognized early [*Glauser, 1966*] due to the similarities in the extent of peak brain growth at the time of birth, the gross anatomy (i.e. gyral pattern, distribution of gray and white matter), and the growth pattern of neonatal porcine brain to that of human infants [*Dickerson & Dobbing, 1966; Thibault and Margulies, 1998*]. Subsequently, a substantial body of evidence has been gathered supporting the wider use of pigs rather than rodents for neuroscience research: through a literature search of the PubMed database using the keywords ''swine brain animal model'' it is possible to reveals four times as many hits in the period 1996–2005 than during the preceding 10-year period.

2.5.1 The swine as a laboratory animal

Agricultural pigs are most commonly used in research due to their ready availability and low price: the most common breeds are Landrace, Yorkshire, Hampshire and Duroc [*Bollen et al., 1999*].

The high body weight of mature animals, which can be as much as 300 kg, presents an obvious disadvantage. A reasonable alternative has been provided by the purpose-bred laboratory minipig, either the Yucatan or Hanford breeds, which have an adult body weight of 70–90 kg, or the Göttingen, Sinclair or Yucatan micropig breeds, which have an adult, final body weight of 35–55 kg.

In experimental research, the use of inbred animals generally serves to diminish variability in the outcome data. Consequently, the number of experimental animals required to reject a null hypothesis can be minimized. Inbred strains of rodents have been available for decades, but such pigs are not widely available. Monkeys have never been inbred, and it is difficult to obtain homogenous and standardized groups of outbreed monkeys.

Thus, the availability of standardization and purpose-bred laboratory pigs is becoming increasing advantageous. Pigs have a long life span (12–15 years), are easily bred, produce large litter size and have a short gestation period (typically 113–115 days) compared to monkeys. Young pigs grow fast, and normally attain puberty at 5–6 months for agricultural breeds, and at 3–4 months for mini and micropigs.

In contrast to rodents, the prolonged period of porcine puberty may present advantages for studies of neurodevelopment and plasticity at the time of puberty. If compared to rodents, pigs require far more space for housing, and costly pig-specific housing facilities are required. However, housing costs of pigs are considerably less than for monkeys.

Occurrence of infectious disease is an obvious detriment to any research study [*Hansen, 2002*]. However, enormous experience on the prevention and treatment of porcine infections, derived from agricultural experience, is readily available [*Hansen et al., 1997*].

Pigs are easily anesthetized and moreover, using positive reinforcement training, pigs can be trained to accept intramuscular injections, change of dressing, without provoking excessive stress, or requiring fixation. For long-term drug infusion, and for prolonged electrophysiological recordings such as electroencephalography (EEG) pigs can be partially immobilized in a sling, which they can be trained to tolerate for several hours if the legs are freely hanging [*Bollen et al., 1999*].

2.5.2 In vivo brain experimental techniques

Studies within the field of neuroscience encompass the use of a wide variety of applied scientific disciplines in order to assess neuroanatomical structures and neurochemical processes which constitute normal brain function, or which contribute to pathophysiology.

Much of the in vivo experimental instrumentation used for preclinical or clinical research has already been applied to pigs (e.g. PET, MRI, fMRI, EEG and EMG). A stereotaxic instrument and a brain atlas with stereotaxic coordinates, essential for the accurate implantation of electrodes and cannulae, and for the placement of injections, are available [*Bjarkam et al., 2004*; *Watanabe et al., 2001*].

The pig has proven to be a superior experimental animal for evoked potential (EP) recordings, a procedure that requires a relatively large brain. As a result, several functional mapping studies have been successfully performed in the pig brain [*Craner & Ray, 1991a,b; Palmieri et al., 1987*]. Basic principles developed for the earliest EP study of pig [*Adrian, 1943*] are still employed [*Fang et al., 2005b, 2006; Okada et al., 1999*]. Recently, it has also been possible to obtain EP scalp recordings in awake, non-sedated pigs [*Arnfred et al., 2004*].

The pig has been used in a wide range of imaging studies for experimental models of traumatic brain injury [*Duhaime et al., 2003; Grate et al., 2003; Munkeby et al., 2004*], Parkinson's disease [*Dall et al., 2002; Danielsen et al., 2000*] and stroke [*Sakoh et al., 2000, 2001*].

The size of the pig brain permits the identification of cortical and subcortical structures by conventional imaging techniques such as MRI [*Jelsing et al., 2005; Watanabe et al., 2001*] and PET (**Figure 3.2**). fMRI have recently been used to assess the development of the pig brain with respect to functional activity after somatosensory stimulation [*Fang et al., 2005b, c, 2006*], and in the course of postnatal myelination [*Fang et al., 2005a*].

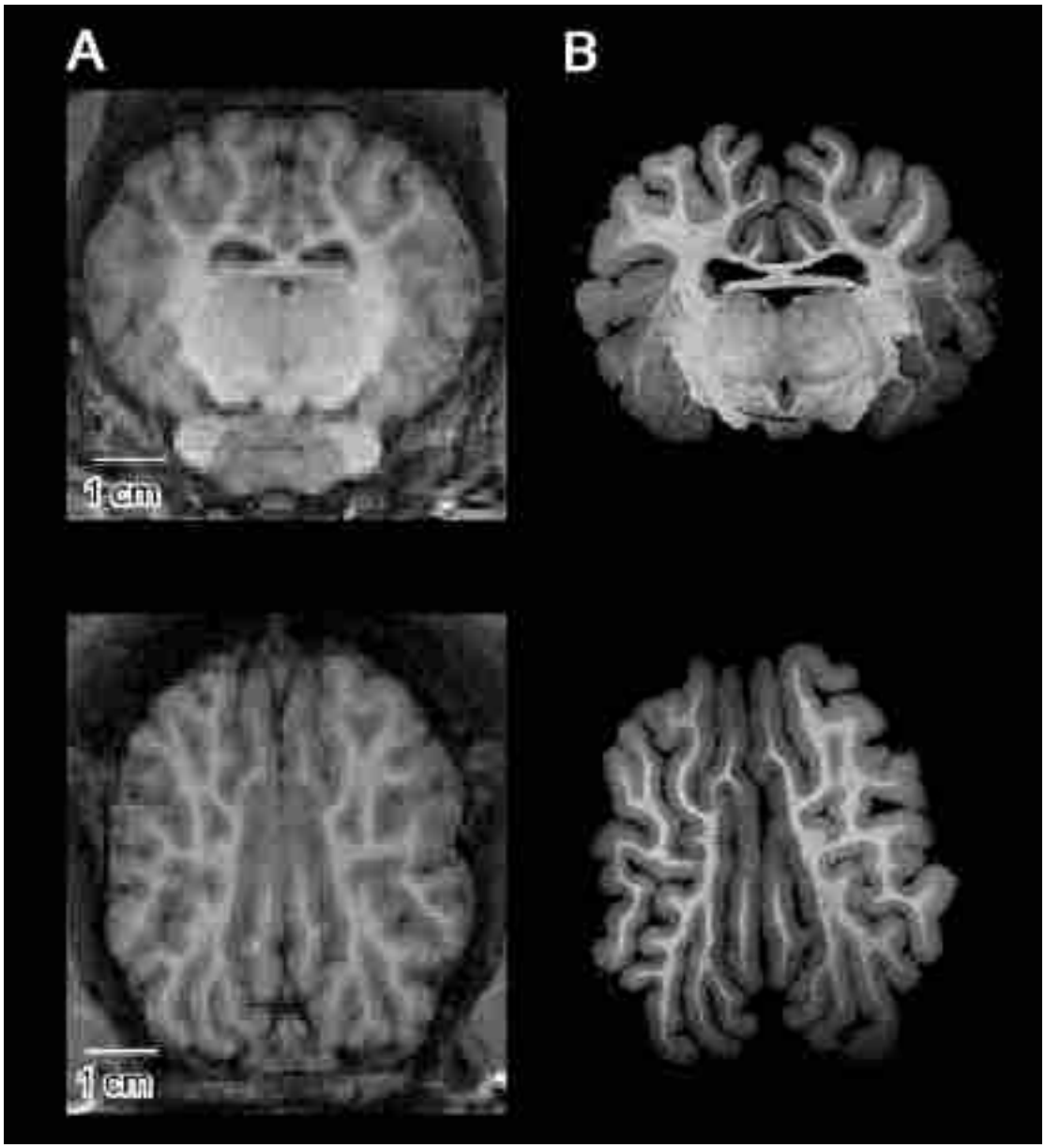
Thus, the pig has in recent years been usefully employed in studies encompassing the entire range of modern imaging techniques.

2.5.3 Anatomy of the porcine brain

Whereas the rat cerebral cortex is lissencephalic, the pig brain cortical surface more closely resembles human gyrencephalic neocortex [*Hofman, 1985*]. Similarities in the gross anatomy of pig brain to that of human brain has also been

demonstrated for the hippocampus, a limbic structure [*Dilberovic et al., 1986; Holm & Geneser, 1989*], as well as for subcortical and diencephalic nuclei [*Felix et al., 1999; Larsen et al., 2004*], and brainstem structures [*Freund, 1969; Ostergaard et al., 1992*]. The sulcal and gyral anatomy the pig brain has been described in detail in a number of studies [*Craner & Ray, 1991b; Jarvinen et al., 1998; Jelsing et al., 2006b; Okada et al., 1999*].

Figure 3.2: Structural MRI obtained in vivo from a young Danish Landrace pig on a Siemens 3 T scanner (**A**) and postmortem in a 4.7 T Varian scanner (**B**).



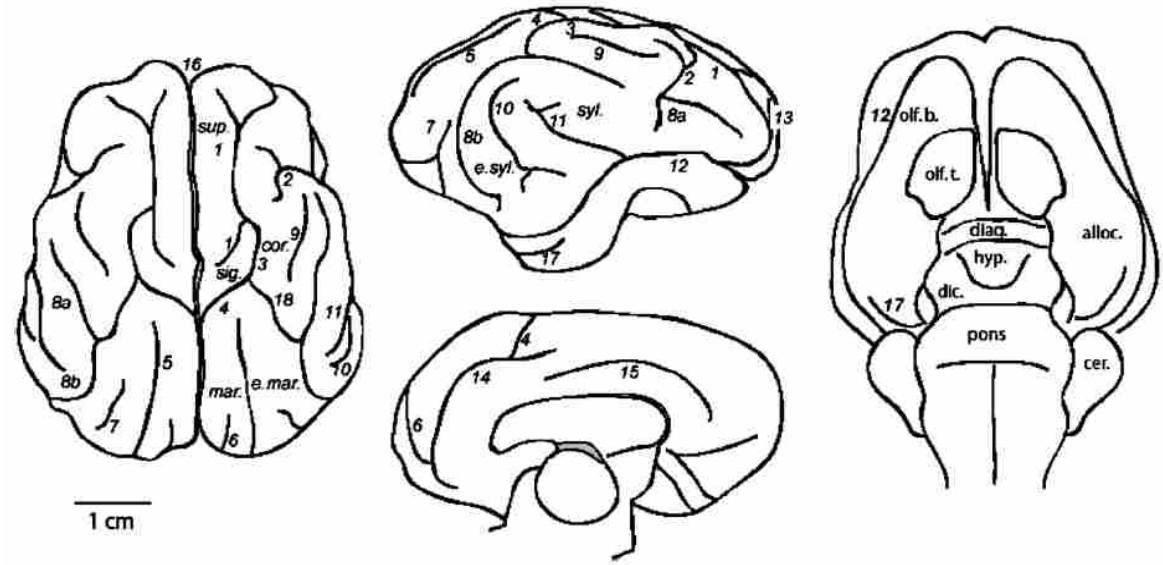
[Adapted from Lind et al. 2007]

The extent and pattern of the cortical convolution pattern show similarities with carnivore gyrification [*Myasnikov et al., 1994*] (**Figure 3.3**).

A number of neuroanatomical studies have been performed on the pig cerebrum including comprehensive descriptions of the allocortex and associated limbic structures [*Kruska & Stephan, 1973*], especially the hippocampus [*Dilberovic et al., 1986*]. There have also been a number of functional/anatomical studies of specific cortical regions, e.g. occipital cortex, auditory temporal cortex [*Plogmann & Kruska, 1990*], somatosensory cortices [*Craner & Ray, 1991a, b*], motor cortex and prefrontal cortex [*Jelsing et al., 2006b*].

Figure 3.3: Schematic illustration of sulci and gyri patterns in the adult Göttingen minipig brain.

Abbreviations: (**1**) cruciate sulcus; (**2**) diagonal sulcus; (**3**) coronal sulcus; (**4**) ansate sulcus; (**5**) lateral sulcus; (**6**) entolateral sulcus; (**7**) ectolateral sulcus; (**8**) suprasylvii sulcus; (a) anterior, (b) posterior; (**9**) sulcus naris; (**10**) ectosylvia sulcus; (**11**) sylvii sulcus; (**12**) rhinal sulcus; (**13**) praesylvii sulcus; (**14**) splenial sulcus; (**15**) cingulate sulcus; (**16**) longitudinal sulcus; (**17**) sagittal sulcus; (**18**) coronal sulcus.
cereb., cerebellum; cor., coronal gyrus; diag., diagonal band of Broca; dienc., diencephalons; e.mar., ectomarginal gyrus; e.syl., ectosylvian gyrus; hyp., hypothalamus; mar., marginal gyrus; olf. b., olfactory bulbus; olf. tub., olfactory tubercle; sig., sigmoid gyrus; sup., superior frontal gyrus; syl., sylvian gyrus.



[Adapted from Lind et al. 2007]

These studies have been based on cytoarchitectonics [*Jelsing et al., 2006b; Kruska, 1970*], electrophysiology [*Craner & Ray, 1991a, b*], and neuronal tract tracing [*Jelsing et al., 2006b*].

The topology of the pig hippocampus, hidden within the temporal lobe, indicates a degree of encephalization lying between that of rodent and primate brain [*Holm and West, 1994*]. The topology of subcortical nuclei are available from stereotaxic atlases of the pig brain [*Felix et al., 1999; Salinas-Zeballos et al., 1986*] and from PET studies in which the distribution of dopamine receptors within the basal ganglia is delineated [*Ishizu et al., 2000; Rosa-Neto et al., 2004*].

The pig brain striatum is similar to that of primates, but in contrast to the rat, is clearly divisible into a caudate and putamen, separated by the internal capsule. The thalamus, subthalamus and hypothalamic nuclei of pig brain have been described in detail, based both upon standard histological stained sections [*Felix et al., 1999*] as well as immunohistochemistry [*Larsen et al., 2004*].

The hypo- and subthalamic nuclei have a high structural correspondence with the homologous structures in rat, non-human primate and man [*Larsen et al., 2004*], whereas the development of the pig thalamus seems more similar to that of primates.

The cerebellum of pig has been investigated in relation to the organization of the cerebellar fissures, the deep nuclei [*Riet-Correa et al., 2003*], and the prenatal and postnatal development [*Jelsing et al., 2006a*]. Furthermore, cerebellum has been considered a key structure for investigations of brain growth [*Pond et al., 1990*), toxicology and comparative morphometry [*Jelsing et al., 2006a*].

The weight of the adult pig brain ranges from 80 to 180 g, depending upon adult body size, breed, as well as duration of domestication [*Herre, 1936*]. This weight is more than 50 times greater than that of rat brain, and is comparable to the brain mass of several non-human primate species used for experimental purposes.

The cortical convolution or gyrification is also more comparable to primate than that of rat. The total number of neocortical neurons is approximately 430 and 325 million respectively in the Danish Landrace pig and in Göttingen minipig [*Jelsing et al., 2006c*]. In comparison, the total number of neocortical neurons in rat brain is 21 million [*Korbo et al., 1993*], versus the 19–23 billion neurons in the human cerebral cortex [*Pakkenberg & Gundersen, 1997*].

Compared with the young individual of rodents, carnivores, and other ungulates, the development of the neonatal pig including the brain appears in many respects to be more like that of the human newborn infant. The rate of intrauterine development of the fetal pig is similar to that of the human fetus, and the pig has consequently become the standard experimental model for human embryology and brain development. The pig brain growth spurt, like that of the human, seems to extend from late prenatal to early postnatal life [*Jelsing et al., 2006c; Pond et al., 2000*], whereas the neurogenesis of rodents occurs almost entirely in the postnatal period.

Furthermore, the maturation of the postnatal pig brain is comparable to that of humans with respect to myelination, composition [*Mayhew et al., 1996; Thibault & Margulies, 1998*] and electrical activity [*Pampiglione, 1971*].

AIM OF THE THESIS

The purpose of this thesis is the production of a swine model for ALS that will allow us to assess if this species may represent a more suitable model than the currently employed transgenic mice in recapitulating the disease. Therefore, the main objective is the birth of healthy piglet carrying the hSOD1^{G93A} ALS related mutation, which was achieved through the following intermediate aims.

The first aim is the production and characterization of ALS expression vectors, carrying the hSOD1^{G93A} mutation, the most and best studied in transgenic rodents.

