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Neural stem cells isolated from amyloid precursor protein-mutated mice for drug discovery

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Abstract

AIM: To develop an *in vitro* model based on neural stem cells derived from transgenic animals, to be used in the study of pathological mechanisms of Alzheimer's disease and for testing new molecules.

METHODS: Neural stem cells (NSCs) were isolated from the subventricular zone (SVZ) of Wild type (Wt) and Tg2576 mice. Primary and secondary neurosphere generation was studied, analysing population doubling and the cell yield per animal. Secondary neurospheres were dissociated and plated on MCM Gel Cultrex 2D and after 6 days *in vitro* (DIVs) in mitogen withdrawal conditions, spontaneous differentiation was studied using specific neural markers (MAP2 and TuJ-1 for neurons, GFAP for astroglial cells and CNPase for oligodendrocytes). Gene expression pathways were analysed in secondary neurospheres, using the QIAGEN PCR array for neurogenesis, comparing the Tg2576 derived cell expression with the Wt cells. Proteins encoded by the altered genes were clustered using STRING web software.

RESULTS: As revealed by 6E10 positive staining, all Tg2576 derived cells retain the expression of the human transgenic Amyloid Precursor Protein (APP). Tg2576 derived primary neurospheres show a decrease in population doubling. Morphological analysis of differentiated NSCs reveals a decrease in MAP2- and an increase in GFAP-positive cells in Tg2576 derived cells. Analysing the branching of TuJ-1 positive cells, a clear decrease in neurite number and length is observed in Tg2576 cells.

The gene expression neurogenesis pathway revealed 11 altered genes in Tg2576 NSCs compared to Wt.

CONCLUSION: Tg2576 NSCs represent an appropriate AD *in vitro* model resembling some cellular alterations observed *in vivo*, both as stem and differentiated cells.

Key words: neural stem cells, Alzheimer's disease, neuron maturation, drug discovery.

Core tip

In this study neural stem cells isolated from Tg2576 mice are characterized as an i*n vitro* model for Alzheimer's disease. These cells represent a robust system for studying pathological mechanisms related to Aβ intracellular accumulation, such as stem cell status, or during differentiation processes. This model could provide a new cell platform for developing and screening new molecules.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, affecting 24.3 million people worldwide[1] with a 10-11% and 14-17% estimated lifetime risk respectively for males and females at age 85[2]. The cost of caring for afflicted patients is enormous and beyond the capability of most developing countries[3] and poses a serious problem also for the gross national product (GNP) of western countries[4]. The prevalence and incidence rates increase exponentially with age[5] and ageing is actually the only risk factor identified with certainty[6].

Today the pathophysiology of this disease is still to be elucidated, resulting in a total absence of disease-modifying therapies. However, the finding of amyloid β (Aβ) peptide deposits in the brains of affected individuals[7] and the study of the causes of the Familial AD (FAD) indicated Aβ peptide production as a possible therapeutic target[8].

The lack of effective therapies leads to the need for new models for drug discovery and screening. In this way stem cells represent a promising tool to create *in vitro* models suitable for studying disease mechanisms, for pharmacological target identification and for drug screening[9].

In particular, the possibility of deriving primary cultures of neural stem cells (NSCs) from animal models of disease at different ages provides an opportunity to derive cell types carrying human mutations. In this context, the Tg2576 mice, expressing the 695 isoform of the human Amyloid precursor protein (APP) gene carrying the Swedish mutation[10], represent a well-characterized model for AD. These mice accumulate Aβ in an age-related manner, particularly in the hippocampus[11], the cerebral cortex[12] and the olfactory bulb [13]. NSCs derived from these animals could be a robust tool for studying the disease mechanisms related to Aβ intra- and extra-cellular accumulation[14]. Cell platforms derived from stem cells can be used at

different stages of the differentiation process, thus acting as a useful tool for pharmacological agents active on proliferation, differentiation, functions and pathways of the mature Wild type (Wt) and pathological phenotype.

In this study we have characterized neural stem cells derived from adult Tg2576 AD mice in terms of self-renewal, gene expression profile, multipotency and differentiation capability, compared to neural stem cells derived from Wt age-matched animals.