The second aim is the production of swine hSOD1^{G93A} transgenic fibroblast colonies (PAF^{G93A}) to use in SCNT experiments. Then, I will assess the hSOD1^{G93A} related effect on the early stage of embryo development, by in vitro culture of NT-blastocyst.

The third aim is the birth of transgenic piglets, in which the transgene expression will be verified.

Finally, possible methodological approaches aimed at the clinical and phenotypic characterization of the ALS swine model will be identified, with particular regards to the comparative aspects between the knowledge coming from rodent models and those from human patients.

MATERIALS AND METHODS

5.1 PRODUCTION OF ALS EXPRESSION VECTORS

5.1.1 Vector construction

The sequence homology analysis and the constructs design were possible thanks to the use of the VectorNTI-11 software (Invitrogen, Carlsband, CA).

Restriction, modification (T4 polymerase and S1 Nuclease) and ligation (T4 ligase) enzymes have been purchased by Fermentas (Vilnius, Lithuania) and used as reported in their manual.

The ligation products, obtained through the use of T4 ligase, were transfected into competent 1SHOT match1 CCDB survival E. coli cells (Invitrogen). The bacterial growth was performed on LB-agar medium (LB medium + 15g / l agar-agar), supplemented with the appropriate antibiotics, at 37° C overnight. The bacterial clones were grown in liquid medium, overnight at 37 ° C in 3 ml of LB medium inocula (10g NaCl / l, tryptone 10g / l, yeast extract 5 g / l, water up to volume) with an addition of the appropriate antibiotics (= Ampicillin 50 mg / ml Kanamycin 50 mg / ml and chloramphenicol 52 mg / ml), aliquoted in 13 ml ventilated sterile tubes. The mini preparation of plasmid DNA (miniprep) involves the alkaline lysis of the culture and the DNA precipitation by the addition of 0.9 volumes of isopropanol to the supernatant obtained by centrifugation after inactivation of bacterial lysis.

All plasmids used in molecular cloning steps were purified by an ion exchange columns kit NucleoBond PC 20 (Macherey-Nagel, Neumann, Germany). The water used in each procedure is microfiltered and sterile without nuclease.

The Avantea research group has previously developed an ubiquitous eGFP expression vector, driven by the pCAGGS hybrid promoter (CMV-IE enhancer + chicken beta actin promoter) [*Niwa et al., 1991*] which is characterized by maintaining high expression level through the F1 generation of pigs [*Brunetti et al., 2008*].

A Destination Vector pMGOrfA5'3'MARpuro5171 (**Figure 6.1**) was created inserting the Multisite Gateway system (Invitrogen) Conversion cassette (OrfA) into the ubiquitous expression vector.

The resulting vector carried the pCAGGS promoter inserted between two insulators (5' MAR of chicken lysozyme gene) [*McKnight et al., 1992*] to prevent various silencing effects (positional or copy number effects). The structure was also provided with a floxed, then removable using the Cre recombinase, SV40-Puro cassette to select the transfected clones.

By restriction of both pcDNA3.0hSOD1^{G93A} and pcDNA3.0hSOD1^{wt} plasmids (**Figure 6.3 a,b**) the *Bam*HI/*Xho*I fragment of hSOD1^{G93A} cDNA and hSOD1^{wt} cDNA, respectively, was obtained. These fragments were inserted into the pENTRL1L2Oligo*Sac*I*Sal*I vector, obtaining either the "EntryClone" pENTRL1L2-hSOD1^{G93A} or the EntryClone" pENTRL1L2-hSOD1^{wt} (**Figure 6.5 a,b**).

On these vectors, by double digestion with the restriction enzymes *Sal*I and *Bam*HI, a deletion has been operated, in order to restore the open-reading frame, obtaining the pENTRL1L2-hSOD1^{G93A}delSB and the pENTRL1L2-hSOD1^{wt}delSB vectors respectively. These vectors are then used, after sequencing, in LR exchange reactions with the Destination Vector pMGOrfA5'3'MARpuro5171. Theses exchange reactions, mediated by the LR Clonase, were used to transform chemically competent *E.coli* cells (One Shot Mach1-Invitrogen).

The resulting pMG5'3'MARPuro5171-hSOD1^{G93A} and pMG5'3'MARPuro5171-hSOD1^{wt} vectors (**Figure 6.8 a,b**) were purified with Plasmid Mini Kit (Qiagen, Hilden, Germany), analyzed by different restriction enzymes, confirmed by sequencing and finally linearized by *Apa*LI (Fermentas). After phenol/chloroform purification, the vectors were precipitated and re-suspended in TE buffer.

5.1.2 Cell isolation and culture

Primary porcine fibroblasts cultures were recovered from adult male ear biopsy (pig adult fibroblasts, PAFs). Biopsy specimens were cut in small pieces

with a scalpel blade and the resulting tissue pieces were distributed on the surface of gelatin-coated dishes containing 1.5 ml of DMEM/TCM199 with 20% of fetal bovine serum (FBS). Culture medium was changed every 3 days.

Cells were allowed to grow until they reached 50% of confluence. Tissue pieces were then removed and the cells sub-cultured until they reached confluence in DMEM/TCM199 with 10% of FBS and growth factors (βFGF). Growth conditions consisted of a 38°C temperature and of an atmosphere composed by 90% N_2, 5% O_2 and 5% CO_2.

Exponentially growing cultures were cryopreserved in DMEM/TCM199 with 20% FBS and 10% DMSO and stored in liquid nitrogen. These batches of cells were used throughout the following experiments.

5.1.3 Fibroblasts Transfection with hSOD1^{G93A} vectors

The day before transfection, passage 3 PAFs were trypsinized, counted, and plated into 60-mm dishes in order to obtain about 1x10^6 cells at 80% confluency in 24 hours. On the transfection day, cells were trypsinized, counted and resuspended in 100μl of nucleofector solution (Basic Nucleofector Kit, Prim. Fibroblasts; Amaxa, Cologne, Germany), and mixed with 5μg of both pMG5'3'MARPuro5171-hSOD1^{G93A} and pcDNA3.0hSOD1^{G93A} linearized vectors. Then PAFs and linearized vector were transferred into the nucleofection cuvettes and transfected with V-24 program (Nucleofector Amaxa).

After nucleofection, cells were plated in 60-mm culture dishes containing fresh culture medium. After 24 hours, the drug (Puromycin: [1 μg/ml], G418 (Neomycin analogue): [1mg /ml]) employed in colonies selection was added. After 4 days (for puromycin) or 15 days (for neomycin), drugs-resistant colonies were picked up using 5-mm cloning discs and transferred into 24-mm well dishes. Cells were then expanded in DMEM/TCM199 with 10% FBS and 5ng/mL of βFGF at a temperature of 38.5°C and in a humified atmosphere containing 5% CO_2 and 5% O_2.

An aliquot was cryopreserved as described above, to be employed in nuclear transfer and the remaining cells were sub-cultured to perform expression analysis.

5.1.4 Immunocytochemistry (ICC)

Cells (transgenic hSOD1^{G93A} PAFs and primary culture cells from ear biopsy obtained from transgenic piglets) were fixed with 4% PFA and then stored in phosphate buffer (PB) until analysis.

In order to detect the expression of human SOD1 by ICC a rabbit polyclonal antibody (07-403 Millipore, concentration 1:200), directed against the full-length wild-type hSOD1 plus an N-terminal methionine is used. Unspecific binding blocking (10% goat serum) was followed by primary antibody incubation (RT, two hours). After 3 washes (PB with 0.2% BSA and 0.05% saponine, 3 minutes each) a secondary FITCH-conjugate antibody incubation (1 h at RT) was performed. Nuclei were counterstained with Hoechst (RT, 15'). After two washes slides were finally mounted in mounting medium (Citifluor).

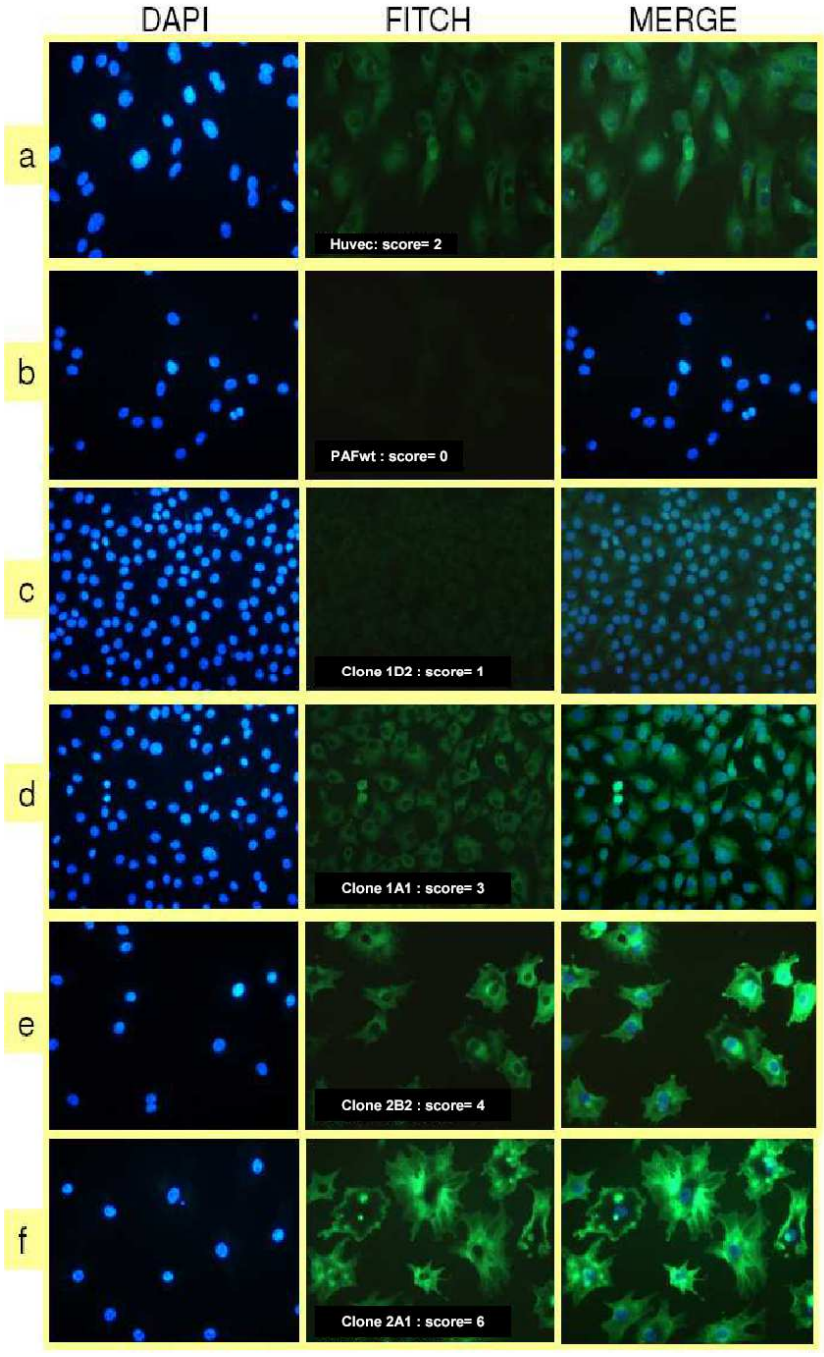
Human Umbilical Vein Endothelial Cells (Huvec) and wild type PAFs were used respectively as positive and negative controls.

Fluorescence was detected by exposing fibroblasts to an epifluorescent light mounted on an inverted microscope (Nikon TE-DH100W) equipped with a FITC (for green) and DAPI (for nuclei) filter and with a digital imaging system (Nikon DIGITAL SIGHT DS-L1).

Subjective assessment of transgene expression level was based on the comparison of the fluorescence intensity revealed in PAFs colonies with that of the controls. The negative control was assigned a Score=0 while the positive a Score=2, as showed in **Figure 5.1**. It should be noted that this is as subjective score attribution and values presented in this study are not objectively measured, neither absolute.

Figure 5.1: ICC on PAF colonies transfected with pMG5'3'MARPurohSOD^{G93A} vector

[a] Human Umbilical Vein Endothelial Cells (Huvec) used as positive control, with score=2 of transgene expression level. [b] wild-type PAF used as negative control (score=0), [c] PAF clone 1D2 with score=1, [d] PAF clone 1A1 with score=3, [e] PAF clone 2B2 with scores=4 and [f] clone 2A1 with a score=6. DAPI is the filter used to highlight the Hoechs nuclear staining. FITCH allows the detection of the fluorophore conjugated with the secondary anti-rabbit antibody and then the transgene expression. MERGE is the overlapping of the two images.



5.2 PRODUCTION OF ALS PIGS

5.2.1 Animal experimental procedures

All procedures involving animals and their care are conducted in conformity with national and international regulations (EEC Council Directive 86/609, OJL358, 1, 12 December 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana 10, 18 February 1992; and Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996) and after the approval of LTR-Avantea Local Ethic Committee. Yucatan Black PAFs were obtained from adult boar ear biopsy, coming from Italian pig farming. Subsequently PAF have been used in cloning procedures to obtain wild type pigs. Experimental pigs, born at the LTR facilities in Cremona, and raised there up to one year of age,are subsequently transferred and allocated at C.I.S.R.A. (Centro Inter-dipartimentale Servizio Ricoveri Animali) of the Faculty of Veterinary Medicine of Turin.

5.2.2 Preparation of nuclear donor cells and SCNT

Transgenic 1A1, 1A2, 1B1, 1C2, 1D1, 1D2, 1E2, 2A2, 2A6, 2B2, 2C1 2C3 pMG5'3'MARPuro-hSOD1^{G93A} and #C5 pcDNA3.0hSOD1^{G93A} fibroblasts colonies were selected, as nuclei donors, according to uniformity and intensity of their expression level.

The day before nuclear transfer, donor cells were induced into quiescence by serum starvation (0.5% FBS). 30 minutes before nuclear transfer, cells were prepared by trypsinization, washed and resuspended in SOF [*Tervit et al., 1972*] supplemented with 25mM HEPES (H-SOF).

Ovaries with *corpora lutea* were collected at local slaughterhouse and carried to laboratory at 31–33°C. Oocytes were aspirated from follicles larger than 3 mm in diameter, washed, and transferred to maturation. DMEM-F12 supplemented with 10% FBS, 110 μg/ml sodium pyruvate, 75 μg/ml ascorbic acid, 100 μg/ml

glutamine, 5μg/ml myoinositol, 0.4mM cystine, 0.6mM cysteamine, ITS liquid media supplement (insulin, transferrin, selenite, Sigma, 1μl/ml), gonadotropins (0.05 IU/ml FSH, and 0.05 IU/ml LH; Pergovet 75, Serono), 100ng/ml IGF1 (recombinant insulin growth factor I analog), 50ng/ml EGF (recombinant epidermal growth factor analog), and 5ng/ml bFGF (human recombinant) was used as maturation medium.

Oocytes were cultured at 38.5°C in 5% CO_2 in humidified atmosphere. After 42 hours maturation, oocytes were denuded of cumulus cells by vortexing in the presence of hyaluronidase in H-SOF and returned to maturation medium. Only oocytes displaying extruded polar body were selected. NT-embryos were reconstructed following a zona-free method [*Lagutina et al., 2005, 2006*; *Oback et al., 2003*]. The zona pellucida of oocytes with extruded polar body was digested with 0.5% pronase in PBS. The oocytes were washed in H-SOF with 10% FBS and returned to maturation medium. All the following manipulations were performed in H-SOF with 10% FBS. Zona-free oocytes were exposed to cytochalasin B (5μg/ml) and Hoechst (5μg/ml) for 5 min prior to enucleation. Metaphase chromosomes were removed under very short exposure to UV light with a blunt enucleation pipette.

After enucleation, zona-free cytoplasts were individually washed for few seconds in 300 μg/ml phytohemagglutinin P in PBS and then quickly dropped over a single donor cell [*Vajta et al., 2003*] settled at the bottom of a microdrop of the diluted donor cell suspension. Forty-six to forty-eight hours after maturation onset, formed cell couples were washed in 0.3 M mannitol (Ca^{2+}free, 100μM Mg^{2+}) solution, fused by double DC-pulse of 1.2 kV/cm applied for 30 μsec and returned to maturation medium. 2 hours later (after 48-50 hours of maturation), NT embryos were activated by double DC-pulses of 1.2 kV/cm for 30 μsec applied in 0.3M mannitol solution, containing 1mM Ca^{2+} [*Cheong et al., 2002*] and 100μM Mg^{2+}.

After activation, embryos were kept in culture maturation medium with 5µg/ml cytochalasin B for 4 hours. NT embryos were cultured in SOF supplemented with essential and nonessential amino acids and with 4mg/ml BSA (SOFaa) in a Well-of-the-Well system (WOW) modification [*Vajta et al., 2000*]. During embryo culture half of the medium was renewed on day 3 and on day 5 with fresh SOFaa.

5.2.3 Embryo transfer and farrowing

Estrus was synchronized by feeding 12 mg of altrenogest (Regumate, Intervet, Peschiera Borromeo, Italy) per sow for 15 days and injecting 0.15mg of PgF2a (Dalmazin, Fatro, Ozzano Emilia, Italy) on the 15th day of regumate treatment and 1000 IU of hCG (Chorulon, Intervet) 96 hours after the last altrenogest administration. The SCNT embryos were transplanted to the sows uterus on day 5 of development. Embryo transplantation was performed 4 days after animals ovulation by midventral laparatomy and pregnancy was examined at day 29, 36, 50, and 62 by ultrasonography. A cesarean delivery was performed at 114th day of gestation.

5.3 TRANSGENE INTEGRATION ANALYSIS

5.3.1 PCR

Genomic DNA from wild type and SOD1^{G93A} swine tissues, was incubated at 55°C overnight with lysis solution (100 mM Tris HCl pH 8.5, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl) plus 100 µg/ml of ProteinaseK/ (Promega, Madison, WI). The lysate was subsequently treated with phenol: clorophorm: isoamilalcool (25:24:1) to remove proteins and finally precipitated with 0.9 volumes of isopropanol by centrifugation (10', 16.100 g) and washed with 500 µl of 70%ethanol (5', 16.100 g). After ethanol removal, pellets were air dried, resuspended in TE buffer. Genomic DNA thus obtained was used in both Polymerase Chain Reaction (PCR) and Southern blot analysis. DNA quantification

was carried out using the fluorimeter Qubit (Invitrogen). The oligonucleotides design for PCR analysis was possible thanks to the use of the VectorNTI-11 software (Invitrogen, Carlsband, CA). For PCR analysis the PX2 thermocycler (Thermo, Dreieich, Gremania), the polymerase GoTaq Flexi-(GoTaq Flexi-Enzime mix - Promega, Madison, WI) in the presence of dNTPs (0.2 mM each), MgCl2 (1.25 mM) and oligonucleotides (1 mM each) were used. Briefly, 20 µl of reaction mixture [1.25mM $MgCl_2$, 1µM Primer-Fw (ATGGACCAGTGAAGGTGTGGG), 1µM Primer-Rv (AGTGTGCGGCCAATGATGC), 0.2mM dNTPs, Buffer 1x, Taq 1U] were added to 5 µl of DNA and then used as template for amplification cicles [1x (94°C, 2''), 35x(denaturation: 94°C, 30''; annealing: 65°C, 30''; elongation:72°C, 20''), 1x(72°C, 7')].

5.3.2 Southern Blot

As regards to Southern blot analysis, the SOD1-DIG probes labeled with digoxigenin were obtained by amplification using the DIG DNA Labeling and Detection kit (Roche), following the same cycling protocol used for PCR. Probes were then precipitated with absolute ethanol and finally resuspended in sterile ultrafiltered water. 10 µg of genomic DNA, extracted from $hSOD1^{G93A}$ swine, were subjected to restriction analysis with *Sal*I + *Bgl*II (10U/µgDNA) enzymes and incubated overnight at 37° C. The next day samples were precipitated with 0.9 volumes of isopropanol by centrifugation (10', 16.100 g) and washed with 500 µl of 70%ethanol (5', 16.100 g). After ethanol removal, pellets were air dried, resuspended in TE buffer and eventually subjected to electrophoresis migration overnight at 15V plus 2h30' at 25V in 0.7% agarose gel. DNA samples were then transferred by capillarity on modified nylon membrane (Hybond-N^+, Amersham) under denaturing conditions (NaOH 0.4N, 5h). The membranes thus obtained were hybridized with the probe SOD-DIG (20ng/ml) at 42°C overnight, and processed the next day (blocking of nonspecific binding, incubation with anti-digoxigenin secondary antibody conjugated with alkaline–

phosphatase, with chemiluminescent substrate and signal detection) following the technical specifications of DIG Luminescent Detection Kit (Roche). Finally membranes were used to impress (3h) photographic plates BIOMAX (Kodak) that subsequently have been developed (5') and fixed (10') in darkroom using GBX (Kodak) chemical reagents.

5.4 FOLLOW UP OF ALS PIGS

Characterization will be performed by investigations on preclinical and symptomatic animals and will consist of clinical and neurological examinations, associated with anatomopathological, neurophysiological, biochemical and neuroimaging analyses.

5.4.1 General clinical assessment

Animals characterization includes evaluation of eventually occurring symptoms: swine health conditions and neurological phenotype will be assessed and serum will be periodically drawn to evaluate cellular/biochemical profile.

5.4.1.1 General clinical examination

Animals will be daily examined to assess their general health condition: food and water consumption, weight, feed conversion rate and stress/distress signs will be monitored.

5.4.1.2 Neurological clinical picture assessment

Specific neurological examination will be monthly performed by a team composed of specialists in human and veterinary neurology and in swine behaviour. If neurological alterations are detected, such examination will be intensified once a week.

5.4.1.3 Serological investigations

Blood samples will be monthly collected and a complete cellular and biochemical profile will be performed. If pathological affections occur, further serological investigations will be set up on affected individuals.

5.4.2 Specific investigations

A 3D Motion Capture system will be designed to evaluate swine locomotor function and its alterations. Moreover, neurophysiological and neuroimaging analysis will be employed to characterize ALS phenotype in the hSOD1^{G93A} swine.

5.4.2.1 Locomotor function assessment

Gait and movement alterations will be analyzed: animals will be evaluated periodically in order to detect alterations in coordination, strength, endurance and balance. A specifically-designed 3D Motion Capture system will be developed to evaluate objectively the locomotion capability of the animals and to detect minimal and precocious gait alterations.

5.4.2.2 Electrophysiological clinical picture

In order to study neuronal damage induced by the mutated protein accumulation, the feasibility of electromyography application on living pigs will be evaluated. Custom-made electrodes and detection systems will be developed to test for the potentialities of superficial EMG techniques in the assessment of the neuromuscular function.

5.4.2.3 Neuroimaging analysis

MRI on the living pigs brain will be performed in a suitable facility during preclinical and clinical phase in order to define physiological and pathological aspect of the hSOD1^{G93A} swine cortico-spinal tract.

5.4.3 Proteomics

The identification and quantification of plasmatic protein differentially expressed by transgenic swine and controls will be performed through a proteomic approach. Definition of a set of candidate proteins that may act as mechanistic/ prognostic ALS biomarkers will be conducted.

5.4.3.1 Protein extraction from plasma

Since plasma is a highly complex mixture of proteins/peptides with a high dynamic range, adequate separation of the sample will improve the success of MS-based proteomic analysis including the identification and quantification of proteins that are present at low levels. To this purpose, depletion of the most abundant plasma proteins will be performed to increase the detection limit of low-abundance proteins.

5.4.3.2 Proteins pre-fractionation

Protein extracts will be run in triplicates on 1-DE gel electrophoresis. Each gel lane will be manually cut into 24 bands of equal height. Excised bands will be crushed into small fragments, processed, submitted to in-gel trypsin digestion and peptide extractions. The final sample will be referred as digest which contains all peptides recovered from the digestion of a single gel-band.

5.4.3.3 Mass-spectrometry-based protein identification and quantification

Aliquot of each digest will be directly analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with an LTQ Orbitrap XL™ interfaced with a 1200 series Agilent capillary pump. The MS/MS data from the 24 gel bands will be submitted to the search engine Mascot (Matrix Science, UK). Scaffold software (Proteome Software Inc., US) will be used to validate MS/MS-based peptide and protein identifications. Peptide and protein identifications will be accepted if they could be established at greater than 99.0% probability with at least two identified peptides.

These filtering criteria establish a false positive identification rate of 0% for the proteome dataset based on the decoy database search strategy. Identified proteins will be quantified using spectral counts directly computed by Scaffold software. Estimation of differential protein abundance will be expressed as fold-change (ratio of the averaged spectral counts in transgenic pigs samples to the averaged spectral counts in the control samples).

5.4.3.4 Differential protein expression and pathways analysis

Multivariate statistical analysis will be performed on proteomic data to find discriminating proteins and systematic variation sources unrelated to protein pattern. In order to map the differentially expressed proteins into biological networks and for functional interpretation of the protein data, an integrated data-mining platform such as MetaCore (GeneGo Inc., USA) will be used. Pathway and protein network analysis will allow for the identification of novel relevant biological entities, represented by proteins sets and for the discovery of highly significant pathways in the pathogenesis of ALS.

5.4.4 Post-mortem analysis

SOD1 aggregation and deposition pattern and histological lesion profile will be evaluated on stillborn piglets or in case of animals death.

5.4.4.1 Tissue banking

Ear biopsy was performed on all piglets, in order to obtain primary PAF cultures and subsequent cryopreservation to establish a cell bank from each individual piglet. Brain, spinal cord, peripheral nerves, muscles and organs were sampled from stillborn or euthanized animals. Tissue samples were both formalin-fixed/paraffin-embedded (FFPE) and paraformaldehyde-fixed. After 24 hours, paraformaldehyde samples were rinsed 2 times in PBS and then exposed to growing sucrose concentrations in order to achieve cryoprotection. Finally, samples were kept for 30 minutes in a 30%sucrose: OCT [1:1] solution and then included in OCT, frozen in isopentane and stored at -80°C. Finally, a part of every tissue was snap-shot freezed in isopentane and stored at -80°C.

5.4.4.2 Western Blot (WB)

Cells (transgenic hSOD1^{G93A} PAF and primary culture cells from ear biopsy obtained from transgenic piglets) were lysed using Laemmli buffer 1X containing β-mercaptoethanol (5%) and boiled for 10 minutes. Total protein was

quantified by Qubit fluorometer (Invitrogen) using the QuantIT Protein quantification kit (Invitrogen). Tissues obtained from stillborn piglets were homogenized (1:5 mg/ml) in lysis buffer (50mM Tris HCl pH8, 150mM NaCl, 5mM EGTA pH8, 1.5mM $MgCl_2$, 10% anhydrous glycerol, 1% Triton, 100µg/ml (=0.57mM) PMSF). Protein quantification was performed by BCA Protein Assay kit (Pierce). Thirteen µg of each sample were loaded onto Glicine-SDS-PAGE (4-12%) and electrophoretically separated for 45 minutes at 200V using the MiniproteanII chamber (Biorad). Blotting onto Immuno-Blot PVDF membrane (Biorad) was obtained after 1h at 100V using MiniproteanII electroblotter (Biorad) according to manufacturer protocol. The resulting PVDF membranes were subsequently processed following the instructions of the chemiluminescent detection system Lumi-LightPLUS Western Blotting Kit Mouse/Rabbit (Roche). Detection of transgenic target was achieved using either a polyclonal Antibody 07-403 (1:1000 Millipore) or a polyclonal Antibody GTX 100659 (1:800 Genetex) that is specifically directed against hSOD1 region within amino acids 75 and 138, which is different between human and swine. Pig β-actin expression was detected using the mAb ab6276 (1:5000 Abcam). The treated membranes were finally exposed, developed and fixed.

5.4.4.3 Immunohistochemistry (IHC)

All FFPE tissues were analysed by IHC. After antigen retrieval (95° bain-marie for 20 minutes), sections were treated with 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase. Sections were incubated in Normal Goat Serum for 20 minutes and then overnight at 4°C in the primary antibody (GTX 100659; 1:250) dissolved in PBS. Afterwards, sections were rinsed 3 times in PBS and incubated with relevant secondary antibody (1:200) for 1 hour. After 3 rinses in PBS, sections were incubated with Avidin-Biotin Complex for 30 minutes. DAB was used as revealing agent. Sections were counterstained with haematoxylin. Ubiquitine-immunoreactive misfolded protein was detected by the

same IHC protocol, but the antigen retrieval step, with a rabbit polyclonal antibody (Dako Z0458, 1:100).

5.4.4.4 Immunofluorescence (IF)

FFPE tissues were further analysed by double immunofluorescence in order to characterize SOD1 deposition pattern. After antigen retrieval (microwave oven in citrate buffer, pH 6,0), sections were washed three times in TBST. Sections were then incubated for two days at 4°C with the primary antibodies (SOD 1 [GTX 100659; 1:250] and NeuN [MAB377; 1:1000]). Afterwards, sections were rinsed in TBST and incubated with Alexa Fluor® (1:200) for 15 minutes and observed with a Nikon Eclipse 80i microscope.

5.5 hSOD1^{G93A} SWINE LINE ESTABLISHMENT

Various assisted reproduction techniques will be used to generate offspring from the transgenic founders animals available.

5.5.1 Pig breeding

5.5.1.1 Semen collection and banking

Semen will be collected from transgenic founder male by manual stimulation or elettroejaculation, a procedure already widely employed in animals with locomotor deficits or in wild/zoo animals. Spermatozoa will be used to inseminate wt sows or to generate embryos, to be transferred in recipient sows, by in vitro fertilization or ICSI in order to generate a F1 offspring to be used to propagate and establish the ALS swine line. By inbreeding of animals obtained from the second and from the subsequent generations, an homozygous ALS swine line will be established.

5.5.1.2 Artificial insemination and embryo production

Depending on the seminal material quality and quantity, as minipigs produce smaller amounts of semen, artificial insemination of wild type sows or in

vitro fertilization techniques, ICSI included, will be employed to establish pregnancies to generate F1 progeny.

As long as artificial insemination is concerned, commercial breed gilts will be synchronized using Regumate and hCG. 30 hours after hCG administration, gilts will be inseminated with fresh semen or, after sedation and general anesthesia, by means of a laparoscopic insemination with frozen semen. If scarce or low quality seminal material doesn't allow insemination, in vitro fertilization/ ICSI will be performed. Oocytes, isolated from ovaries collected at the local slaughterhouse, will be matured in vitro for 40 hours and then subjected to in vitro fertilization or ICSI. Five days later, the resulting blastocysts will be transferred by laparoscope to the uterus of synchronized gilts.

5.5.2 F1 characterization and back crossing

Newborn piglets will be screened by PCR to identify the transgenic animals (50% of the offspring are expected to carry the transgene). Transgenic animals will be subjected to an ear biopsy to establish a cell line to be employed in ICC to assess transgene expression level and in cell-banking. At puberty, most promising females will be back crossed with their male siblings to bring the transgene to homozygosity and to stabilize the ALS swine line using animals with high expression levels.

5.5.3 Re-cloning of the most promising phenotypes

Animals displaying a promising or valuable phenotype, which could also be identified after death or sacrifice, will be regenerated by re-cloning by Somatic Cell Nuclear Transfer in order to increase the number of animals available. A cell line from every transgenic animal born will be conserved in a dedicated cell bank.

5.6 RESULTS COMPARISON WITH BOTH OTHER ALS MODELS AND PATIENTS

Results obtained from the characterization of the hSOD1^{G93A} swine will be evaluated in order to assess if the pig may be considered a suitable species in which ALS can be faithfully reproduced. Moreover our results will be compared with literature data both in other ALS model and in human patients in order to identify similarities and differences and eventually strong point of our swine model. As far as statistical analysis is concerned, the study design will be a single-blinded, randomized clinical trial.

RESULTS

6.1 VECTOR CHARACTERIZATION

On the following pages both graphical representations and restriction analyses of vectors realized in this work are listed (**Table 6.1**). After restriction analysis the open reading frame of each vector was confirmed by sequencing.

Figure 6.1: The destination vector pMGOrfA5'3'MARpuro5171.

This vector is able to replicate in competent bacterial strains, to which it confers the ability to grow on agar containing Ampicillin. It contains sequences able to promote its recombination with "Entry-Clone" vectors, in addition to sequences essential for its insertion into the genome of a eukaryotic cell in highly transcribed regions.

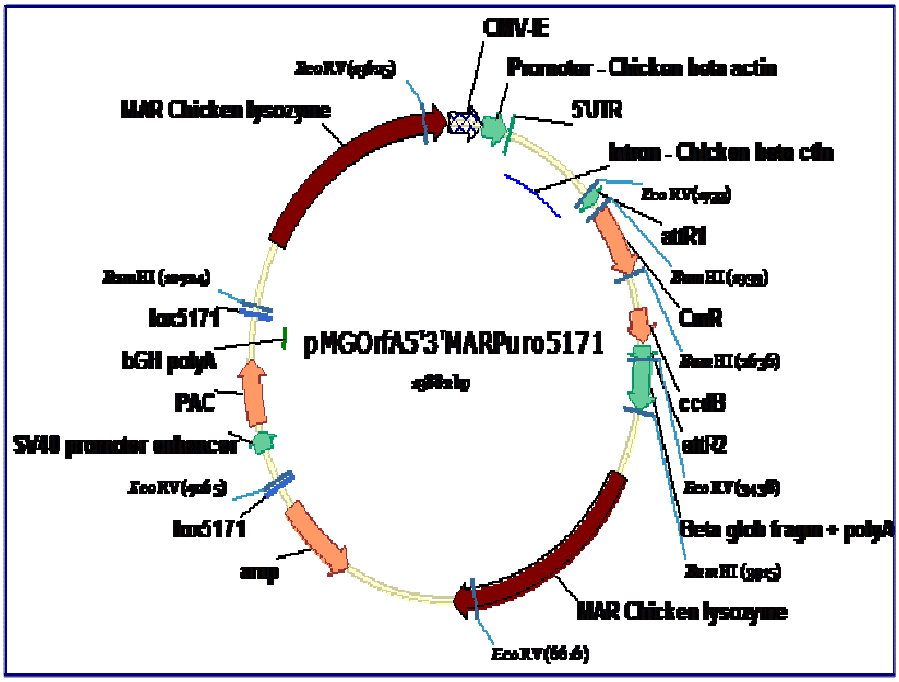


Figure 6.2: *Bam*HI restriction analysis of the destination vector pMGOrfA5'3'MARpuro5171.