We derived NSCs from the subventricular zone (SVZ). Neuroblasts generated in this area migrate to the olfactory bulb, renewing inhibitory interneurons in the primary olfactory nucleus and olfactory glomerula, thus contributing to the preservation of the olfactory function. The interest in studying these cells derives from the early olfactory impairment in AD patients [15], and from the possibility to use olfaction as a response marker for novel treatments [16]. Moreover, the neurosphere assay is almost exclusively used in the SVZ, while neuroblast and neural stem cells derived from the subgranular zone of the dentate gyrus of the hippocampus (e.g. the second brain area for constitutive neurogenesis) are predominantly cultured as adherent cells [17].

The term neural stem cell (NSC) has been used throughout the text to refer to a heterogeneous population of neural stem cells (NSCs), neural precursors and progenitor cells (NPCs)[18].

MATERIAL AND METHODS

Isolation, generation and expansion of neurospheres

Tg2576 mice and their non-transgenic littermates (001349-W) were purchased from Taconic Europe (Lille Skensved, Denmark). Animal care and treatment were in accordance with the EU Directive 2010/63/EU for animal experiments and in conformity with protocols approved by the Ethical Committee of Animal Experimentation, University of Bologna.

Adult NSCs were isolated following the Ahlenius and Kokaia protocol[19] with some modifications[20]. Brains from six month old mice were collected in a 50 ml tube containing ice-cold HBSS (Life Technologies, Milan, Italy).

Using a lancet, the olfactory bulbs were removed. Two 1 mm thick coronal slices were prepared from the rest of the brain and the SVZ was isolated and triturated in cold PBS using scissors. SVZ tissues were transferred to a 15 ml tube and allowed to settle. The PBS was then removed and the tissues incubated with the dissociation buffer consisting of: 1x HBSS; 5.4 mg/ml D-Glucose (SIGMA, St. Louise, MO, USA); 15mM HEPES (Life Technologies); 1.33 mg/ml Trypsin (SIGMA); 0.7 mg/ml hyaluronidase (SIGMA); 80U/ml DNase (SIGMA). After 15 minutes incubation at 37°C tissues were pipetted several times to favour dissociation and incubated again at 37°C for 10 minutes. In order to remove the undissociated tissue fragments, the solution was filtered through a 70 µm filter paper and then centrifuged at 400xg for 5 minutes. The resulting pellet was washed twice, first with a sucrose-HBSS solution (HBSS 0.5x; 0.3 g/ml sucrose), 500xg 10 minutes, then with a solution consisting of BSA (40 mg/ml), HEPES (0.02 M) in EBSS. After 7 minutes centrifugation at 400xg, the cellular pellet was resuspended in serum-free medium (DMEM/F12 GlutaMAX 1x; 8 mM HEPES; 100 U/100 µg Penicillin/Streptomycin; 0.1x B27; 1x N-2; 10ng/ml bFGF; 20ng/ml EGF) and, after cell count, cells were plated at a density of 50 cells/µl in a final volume of 3 ml in low-attachment 6-well plates (NUNC). Medium was changed every three days, centrifuging the cell suspension at 300xg for 5 minutes and gently resuspending the cellular pellet in fresh medium.

To obtain secondary neurospheres, cells were centrifuged at 300xg for 5 minutes and incubated in a 0.5 mg/ml trypsin – 0.2 mg/ml EDTA solution in HBSS at 37°C for 15 minutes. After inhibiting trypsinization and subsequent centrifugation, the cellular pellet was resuspended in half fresh/half old medium. Cells were counted and re-plated at the same density.

Three different cultures were prepared; all experiments were performed in duplicate.

For population doubling, cell yields and mRNA analysis, undifferentiated neurospheres were used, while for morphology studies, secondary neurosphere derived cells were analysed during spontaneous differentiation.

Cell count and population doubling

Cells were counted from primary and secondary neurospheres 3, 4 and 5 days after plating. Counting procedure was performed taking images of all neurospheres and statistically calculating the cell number based on single cell and sphere area using Image ProPlus software (Media Cybernetics Inc, Bethesda, MD, USA).

Population doubling was calculated using the following formula[21]:

$$PD = \log_{10}(N/N_0)*3.33$$

Where PD is the Population Doubling, N and N_0 are the final and initial number of cells, respectively.

RNA extraction and PCR array

The RNeasy Micro Kit (QIAGEN) was used for total RNA extraction and 300ng were retrotranscribed using the RT2 First Strand Kit (QIAGEN) following the manufacturer's instructions.