M= 1Kb ladder (Fermentas)

B= *Bam*HI restriction analysis

Expected fragment (bp)

//*Bam*HI= 7009 + 4891 + 1279 + 703

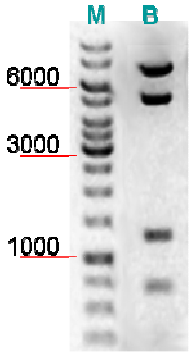


Table 6.1: List of vectors used in the present work.

pcDNA3.0hSOD1^{G93A}	pcDNA3 plasmid (Invitrogen)contains the human mutated G93A SOD1 cDNA sequence. Was used to obtain the hSOD1^{G93A} cDNA sequence and for PAFs transfection experiments.
pcDNA3.0hSOD1^{wt}	pcDNA3 plasmid (Invitrogen)contains the human wild type SOD1 cDNA sequence. Was used to obtain the hSOD1^{wt} cDNA sequence.
pENTRL1L2Oligo*SacISalI*	This plasmid is part of the Multisite Gateway system (Invitrogen) and was used in ligase reactions with both hSOD1^{G93A} cDNA and hSOD1^{wt} cDNA.
pENTRL1L2-hSOD^{G93A}	This vector was obtained from ligase reaction between the *Bam*HI/*Xho*I fragment of hSOD1^{G93A} cDNA and the pENTRL1L2Oligo*SacISalI* plasmid
pENTRL1L2-hSOD^{wt}	This vector was obtained from ligase reaction between the *Bam*HI/*Xho*I fragment of hSOD1^{wt} cDNA and the pENTRL1L2Oligo*SacISalI* plasmid
pENTRL1L2-hSOD1^{G93A} delSB	This plasmid was obtained trough double digestion of pENTRL1L2-hSOD^{G93A} with the restriction enzymes *Sal*I and *Bam*HI and was used in LR exchange reactions with the pMGOrfA5'3'MARpuro5171 vector.
pENTRL1L2-hSOD1^{wt}delSB	This plasmid was obtained trough double digestion of pENTRL1L2-hSOD^{wt} with the restriction enzymes *Sal*I and *Bam*HI and was used in LR exchange reactions with the pMGOrfA5'3'MARpuro5171 vector.
pMGOrfA5'3'MARpuro5171	This destination vector is used in LR exchange reactions with both pENTRL1L2-hSOD1G93A delSB and pENTRL1L2-hSOD1wtdelSB vectors.
pMG5'3'MARPuro5171-hSOD1^{G93A}	This vector results from the LR exchange reactions between the pMGOrfA5'3'MARpuro5171 Destination Vector and the pENTRL1L2-hSOD1^{G93A} delSB vector and is used in transfections experiments
pMG5'3'MARPuro5171-hSOD1^{wt}	This vector results from the LR exchange reactions between the pMGOrfA5'3'MARpuro5171 Destination Vector and the pENTRL1L2-hSOD1^{wt} delSB vector

Figure 6.3: The pcDNA3.0hSOD1^{G93A} (**a**) and pcDNA3.0hSOD1^{wt} (**b**) plasmids

These plasmids contain the neomycin selection cassette under the SV40 promoter, while the transgene, consisting of the cDNA encoding or the mutated form of the human SOD1 (a) or the wild-type one (b), is placed under the control of the CMV promoter.

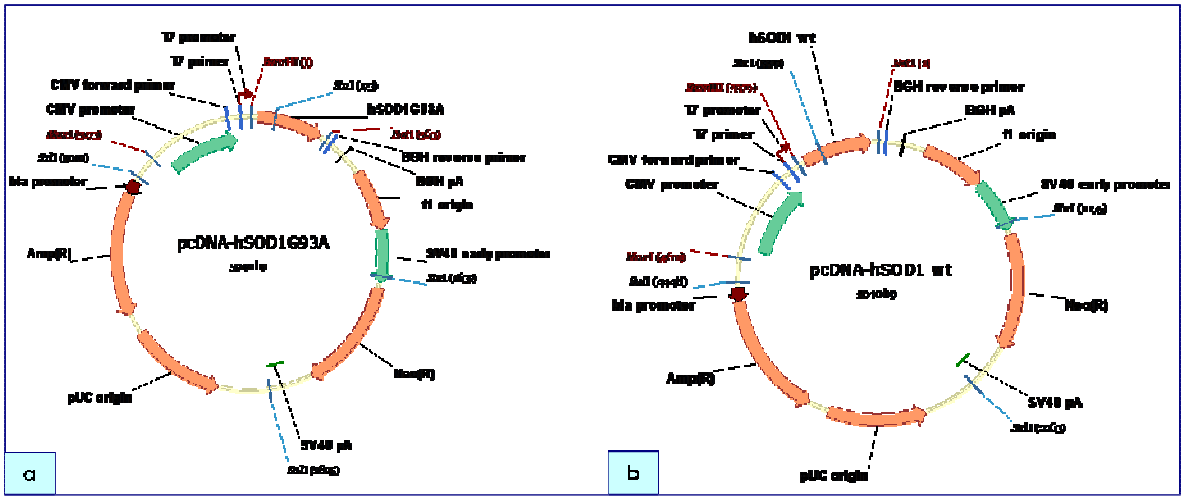


Figure 6.4: The restriction analysis of pcDNA3.0hSOD1^{G93A} plasmid

M= 1Kb ladder (Fermentas)

Mu= *Mun*I restriction analysis

Stu= *Stu* I restriction analysis

Expected fragment (bp)

*//Mun*I= 5940

//Stu I = 4476 + 1464

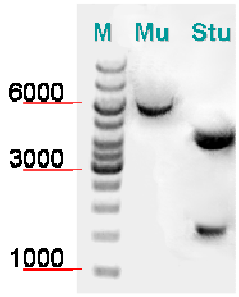


Figure 6.5: The "EntryClone" pENTRL1L2-hSOD^{G93A}(a) and pENTRL1L2-hSOD^{wt} (b)

These vectors, in which was inserted the cDNA encoding or the mutated form of the human SOD1 (a) or the wild-type one (b), are able to replicate in competent bacterial strains, to which confer the ability to grow on agar containing Kanamycin.

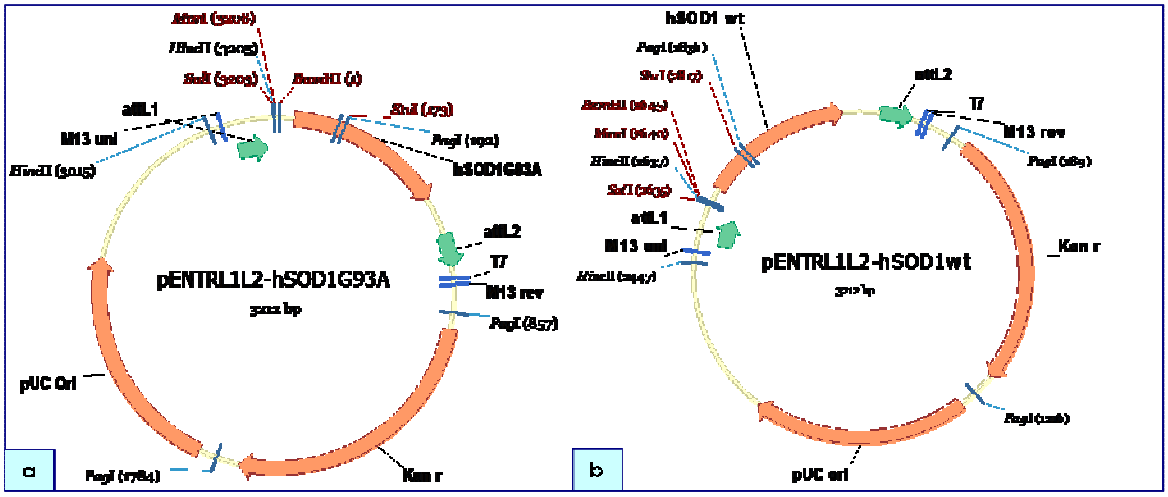
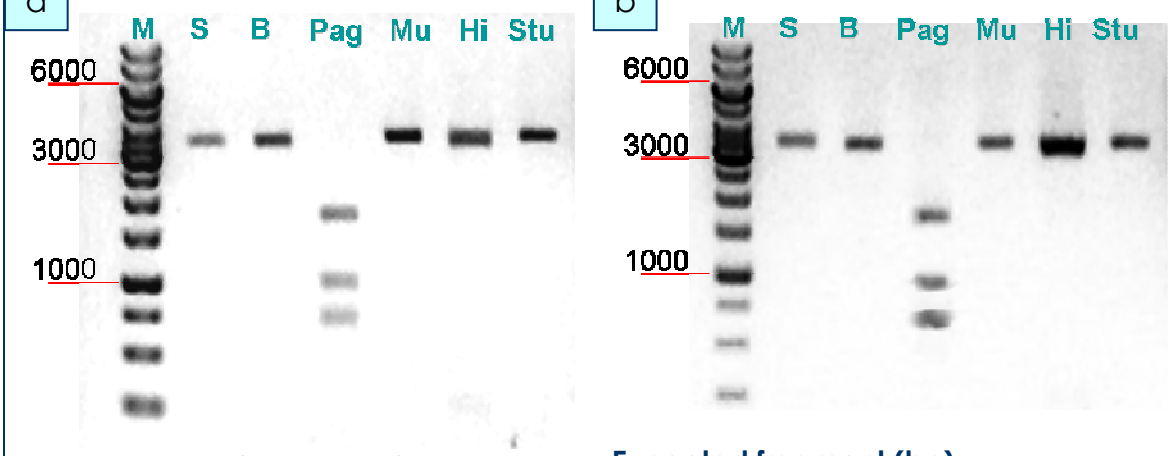


Figure 6.6: Restriction analysis of pENTRL1L2-hSOD^{G93A}(a) and pENTRL1L2-hSOD^{wt} (b)



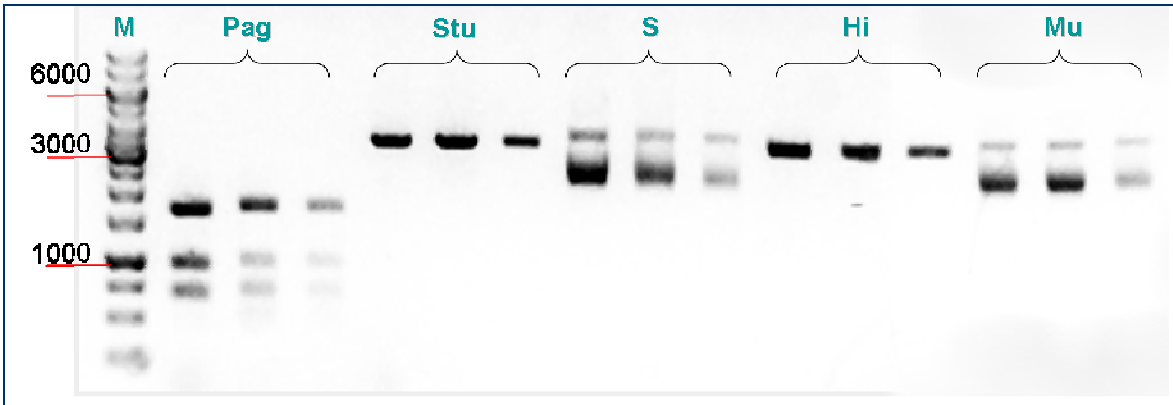
M= 1Kb ladder (Fermentas)

S= *Sal*I restriction analysis
B= *Bam*HI restriction analysis
Pag= *Pag*I restriction analysis
Mu= *Mun*I restriction analysis
Hi = *Hin*cII restriction analysis
Stu= *Stu* I restriction analysis

Expected fragment (bp)

// *Stu*I = 3212
// *Mun*I = 3212
// *Sal*I =3212
// *Bam*HI =3212
// *Pag*I = 1620+952+640
// *Hin*cII = 2997+215

Figure 6.7: Restriction analyses of pENTRL1L2-hSOD1^{G93A}delSB vector. Same results are obtained from the restrictions of pENTRL1L2-hSOD1^{wt}delSB vector



M= 1Kb ladder (Fermentas)
S= *Sal*I restriction analysis
Pag= *Pag*I restriction analysis
Mu= *Mun*I restriction analysis
Hi = *Hin*cII restriction analysis
Stu= *Stu* I restriction analysis

Expected fragment (bp)

// *Stu*I = not digested
// *Mun*I = not digested
// *Sal*I = not digested
// *Pag*I = 1620+927+640
// *Hin*cII = 2997+190

Figure 6.8: The pMG5'3'MARPuro5171-hSOD1^{G93A} (a) and the pMG5'3'MARPuro5171-hSOD1^{wt} (b) expression vectors

These vectors are capable of promoting the transgene expression within eukaryotic cells through the action of the pCAGGS promoter. They are also able to confer to cells the ability to grow and replicate in medium containing puromycin, because of action of their puromycin-selection cassette under the control of SV40 promoter.

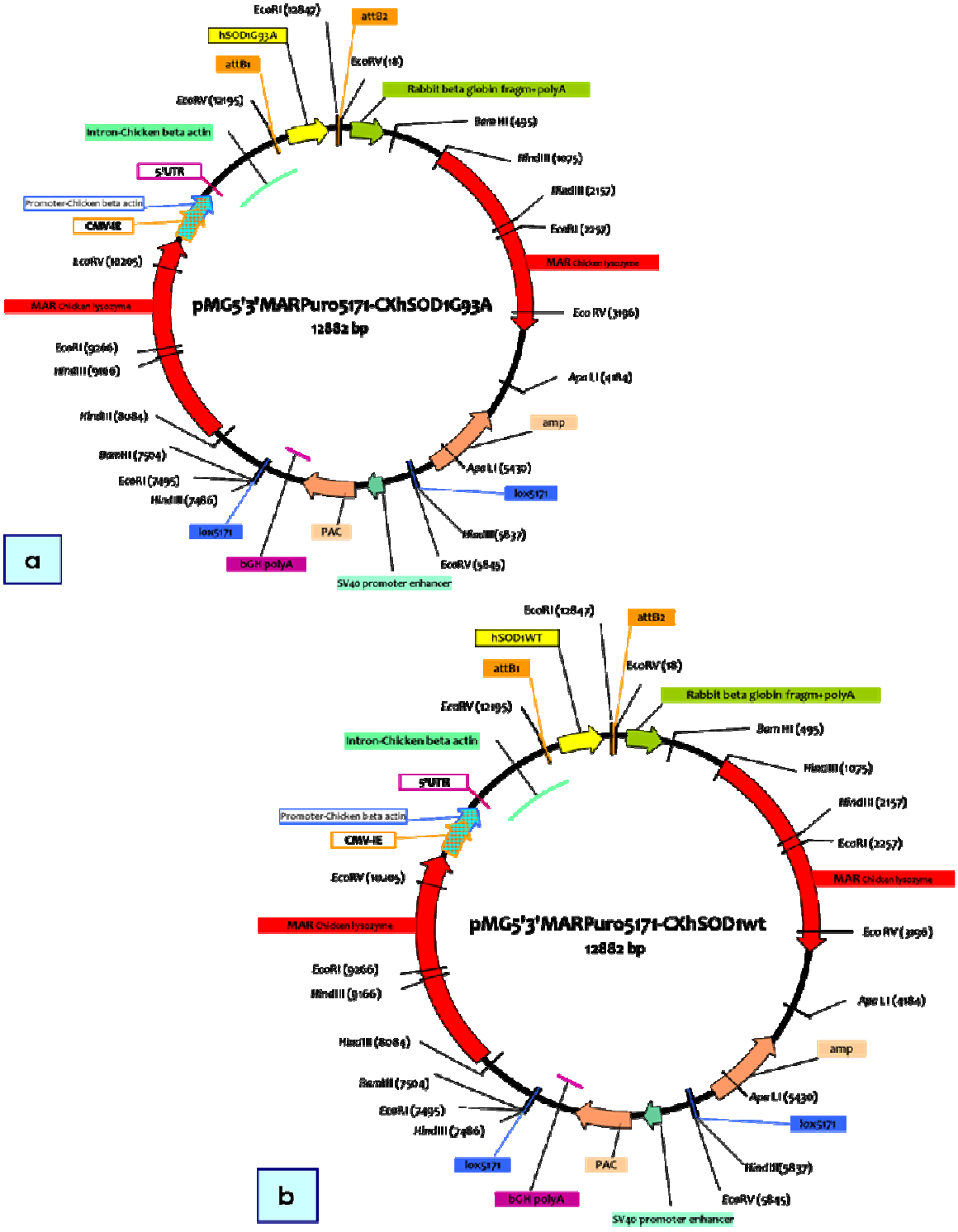
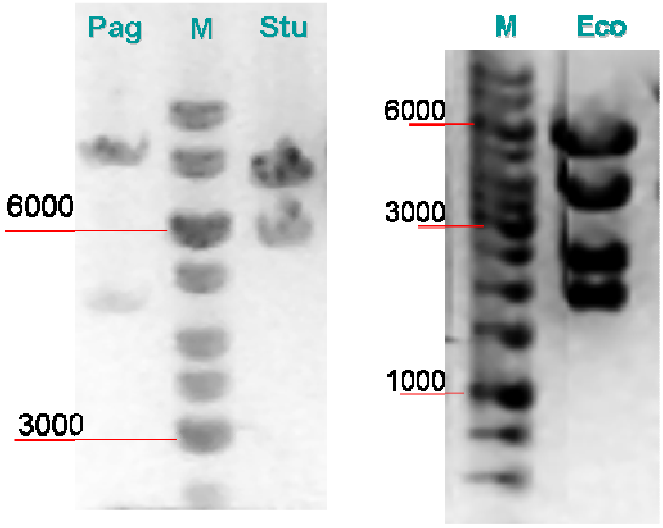


Figure 6.9: Restriction analysis of pMG5'3'MARPuro5171-hSOD1^{G93A} expression vector. Same results are obtained from pMG5'3'MARPuro5171-hSOD1^{wt} vector restrictions.



M= 1Kb ladder (Fermentas)
Pag= *Pag*I restriction analysis
Eco= EcoRI restriction analysis
Stu= *Stu* I restriction analysis

Expected fragment (bp)
// *Pag*I = 7849+4527+506
// EcoRI = 5238+3581+2292+1771
// *Stu*I = 7202+5680

6.2 TRANSFECTION OF VECTORS INTO PIG ADULT FIBROBLASTS AND RELATED ANALYSIS

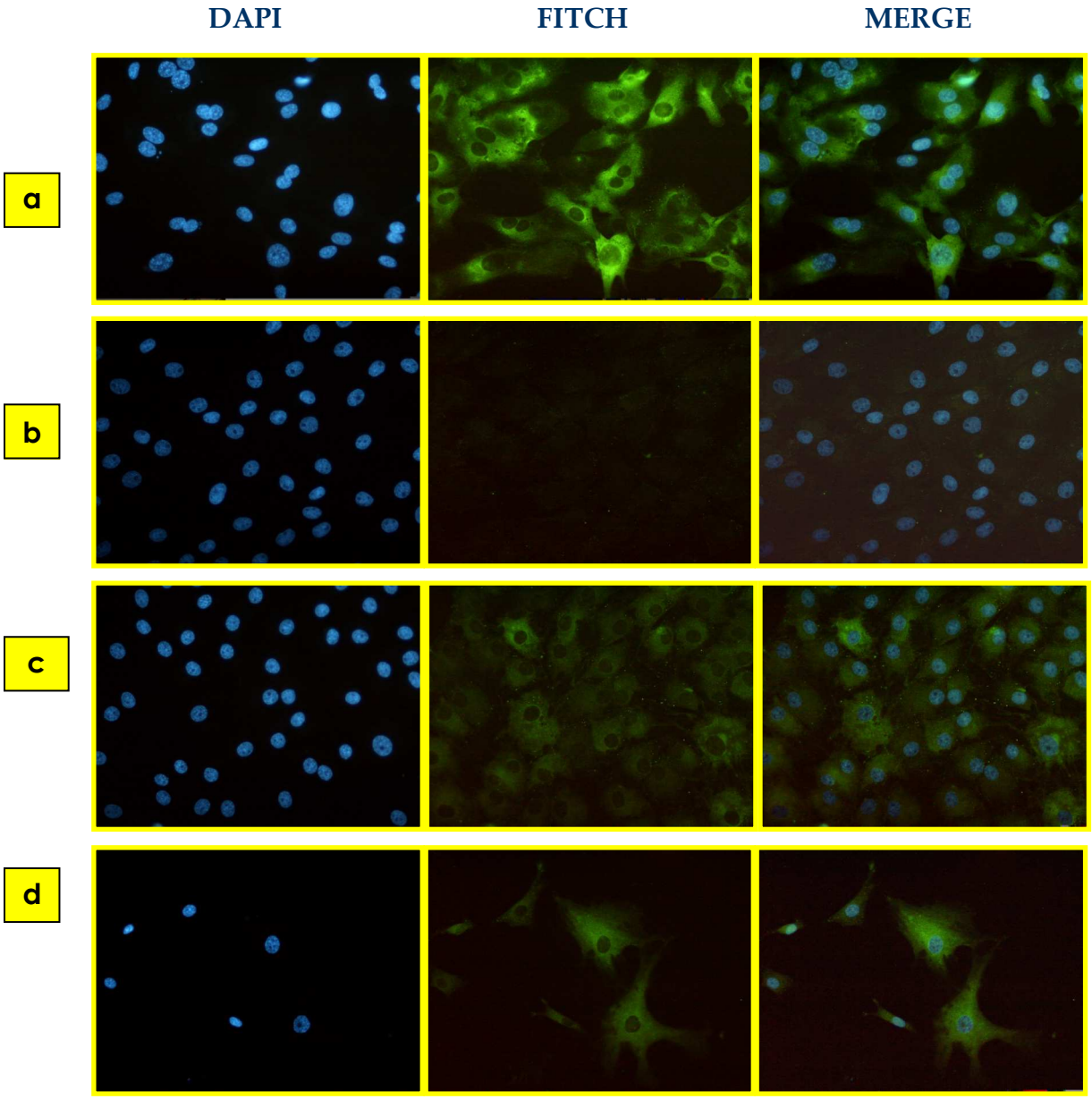
6.2.1 pcDNA3.0hSOD1^{G93A}

The pcDNA3.0hSOD1^{G93A} vector, carrying the mutated SOD gene, has previously been used by our group to transfect mouse astrocyte cell lines, obtaining a high transgene expression level. A pcDNA3.0hSOD1^{G93A} mouse astrocyte lysate was used as a further positive control in WB analysis. To obtain comparison data, we decided to use the same construct for transfection of wild type PAFs. Thus we obtained 17 colonies of pcDNA3.0hSOD1^{G93A} PAFs that were analysed by both WB and ICC, revealing the SOD1^{G93A} protein expression in 15 of 17 PAFs colonies.

Among the hSOD1^{G93A}PAFs colonies some presented a transgene expression pattern rather uniform while others showed a variegate expression pattern (in the same colony some cell showed a high expression and some were completely off!) (**Figure 6.10**). To explain this phenomenon, it is acceptable to assume a transgene position effect, due to its integration site in the host cell genome.

Figure 6.10: ICC conducted with rabbit polyclonal Ab 07-403 (Millipore) on pcDNA3.0hSOD1^{G93A} PAFs colonies.

[**a**] Human Umbilical Vein Endothelial Cells (Huvec) used as positive control. [**b**] wild-type PAF used as negative control. [**c**] PAF clone #C5 with uniform expression of human protein [**d**] PAF clone #B3 with variegate expression pattern. DAPI is the filter used to highlight the Hoechs nuclear staining. FITCH allows the detection of the fluorophore conjugated with the secondary anti-rabbit antibody and then the transgene expression. MERGE is the overlapping of the two images.



6.2.2 pMG5'3'MARPuro-hSOD1^{G93A}

The transfection experiments conducted with the pMG5'3'MARPuro-hSOD1^{G93A} vector led to the isolation of 26 PAF clones, which were analysed by WB and ICC. All clones, as shown in **Table 6.2**, revealed transgene expression, to which a score was assigned. Four clones showed a transgene expression level lower than the control one (score 2), and three clones achieved the same score as the control. The remaining 19 clones showed a higher expression (Score ranging from 3 to 6) (**Table 6.2 and Figure 5.1**).

Overall, the PAF colonies obtained by transfection of pMG5'3'MARPuro hSOD1^{G93A} vector showed a transgene expression pattern higher and more uniform than those shown by the colonies obtained by transfection of the vector pcDNA3.0hSOD1^{G93A}: this phenomenon, is attributed to the greater effectiveness of the pCAGGS promoter to induce the expression of the human transgenic protein in the swine genome.

Table 6.2: List of PAF clones transfected with pMG5'3'MARPuro5171-hSOD1-G93A vector.

Clone	Score
1A1	3
1A2	6
1A4	1
1A5	5
1B1	3
1B3	4
1B6	6
1C1	1
1C2	2
1C6	4
1D1	5
1D2	1
1D5	4
1D6	5
1E1	6
1E2	4
2A1	6
2A2	6
2A3	5
2A4	1
2A5	4
2A6	4
2B2	4
2C1	5
2C2	5
2C3	4

In this table clones obtained by pMG5'3'MARPuro5171-hSOD1-G93A vector transfection in wild-type PAF are reported. A score indicating the transgenic protein expression level, compared to the positive control one (score=2), was assigned to each clone.

6.3 Somatic Cell Nuclear Transfer (SCNT)

The hSOD1^{G93A} PAF colonies to be used for NT experiments were chosen depending on protein expression level shown. In particular, the 1A1, 1A2, 1B1, 1C2, 1D1, 1D2, 1E2, 2A2, 2A6, 2B2, 2C1 and 2C3 pMG5'3'MARPuro-hSOD1^{G93A} PAFs colonies and the # C5 pcDNA3.0hSOD1^{G93A} Paf colonies were selected. Eight cloning experiments were conducted. In the first and second SCNT experiment the colony #C5 has been employed, as nuclei donor. In the third and fourth experiment, a pool of clones (1A1, 1C2, 1D2 and 1E2) was used as a nucleus donor. In the fifth and sixth experiment a pool composed of 1B1, 1D1, 2C1 and 2B2 clones was employed, while a pool composed of 1A2, 2A2, 2A6 and 2C clones was employed in the seventh and eighth experiment. All the SCNT experiments conducted are listed in **Table 6.3** where viable embryo percentages (ranging from 31,58% to 50,50%) can be appreciated. These results are in line with those obtained from similar experiments conducted with other transgenes (data not shown).

Table 6.3: List of cloning experiments and in vitro embryo development data

The "ID clones" column shows the names of hSOD1-G93A PAF clones that composed the cells pool, used as nuclei donors. In "N° SCNT" column the number of SCNT experiments is reported. The "average score" column indicates the transgene expression level owned by the pool of PAF clones. "N" is the number of pairs cytoplasts/PAF formed after the fusion. "CI" is the number of segmented embryos and its value is compared to the total percentage of reconstructed embryos. "Mc/Bl" indicates the number of compact morulae and blastocysts at the sixth day of in vitro culture. "Tot embryo" indicates the number of viable embryos, with the relative percentage value obtained by comparison with the initial number of manipulated oocytes. "Piglets" indicates the pregnancy outcome, with the number of vital and stillborn piglets obtained.

ID clones	N° SCNT	average score	N	CI	%	Mc/Bl D6	TOT embryo	%	Piglets Vital/stillborn
#C5	1	1	209	189	90,43%	10 mc,56 bl	66	31,58%	No pregnancy
#C5	2	1	206	195	94,66%	78 bl	78	37,86%	No pregnancy
A1,E2,C2,D2	3	2	195	181	94,66%	8 mc, 61 bl	69	35,38%	No pregnancy
A1,E2,C2,D2	4	2	204	185	92,82%	11mc, 63 bl	74	36,27%	No pregnancy
B1, 2B2, 2C1,D1	5	3	200	172	86,00%	21 mc, 54 bl	75	37,50%	6/4
B1, 2B2, 2C1,D1	6	3	209	151	72,25%	31 mc, 47 bl	78	37,32%	5/2
1A2,2A6,2A2, 2C3	7	5	205	179	87,32%	96 bl	96	46,83%	3/1
1A2,2A6,2A2, 2C3	8	5	202	178	88,12%	102 bl	102	50,50%	2/7

6.4 Development to term of SCNT embryos derived from hSOD1^{G93A} PAFs

The transfer of 638 embryos to eight recipients sows resulted in four pregnancies. The 4 pregnant sows received 75, 78, 96, 102 embryos obtained from the fifth, sixth, seventh and eighth SCNT experiment respectively (**Table 6.3**). Pregnancies were developed to term and resulted in the birth of 10, 7, 4 and 9 piglets with a mean efficiency of blastocyst development to term of 8,78%. Among produced piglets 16 were vital and 12 were stillborn. Five piglets survived artificial hand raising and weaning and developed normally. The remaining 11 piglets died within 48–96 h from birth due to events commonly reported in commercial herds (i.e. some piglets were not nursing, others developed diarrhoea, pneumonia etc). In **Table 6.4** the ID that was assigned to the transgenic piglets are summarized.

Table 6.4: hSOD1^{G93A} piglets ID.

N° SCNT	Piglet ID	Healt Status
5	052-1	stillborn
5	052-2	stillborn
5	052-3	stillborn
5	052-4	stillborn
5	165	Dead at 3 days
5	166	Dead at 15 days
5	167	Dead at 15 days
5	168	Alive
5	169	Dead at 6 days
5	171	Dead at 5 days
6	053-1	stillborn
6	172	Dead at 4 days
6	173	Alive
6	174	Alive
6	175	Dead at 5 days
6	176	Dead at 6 days
7	201	Dead at 3 days
7	202	Dead at 9 days
7	203	Dead at 1 days
7	137-1	stillborn
8	204	Alive
8	205	Alive
8	143-1	stillborn
8	143-2	stillborn
8	143-3	stillborn
8	143-4	stillborn
8	143-5	stillborn
8	143-6	stillborn
8	143-7	stillborn

6.5 Transgene Integration Analysis

Trough PCR analysis it has been possible to detect the transgene presence in the swine genome. In **Figure 6.11** it is possible to appreciate the 290pb lane corresponding to transgene amplification in piglet 168 genomic DNA (α). Lanes β-λ show the pENTRL1L2-hSOD1^{G93A}delSB DNA plasmid amplification in serial dilution, while μ show no amplification signal in swine wild type genomic DNA.

With the aim to establish the integration pattern of the construct containing the ALS-related human SOD1^{G93A} mutated protein in the swine genome, Southern blot analysis was performed on DNA extracted from all piglets obtained during the project. In particular, **Figure 6.12** shows the results obtained on DNA from piglets born after the fifth and sixth embryo transfer experiments in which B1, 2B2, 2C1 and D1 PAFs-SOD1^{G93A} colonies was employed as nuclei donors. The analysis revealed four different integrations patterns (identifiable with blue ♠,♣,♦,♥ symbols), corresponding to the four different PAFs-SOD1^{G93A} colonies. **Figure 6.13** shows the results obtained on DNA from piglets born after the seventh and eighth embryo transfer experiments in which nuclei come from 1A2, 2A6, 2A2, and 2C3 PAFs-SOD1^{G93A} colonies. Similarly to that observed in figure 6.13, it is possible to appreciate four different transgene integration patterns, that are indicated with red ♠,♣,♦,♥ symbols and correspond to 1A2, 2A6, 2A2, and 2C3 PAFs-SOD1^{G93A} colonies.

Figure 6.14 shows an overview of data obtained from SB analysis: the alive pigs ID, who have reached one years of age, are highlighted with green color. Among the five live pigs are represented four transgene integration patterns. The increasing integrations numbers is also reported.

Figure 6.11: PCR analysis.

The picture show the results obtained from PCR analysis of various templates. **M**= molecular weigh; **α**= 168 piglet genomic DNA at [1ng/µl] of concentration; **β**, **γ**, **δ**, **ε**, **ζ**, **η**, **θ**, **ι**, **κ**, **λ**= pENTRL1L2-hSOD1^{G93A}delSB plasmid at serial dilution ranging from [1ng/µl] to [1ag/µl] **µ**=Wild type swine genomic DNA. **W**= water.

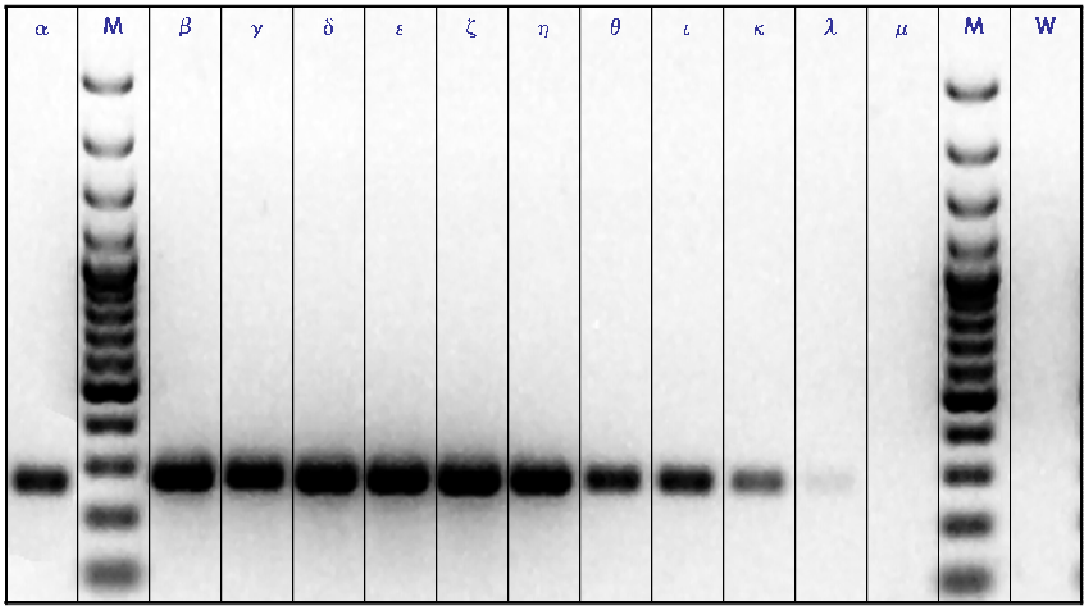


Figure 6.12: Southern blotting of DNA from transgenic piglets born after fourth e fifth cloning experiments.

M = λ//*Hind*III ; **165,166,167,168,169,171,052-1, 052-2, 052-3, 052-4, 172, 173, 174, 175, 176** = piglets ID. **WT** = wild type pig. The blue ♠,♣,♦,♥ symbols correspond to the four different transgene integration patterns obtained in transgenic piglets born after fifth and sixth cloning experiments

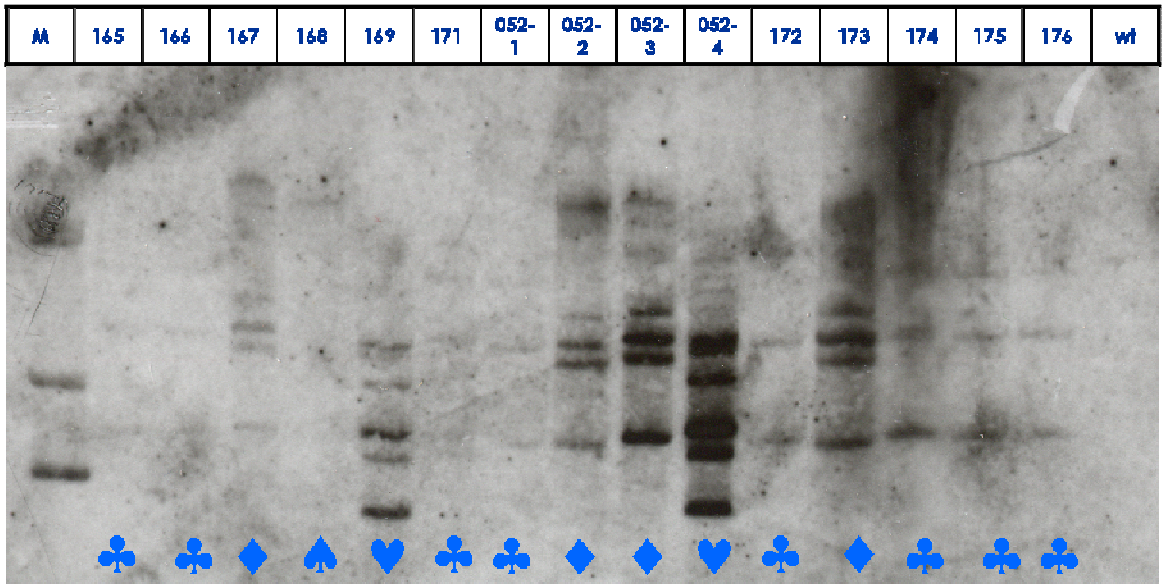


Figure 6.13: Southern blotting of DNA from transgenic piglets born after seventh and eighth cloning experiments.

M = λ//*Hind*III ; **201, 203, 202, 143-1, 143-5, 143-4, 137-1, 204, 204, 205, 143-6, 143-7** = piglets ID. **WT** = wild type pig. The red ♠,♣,♦,♥ symbols correspond to the four different transgene integration patterns obtained in transgenic piglets born after seventh and eighth cloning experiments.

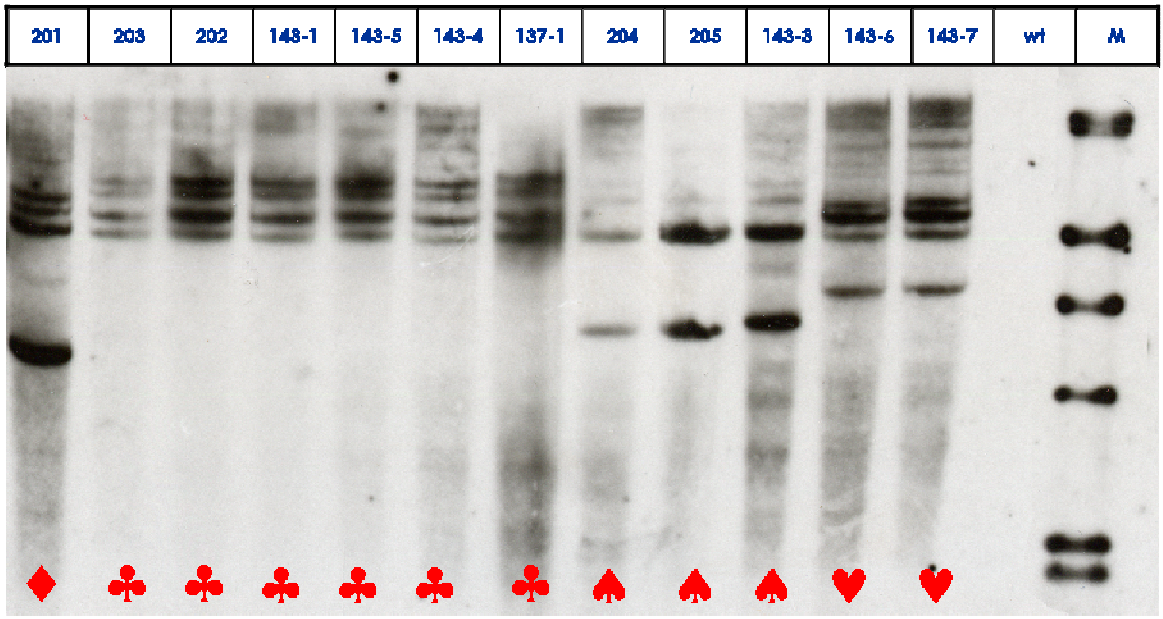
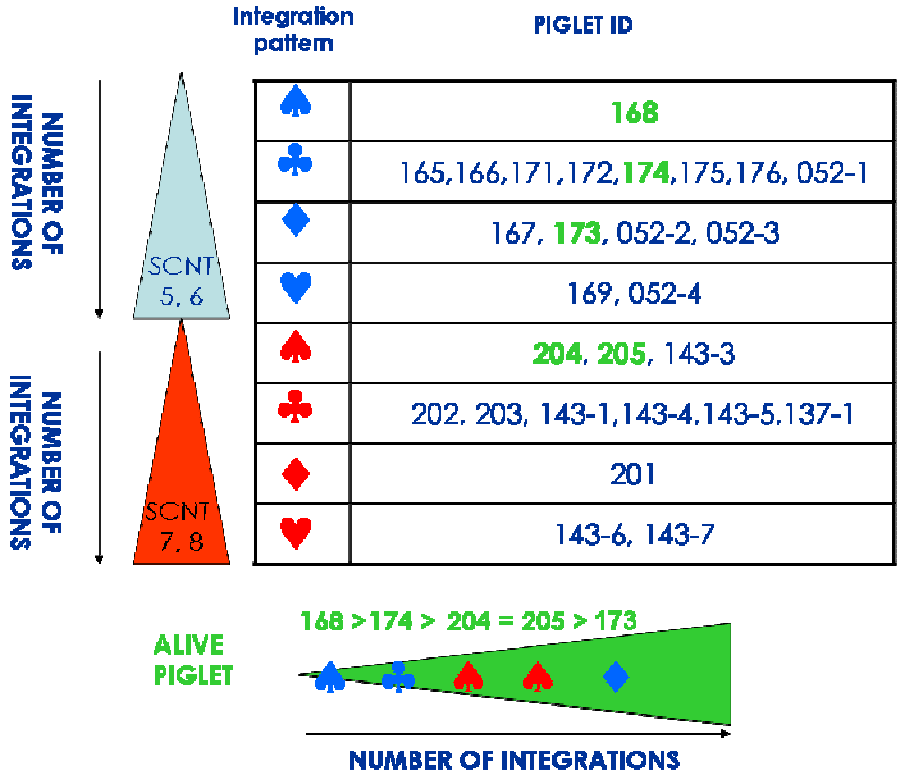


Figure 6.14: Transgene integration pattern summarizing chart.

Alive piglets ID is indicate with green. The growing order of transgene integrations number relative to piglets that have reached adulthood is reported.



6.6 Detection of hSOD1^{G93A} expression in stillborn piglets and living animals

Fibroblasts obtained from ear biopsy of both vital and stillborn piglets, were analyzed by ICC and revealed a transgene expression level comparable with that of PAF used as nuclei donors (**Figure 6.15**).

Furthermore, IHC and WB analysis were performed on dead and stillborn piglets tissues. Immunohistochemistry (**Figure 6.16**) was performed on coronary sections of all FFPE samples. Analysis performed by GTX 100659 revealed granular mutant protein aggregates in neurites and perikarya in brain (from area *hypothalamica lateralis* to the third ventricle), spinal cord (especially at the thoracic level), peripheral nerves (brachial plexus and sciatic nerve) and in the Enteric Nervous System. The same brain areas also revealed ubiquitine immunoreactivity characterized by intracytoplasmatic aggregates, while we couldn't detect any ubiquitin staining in spinal cord and in peripheral nerves.

Double immunofluorescence analysis revealed NeuN reactivity in cells displaying SOD1 aggregates, confirming mutated protein accumulation in neurons (**Figure 6.17**). Anyway, considering the extremely young age of our piglets and the brain areas involved, the pathogenic role of these protein aggregates in the hSOD1G93A swine requires further investigations.

Snapshot spinal cord samples were homogenized and analysed by WB. The transgenic protein was revealed using both antibodies (07-403 Millipore and GTX 100659 Genetex). In **Figure 6.18** it is possible to see two bands, corresponding to the two SOD1 isoforms: the endogenous swine protein displaying a lower molecular weight (16 KDa) and the human transgenic one, with a higher molecular weight (18 KDa).

Figure 6.15: ICC on PAF biopsied from the 5 transgenic living pigs.

Picture shows FITCH signal obtained with Millipore 07-403 antibody staining. hSOD1-G93A expression is revealed in all animals analyzed. [**A**] and [**B**] display staining in wild-type PAF and Huvec cells, respectively used as negative and positive controls. [**C**] Pig 168 is characterized by a moderate cytoplasmic and perinuclear staining. [**D**] Pig 173 displays an intense cytoplasmic labelling along with a perinuclear ring, noticeable in some cells. [**E**] and [**F**] show nuclear labelling with faint cytoplasmic staining detected in pigs 204 and 205 respectively. [**G**] Pig 174 displays a faint cytoplasmic staining. Perinuclear rings and faint nuclei labelling can also be observed in some cells. [**H**] shows intense cytoplasmic labelling detected in cells from stillborn piglets 169.

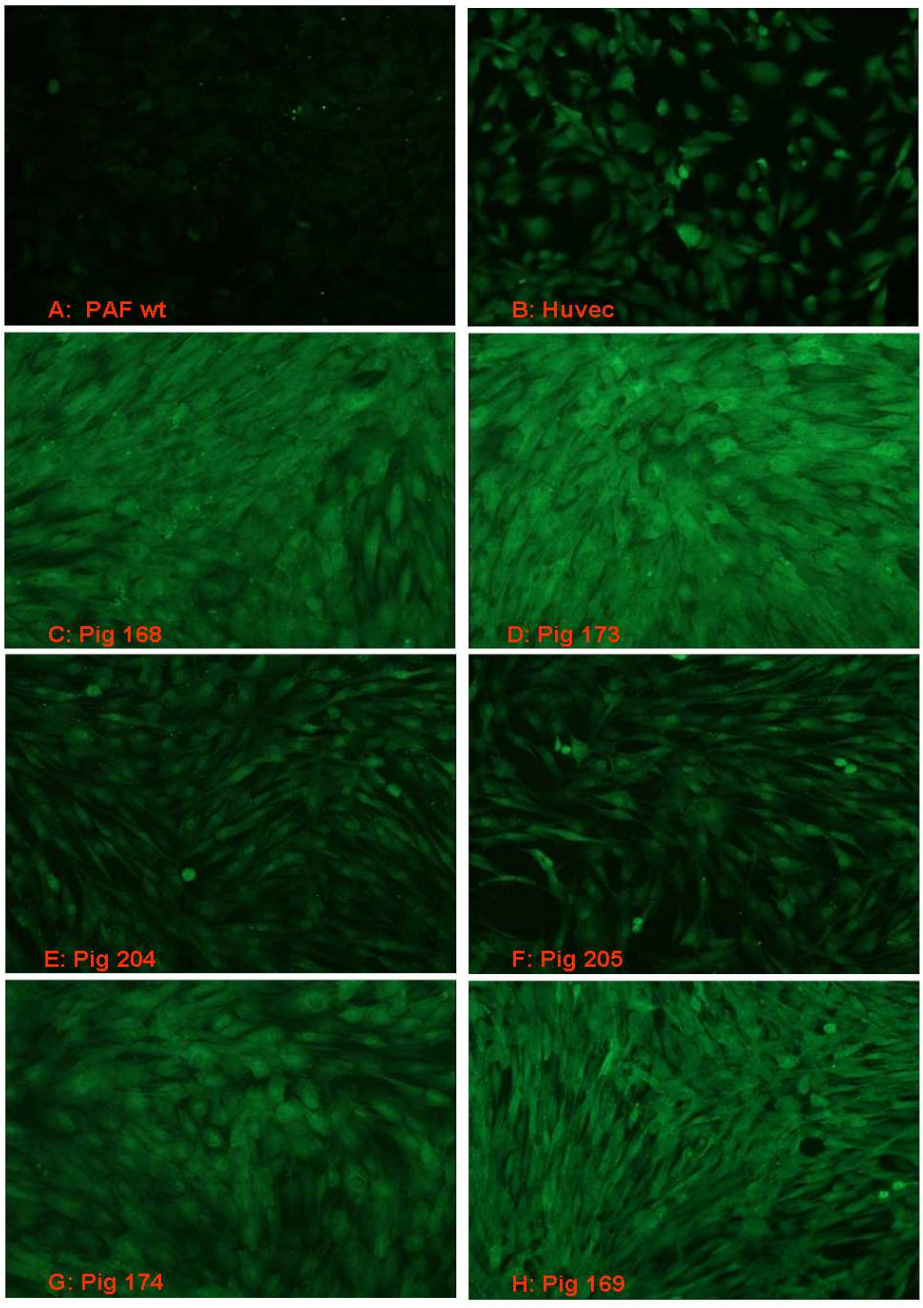


Figure 6.16: Genetex GTX 100659 IHC on FFPE piglet samples.

In panel A it is possible to appreciate hSOD1-G93A staining in the ileum with clusters of positive cells next to the *muscolaris mucosa*. Panels B and C show deposition pattern at the level of *area Hypothalamica lateralis*. In the same area (panel D), it is also possible to appreciate isolated cells displaying hSOD1-G93A staining. Panel E shows a bundle of positive cells lining the optic tract. Panel F displays deposition pattern in the spinal cord: it is possible to appreciate granular aggregates along fibres.

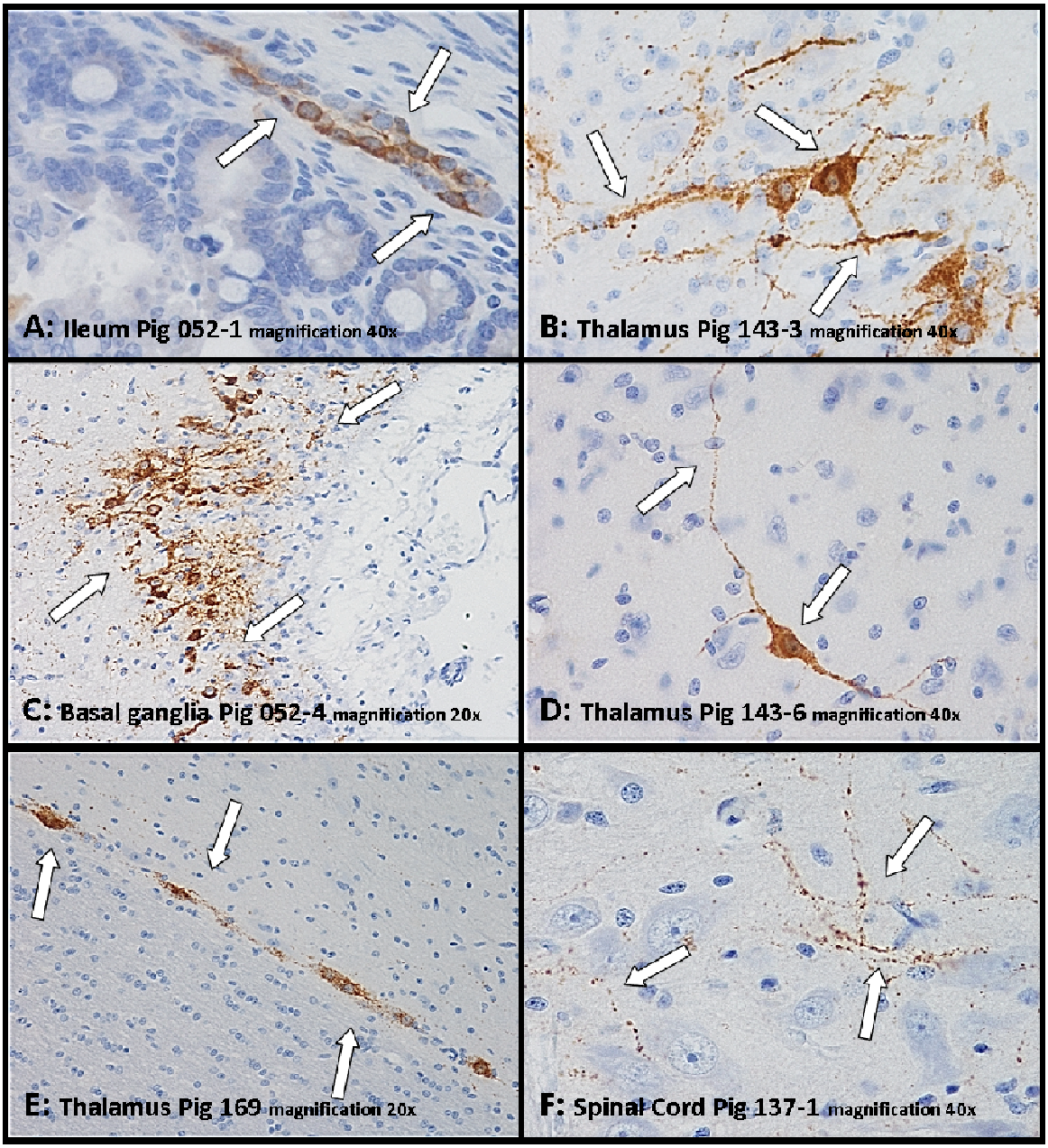


Figure 6.17: Double IF on hSOD1^{G93A} swine spinal cord.

Double immunofluorescence analysis reveals NeuN reactivity (red) in cells displaying SOD1 aggregates (green), pointing out mutated protein accumulation in neurons.

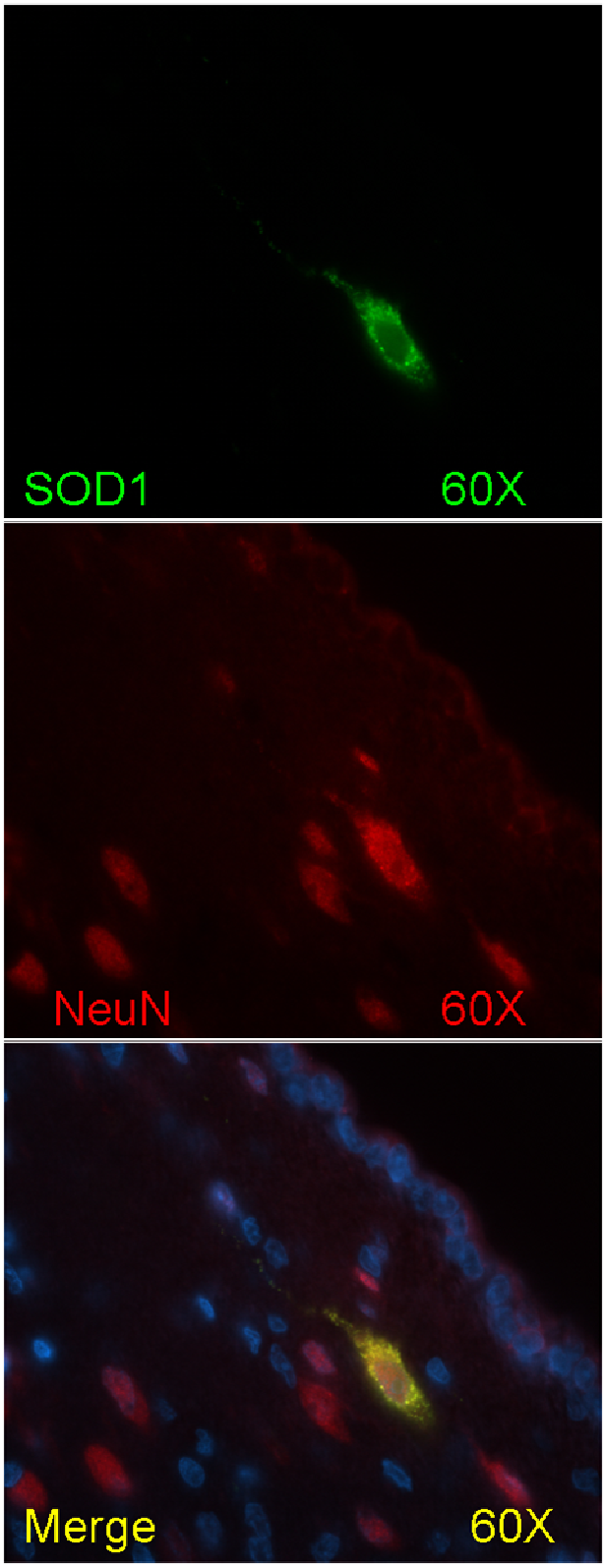
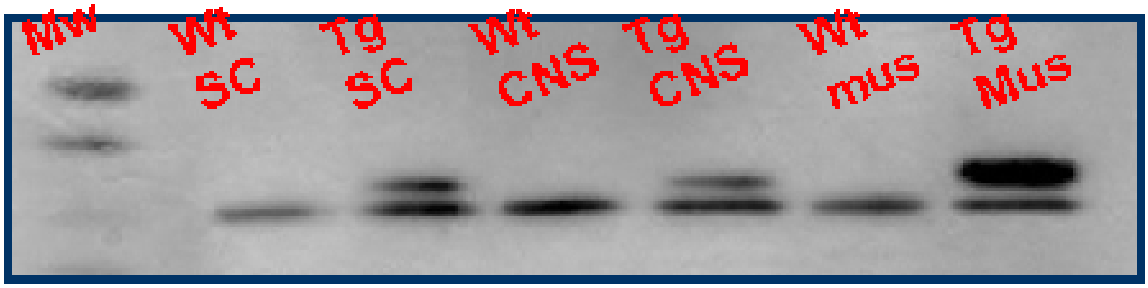


Figure 6.18: WB on stillborn piglets spinal cord.

The figure displays results obtained with Millipore 07-403 at the dilution 1:1000. In all hSOD1^{G93A} piglet tissues (spinal cord, brain, muscle) it is possible to appreciate two lanes, corresponding to the two SOD1 isoforms: the endogenous swine protein displaying a lower molecular weight (16 KDa) and the human transgenic one, with a higher molecular weight (18 KDa). On the contrary on tissues homogenate from non-transgenic pigs, used as negative control, only the band with lower molecular weight is present (16 KDa). Mw: molecular weight; Wt: wild type; Tg: transgenic; SC: spinal cord; CNS: Central Nervous System; Mus: muscle.



6.7 Phenotypic characterization and hSOD1^{G93A} swine line establishment

At the time of writing, the five transgenic swine have reached the age of one year and four of them are healthy, since one of them recently died from a bacterial infection.

Clinical and Neurological picture assessment

As far as characterization of the hSOD1^{G93A} swine is concerned, a complete examination is ongoing by simultaneous evaluation of general clinical and neurological aspects, by biochemical and proteomic investigations and, by electrophysiological and neuroimaging analysis. Animal's phenotype will be analyzed as thoroughly as possible by means of repetitive evaluations along the whole time course of the disease/project. Actually, a careful clinical monitoring on the four living swine, conducted using the protocols described above is ongoing, in order to recognize the ALS symptomatic onset. Periodical blood samples are taken from hSOD1^{G93A} pigs and controls and analyzed with a standard biochemical profile, with no variation between controls and transgenic pigs has yet been detected.

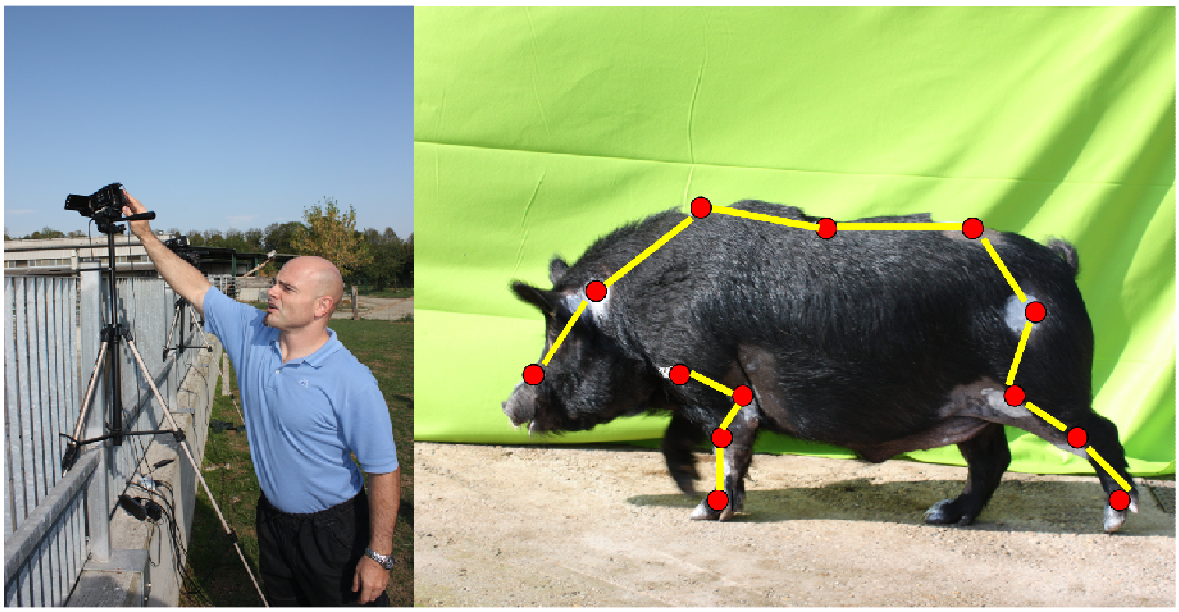
Locomotor function assessment, electrophysiological and neuroimaging analysis.

Since in G93A mice an early ALS clinical manifestation is the development of locomotor deficits, our aim is to promptly detect gait alterations in the hSOD1^{G93A} swine. A specifically-designed 3D Motion Capture System has been developed, in collaboration with Prof. A. Rainoldi (SUISM, Turin University Physical Education Research Center), to evaluate objectively the locomotion capability of animals and to detect minimal and precocious gait alterations (**Figure 6.19**).

So far, no gait alteration has been detected. This procedure will be continued for the entire duration of the experiment and, in case of encouraging results of the 3D motion capture Pilot-study, this analysis will be implemented.

Figure 6.19: 3D motion Capture.

The figure shows a 3D Motion Capture technique: the animals are subjected to trichotomy and marked with withe atoxic coluourant in the main joints area. Subsequently pigs are filmed during gait: the green background is needed for the next computer movie processing. Through a software it is possible to create a objective gait profile of each pig and thus possibly highlighting gait impairments.



With regard to the electrophysiological analysis, in collaboration with Prof. R. Merletti (Laboratory for Engineering of the Neuromuscular System, Politecnico, Turin), an EMG protocol is in progress of development. With regard to the MRI

analysis, we are looking to obtain the necessary permissions to use the scanner located at Neuroradiology Department of CTO Hospital, Turin.

Proteomic investigations

Plasma samples have been collected at one year of age, and then every four months, in order to assess whether proteome profile changes are detectable in the hSOD1^{G93A} swine. A global proteomic approach to characterize as much as possible the entire protein repertoire in animal plasma is ongoing and includes a mono-dimensional electrophoresis (1DE) approach for protein pre-fractionation and high-accuracy mass spectrometry (MS) for protein identification.

This strategy, called label-free quantitative proteomics, provides the most abundant protein identifications because it overcomes the limited dynamic range of protein separation inherent to bi-dimensional electrophoresis-based proteome analysis. Moreover it is capable of accurately identifying and quantifying thousands of peptides simultaneously, allowing a comprehensive protein profile with relative quantitative information for all the proteins identified in a sample using the mass spectral output. In this study we used spectral counting, which provides an estimation of the relative amounts of each identified protein in the different samples. Because of the low number of transgenic animals, this proteomic approach is a pilot investigation to assess whether proteome profile changes in plasma are detectable in a transgenic swine model for ALS when compared to the healthy counterpart. To date no differently expressed proteins between hSOD1^{G93A} swine and controls have been yet identified.

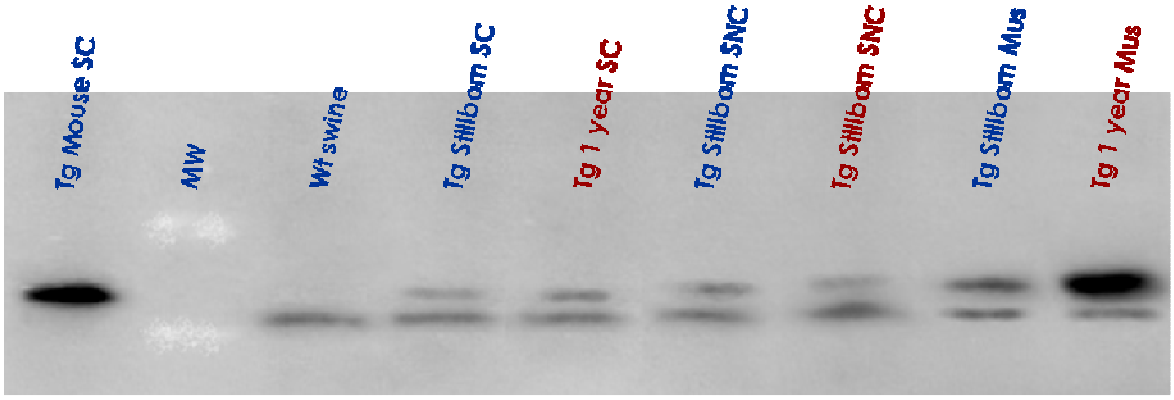
Post-mortem analysis

On tissue samples from the one year-old died hSOD1^{G93A} pig a complete banking has been performed. Homogenates coming from various tissues have been analyzed by WB. Interestingly, it was found that the one year-old pig muscle shows an increased accumulation of hSOD1^{G93A}, when compared to that of stillborn piglets, and that this is reminiscent of what occurs in transgenic mice, as

shown in (**Figure 6.20)**. Confirming this phenomenon, also the IHC analysis shows a hSOD1^{G93A} deposition pattern overall slightly more pronounced.

Figure 6.20: WB on tissues from stillborn piglets and on one year old pig.

The figure displays the results obtained with Millipore 07-403 at the dilution 1:1000 on tissues from the spinal cord of G93A mouse, wild type swine, stillborn piglets and from one year old pig. Mw: molecular weight; Wt: wild type; Tg: transgenic; SC: spinal cord; CNS: Central Nervous System; Mus: muscle.



hSOD1^{G93A} swine line establishment

As far as semen collection and banking is concerned, three founder boars have been generated and characterized for different transgene integration sites. Two boars are one year old and one is 10 months old. They are trained for artificial collection of semen over a dummy. Since they are cloned transgenic minipigs, training is more difficult than in commercial breeds and animals don't adapt to this procedure: to overcome this problem seminal material has been collected by electro-ejaculation. The collected semen has been both used in artificial insemination and frozen to be used later and to maintain a "back-up" of the genetic line. Two wild type sows have been artificially inseminated by laparoscopy and one of them is currently pregnant. This encouraging result gives hope that the ALS swine model can be maintained by sexual reproduction.

DISCUSSION AND CONCLUSIONS

In the decades since the first generation of transgenic mice produced by pronuclear injection [*Gordon et al., 1980*], transgenic animals have become indispensable tools for studying gene function, development, and regulation. Transgenic animals have already provided key insights into the brain and behavioral processes of relevance for human brain disorders.

The production of genetically modified live domestic animals remains a demanding task, especially for pigs (for review, *Lavitrano et al., 2006*; *Nagashima et al., 1995*; *Niemann et al., 2005*). Pronuclear injection and retroviral infection approaches were much less efficient in pigs than in mouse [*Hammer et al., 1985*; *Pursel et al., 1989*; *Wheeler & Walters, 2001*]. Although lentiviral transgene delivery offers an efficient method for the generation of transgenic pigs [*Whitelaw et al., 2004*], the use of lentivirus (HIV-1; EIAV) poses many safety and ethical concerns, due to the their proven ability of activating oncogenes as well as to the possibility of re-acquiring their pathogenic characteristics.

The recent introduction of SCNT [*Campbell et al., 1996*; *Wilmut et al., 1997*] has opened a new way for gene targeting in domestic animals, which may prove useful for the pig. Here, genetic manipulation is performed in somatic cell cultures, and the modified cells are later used as donors for nuclear transfer. In spite of the inefficiency of nuclear transfer, the first live offspring produced by porcine SCNT were obtained only 4 years after the birth of Dolly, simultaneously in three independent groups [*Betthauser et al., 2000*; *Onishi et al., 2000*; *Polejaeva et al., 2000*]. During the past 5 years, birth of somatic cell cloned piglets has been reported in laboratories of eight countries: UK, USA, Japan, Australia, Germany, Italy, Korea and China.

The overall efficiency remains low; only 1–2% of the transferred embryos develop to term, and the establishment of pig cloning as a routine procedure is a formidable task. Nonetheless, cloning of pigs has been more successful than rat cloning, which has only been accomplished once, by SCNT [*Zhou et al., 2003*].

Cloning of monkeys has likewise been reported only once [*Chan et al., 2001*], a procedure which has raised particular ethical considerations [*Gagneux et al., 2005*].

The use of pigs in experimental brain research was advocated more than 30 years ago. However, factors favouring the continuing and nearly exclusive use of rodents include the ease of housing and handling of small laboratory rodents. In contrast, the use of non-human primates was favoured due to the inherently greater similarity to human brain function. Nonetheless, the use of pigs within neuroscience has increased in the past decade to an extent, which far exceeds that of other farm large animals, such as, for instance sheep. However, basic knowledge about the anatomy, physiology and development of the pig brain is still scanty. At the same time, the continued extensive use of non-human primates in neuroscience research has become problematic due to their particular requirements for housing and handling, the lack of uniform quality with respect to microbiological status and group comparability and, importantly, ethical considerations. In a 'relational perspective' where emotional bonds between humans and animals are recognized, the use of pigs in neuroscience will probably become more ethically acceptable than is the use of primates, but may remain less acceptable than the use of rodents.

As reviewed above, pigs fulfil many of the requirements for a good experimental animal species for studying brain and behavioral processes and could eventually replace the use of monkeys for many neuroanatomy and neurochemistry studies. Wider use of pigs in research could facilitate extrapolation of preclinical findings to humans, especially in important research areas wherein obvious dissimilarities in brain structure and function render rodents less comparable to humans. Many of the prerequisites for conducting neuroscience research are fulfilled for pigs, including the need for standardized and inbred laboratory breeds, and an advanced knowledge of the general anatomy and physiology, housing, handling and experimental procedures.

The recent advent of imaging technology for studying brain function and structure in vivo has benefited from the relatively large size of the pig brain. Availability of a high quality normalized cDNA library for porcine brain has recently made possible microarray technologies and functional genomics for studies of brain function in pigs [*Nobis et al., 2003*]. To date, several specific pig models of brain disorders have been suggested or established.

Current developments in organ transplantation indicate that the pig may be suitable as brain tissue donor for humans afflicted by neurodegenerative diseases [*Deacon et al., 1997*]. The model of acquired Parkinsonism in Göttingen minipigs [*Mikkelsen et al., 1999*], and a model of diffuse brain injury in pigs with relevance for Alzheimer's disease have been described [*Smith et al., 1999*].

Rapid progress in basic biomedical research, mostly conducted on small laboratory rodents, has generated a huge amount of experimental data. However, before this newly gained knowledge can find its way into designs of new therapies, we need to validate it on animal models more closely related to humans. On the basis of selection of examples adduced in this thesis, we are convinced that the laboratory miniature pig can represent such a model. In spite of the disadvantages presented by housing requirements and large body size, the advantages of using pigs as an experimental animal for modelling human brain disorders has become increasingly evident. Compared to non-human primates, the primary candidate species for bridging this gap, pigs are cheaper and easier to maintain in controlled conditions. A considerable amount has been learned about pig brain anatomy and neurochemistry. The swine brain is gyrencephalic and more similar in anatomy, growth and development to the human one rather than those of common small laboratory animals. Moreover its large size allows detailed identification of cortical and subcortical structures by imaging techniques. Furthermore, swine offers numerous opportunities regarding transgenic manipulations of neural genes.

Their human-like physiology assures a high relevance of the data obtained in this species for human-related therapeutic research. With the miniature pig gestation period of 114 days and litters up to 12 piglets, enough experimental animals can be obtained, and a lifespan of 12–18 years allows long-term experiments evaluating the safety and efficacy of possible therapies.

As described in this text, miniature pigs are already extensively used in several fields of biomedical research, and we firmly believe that the importance of these animals as a biomedical model will increase even further in the near future. Thus, the development of animal models of human brain disorders in pigs can benefit from the full range of neuroscience techniques, as an alternative to research exclusively in rodents and non-human primates.

The present thesis reports the creation of a novel animal model, consisting in a transgenic swine carrying the human SOD1 gene with the G93A mutation responsible for the onset of ALS. This model is useful in studying such a disease and to develop novel diagnostic markers and therapeutic approaches for the human species. To achieve this goal, we were able to obtain a stable expression of exogenous gene ($hSOD1^{G93A}$) in a consistent, reproducible way and in the long term in living animals. Our experimental approach turned out to be successful and we managed to produce a large number of piglets (mean efficiency of blastocyst development to term of 8,78%), five of which reached the adulthood. All pigs generated express $hSOD1^{G93A}$, thus allowing us to exclude a $hSOD1^{G93A}$ expression-related toxic effect and simultaneously to confirm the effectiveness of our SCNT knowhow with obtaining a percentage of born animals far higher than that reported in the literature. Since $hSOD1^{G93A}$ expression was revealed in tissues from dead and stillborn piglets, our vector proved to be a suitable cassette for the expression of multiple genes relevant to human inherited diseases as far as tissue specificity is not required.

We decided to use in SCNT experiments a pool of donor cells showing different transgene expression level in order to minimize the risk of using cell clones unable to generate a viable animal. As a matter of fact, since this is the first hSOD1^{G93A} swine model produced so far, no data are available about the toxicity related to transgene expression level in the early stage of porcine embryonic development and after embryo transfer in sows. Since donors cells with different expression levels have been employed in SCNT, the cloned piglets present variable transgene expression levels depending on the particular cell from which they have been created. Among the 4 boars available, 3 different transgene integration sites have been identified. Semen collected from these animals has been used in breeding experiments to fertilize two wild type sows, and one of them is currenly pregnant. After farrowing the F1 progeny, of which, accordingly to Mendelian laws, 50% will inherit the disease, will be assessed for transgene expression levels and, by further inbreeding, the ALS swine line will be brought to homozygosity. Expected generation interval is about 12-15 months.

The SOD1 protein is an enzyme with antioxidant function acting by reducing the superoxide ion (O^{2-}) level, a toxic free radical product during the oxidative cellular metabolism. The superoxide ion is capable of altering proteins, membranes and DNA. The involvement of a mutated protein in its pathogenesis leads to include ALS in the proteinophaty family. Studies on transgenic rodent models were aimed at understanding the mechanisms by which mutated SOD1 gene leads to the onset of ALS: they have ruled out that the motor neuron degeneration is the result of loss of dismutase activity and allowed to detect the formation of aggregates of ubiquitinated proteins in affected tissues, among other containing the mutant SOD1: it is assumed that these inclusions protein play a role in the interruption of cell functions damaging mitochondria, proteasomes, mechanisms of protein folding or other proteins.

Unlike rodent models that show an extremely high transgene expression level and a rapid disease course [*Bendotti & Carrì, 2004*], our swine model presents an expression level comparable to that of human patients, where a single allele mutation results in the aforementioned toxic gain of function.

Piglets expressing the hSOD1-G93A protein, which already express the mutated protein at birth, are expected to show the full degeneration of upper and lower motor neurons, leading to muscle weakness, atrophy and evolving to complete paralysis, with times and modalities similar to those that occur in ALS patients.

On one hand this could result in a longer pre-clinical phase and in an increase in animal maintenance costs, on the other hand the present hSOD1^{G93A} swine represents an invaluable opportunity to find early biomarkers and a closer and more faithful model to reproduce human pathology since ALS is typically an adult-onset disease.

Currently, an animal model recapitulating all the ALS crucial aspects has not yet been produced. However, since increasing difficulties are emerging in translating information gained from rodent models into therapeutic options for ALS patients, there is an urgent need for an intermediate research system. We believe that a swine model provides this essential bridge between insights gained from rodent models and the reality of treating a human disease.

As a matter of fact, most preclinical studies on ALS are carried out in rodents, such as mice and rats overexpressing mutant SOD1. They have provided important information regarding the pathogenesis of the disease, nevertheless, one of the limit of these models is that they exhibit a loss of spinal but not of cortical motor neurons and the cortical spinal tract is poorly represented in these animals. In this respect, the anatomy of the pig motor cortex shows more similarities with that of humans, allowing investigating whether the loss of primary motor neurons is a cause or a consequence of the loss of spinal motor neurons.

Another advantage of the pig as a model of ALS is the possibility to work on a large amount of nervous system material which allows a series of analyses from the same animal. At present ALS pathogenetic mechanisms are poorly understood. *In primis* the availability of a swine model capable of reproducing the disease could allow to disclose still unknown ALS traits.

Particularly we will be able to study the clinical presentation of the disease, evaluating similarities and/or differences with the human condition. By a detailed examination of the clinical symptoms of the experimental swine hypotheses on the pathogenesis of the disease will be put forward. Consequently, we will try to delineate and characterize the involved pathogenetic mechanisms by investigating the supposed molecular pathways at their basis.

Since this is the first swine ALS model produced so far, the amount of data, obtained from animals characterization, will be enormous and hopefully, unexpected results or ground-breaking ideas could rise. We are expecting to reach an exhaustive characterization of this new animal model. Specific clinical aspects, such as neurological clinical picture, neuromuscular function and blood biochemical parameters, will be defined as thoroughly as possible by means of repeated evaluations along the whole time course of the disease/project.

All pigs will be neurologically examined according to an optimized protocol. Each animal will be examined by the same veterinarian. A standardized data collection form will be filled. Neurological examination will follow a standard procedure to assess mental status, posture, gait, postural reactions and proprioception, cranial nerves, spinal reflexes and sensitivity. In each pig the hind limbs' proprioceptive positioning reaction (knuckling over), the wheel barrowing reaction (with and without the neck extended) will be tested to evaluate postural reactions and proprioception. Spinal reflexes will be assessed by the standard test for flexor reflex, wherein the reflex is stimulated by pinching the foot with a forceps in the standing pig. If the response will be diminished or absent, the pig will be

examined in the lateral recumbent position, checking on each side the flexor, patellar and cranial tibial reflexes on the hind limbs and the flexor and extensor carpi radialis reflexes on the front limbs.

ALS patients show different onset, regarding both body side and age, and may present different courses of disease. The clinical management is based on neurological examination and functional rating scales. In daily clinical practice of neurologists trained in ALS this scales demonstrated to be reliable and useful.

We will attempt to correlate these clinical scales to ALS swine models and create a clinical reliable instrument within the global characterization, finding analogies with the disease in man and pig. Specifically, the ability to swallow will be assessed using direct and indirect tests and respiratory failure will be evaluated clinically, by performing blood gas analysis.

Periodical analyses on blood, urine and CSF from experimental pigs will hopefully confirm the already known role of some molecules in the pathogenesis of the disease and lead to the discovery of other probable biochemical markers characterizing ALS neurodegenerative process. Finding a model more similar to man will be fundamental for clinical and preclinical trials in ALS.

The identification of previously unraveled diagnostic markers of ALS will enable to recognize new therapeutic targets for the disease. This would further allow starting in the future clinical trials for anti-ALS drugs in the swine model, in the hope that possible positive outcomes obtained in this species may be efficaciously transferred to man.

We must actually remember that the majority of the preclinical trials for ALS performed until recently have been carried out in the mouse model and, even if successful in this species, they further failed to prove their efficacy in human beings. We are instead confident that the findings from the drug tests in the swine model, which is closer to man's physiology than the mouse, could really be transferable to ALS affected patients.

On 4th May 2012 our research group filed a patent application with title: "Novel transgenic animal model of Amyotrophic Lateral Sclerosis" that is become advantageously available for ALS researchers (**Figure** 7.1).

Figure 7.1: The patent application: "Novel transgenic animal model of Amyotrophic Lateral Sclerosis".

Verbale di Deposito Domanda di Brevetto per Invenzione Industriale
numero domanda: TO2012A000402

CAMERA DI COMMERCIO INDUSTRIA ARTIGIANATO E AGRICOLTURA DI TORINO

Camera di Commercio Industria, Artigianato e Agricoltura di TORINO

Verbale di Deposito
Domanda di Brevetto
per INVENZIONE INDUSTRIALE

Numero domanda: TO2012A000402
CCIAA di deposito: TORINO
Data di deposito: 04/05/2012

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LIST OF PUBBLICATIONS

List of scientific publications produced by Dr. Chieppa Maria Novella as author or co-author during the XXV cycle of "Patologia e Neuropatologia Sperimentali" PhD course, period 2009- 2012.

C.Porcario, F. Martucci, B.Iulini, E. Vallino-Costassa, D. Corbellino, **M.N. Chieppa**, M.D. Pintore, M.Mazza, P.L. Acutis, C.Casalone, C.Corona. - "coinvolgimento del sistema olfattivo in corso di scrapie naturale" - Large Animal Rewiew 2010; 6:21-32

M.N. Chieppa, A. Perota, D. Brunetti, C. Porcario, G. Lazzari, C. Bendotti, C. Corona, F. Lucchini, C. Casalone, C. Galli – "*Creation of an ubiquitous vector for expression of hSOD1G93A in pigs*" – Transgenic Research – vol 19 Number 2 April 2010- pag 326. 9th Transgenic Technology Meeting (TT2010) 22-23 March 2010 Berlin, Germany.

E. Vallino-Costassa, C.Porcario, D.Corbellini, **M.N.Chieppa**, A.Z.Perrazzini, T.Avanzato, F.Pulitano, M.D.Pintore, B.Iulini, C.Casalone and C.Corona. – "*Immunohistochemical characterization of BASE plaques by Confocal microscopy*" – Prion 2010 pg 177, 8-11 September 2010 Salzburg, Austria.

A.Schmädicke, L.Gasperini, D.Motzkus, C.Corona[3], **M.N.Chieppa**, C.Porcario, S.Gustincich, C. Casalone, G. Salinas-Riester, L. Opitz and G.Legname – "*Differential gene expression in BSE-inoculated macaques*" –Prion 2010 pg 172, 8-11 September 2010 Salzburg, Austria.

C.Corona, C.Porcario, E. Vallino-Costassa, M.Maria, F.Martucci, B.Iulini, M.Gallo, F.Paterlini, **M.N. Chieppa,** L.Dell'Atti, C.Maurella, S.Peletto, P.Acutis, M.Caramelli, G.Zanusso, and C.Casalone. – "*Phenotypic Variability of Italian BASE Affected Cattle*" - Prion 2011 pg37, 16-19 May 2011 Montreal, QC Canada.

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M.N.Chieppa, A.Perota, I.Lagutina, E.Vallino-Costassa, A.Grindatto, C.Palmitessa, D.Corbellini, M.Tortarolo, S.Colleoni, R.Duchi, G.Lazzari, C.Corona, F.Lucchini, C.Bendotti, C.Galli, C.Casalone. -"*Generation of pre-implantation pig SCNT embryos harboring the amyotropic lateral sclerosis- related hSOD1G93A gene*" - Prion 2012 pg 84. 9-12 May 2012 Amsterdam, The Netherlands.

E.Vallino-Costassa, G.Zanusso, E.Baioni, S.Peletto, P.L.Acutis, **M.N.Chieppa**, A.Grindatto, M.Gallo, M.Fiorini, M.Catania, O.Paciello, F.Tagliavini, M.Caramelli, C.Casalone and C.Corona. - "*Characterization of beta amyloid deposition in cattle brain*". Prion 2012 pg 82, 83. 9-12 May 2012 Amsterdam, The Netherlands.