For the study of NSC gene expression, the 96-well QIAGEN PCR array for neurogenesis was used in combination with the RT2 SYBR Green qPCR Mastermix (QIAGEN), using 10 ng of cDNA per well.

Immunocytochemistry

In brief, 5 day *in vitro* (DIV) secondary neurospheres were dissociated as described above and plated on 0.25 mg/ml MCM Gel 2D Cultrex (TREVIGEN, Helgerman Court, Gaithersburg, MD USA) in 24-well plates at a density of $1x10^4$ cells/cm^2. Cells were grown in the same culture medium without mitogens. After this, 6 DIV cells were fixed (cold 4% paraformaldehyde, 20 minutes), washed (two PBS washes, 10 minutes each) and incubated overnight at 4°C with primary antibodies diluted in PBS/0.3% Triton x-100. After two washes, incubation with the secondary antibodies was performed at 37°C for 30 minutes. Cells were then washed and incubated with Hoechst 33258 (1 µg/ml in PBS/0.2% Triton x-100) for 30 minutes at RT. Finally, cells were washed again and mounted with phenylendiamine solution (0.1% 1,4-phenylendiamine -Sigma-, 50% glycerine -Sigma-, carbonate/bicarbonate buffer pH 8.6). Controls were always performed on secondary antibodies. The primary and secondary antibodies used are described in table 1, as well as the species in which they were produced, the manufacturers and the working dilutions.

In order to study the development of the filaments net of differentiated neurons, cells positive for the β-tubulin marker (TuJ-1) were analysed using the NIS-Elements Microscope Imaging Software (NIKON). Three random fields from each well were analysed, counting total neurite length and the number of branches per neuron. Hoechst 33258 nuclear staining was used to identify the total number of cells. At least 20 TuJ-1 positive cells per group were analysed.

To quantify the percentage of GFAP, CNPase and MAP2 positive cells, three random fields per well were considered.

In order to identify the expression of the Aβ/APP transgenic protein, the 6E10 antibody was used. This antibody recognizes the APP whole protein, its cleavage products Aβ (40 and 42) and the CTFβ (C-terminal fragment β),

although it is specific for the human protein, identifying in this case only the transgenic protein [22].

Statistical analysis

Student's *t*-test was used to statistically analyse data obtained from Wt and Tg2576 stem cell cultures. Data were considered significant when $p<0.05$.

RESULTS

Population doubling and cell morphology

Cells from Tg2576 mice neurospheres express the human transgenic protein APP/Aβ visualized using the 6E10 antibody (Fig. 1 A) whereas cells differentiated from Wt neurospheres do not (results not shown).

In order to characterize the neural stem cell model, the proliferation and differentiation capability of cultured NSCs derived from Tg2576 compared to Wt cultures were studied. Up to two generations of neurospheres, primary and secondary, were derived from Wt and Tg2576 animals in the presence of EGF and bFGF, and the population doubling calculated. When comparing Wt versus Tg2576 derived neurospheres, a statistically significant impairment was observed in population doubling of Tg2576 primary neurospheres (Fig. 1 B). A decrease in the yield of cells per animal in Tg2576 secondary neurospheres (Table 2) was also observed, even if the population doubling rate was restored during the generation of secondary neurospheres (Fig. 1 C).

Specific markers for neurons (Tuj-1 and MAP2), oligodendrocytes (CNPase) and astroglial cells (GFAP) were used to study the differentiation capability of NSCs from primary and secondary neurospheres after mitogen withdrawal (Fig. 2). Significant differences were found in the expression of GFAP (glial fibrillary acidic protein) and MAP2 (microtubule associated protein 2) between Wt and Tg2576 differentiated cells, with a lower number of MAP2-positive cells and a higher number of GFAP-positive cells derived from Tg2576 mice (Fig. 2 E) being observed. Differentiated neurons from these animals also show a lower number (Fig. 2 F) and length (Fig. 2 G) of neurites marked using an antibody against the β-III-tubulin (Tuj-1).

Gene expression analysis

In order to detect gene expression alterations between Tg2576 and Wt derived NSCs, a QIAGEN PCR array for neurogenesis was used.

Different genes are affected by the transgenic human mutated APP gene expressed in Tg2576 mice. According to the manufacturer's instructions, a

difference two times greater is considered biologically significant (Table 3). All the genes differently expressed in the two genotypes are overexpressed in Tg2576 cells. These include genes involved in neurotransmission (Acetylcholinesterase and Dopamine receptor D2), growth factors (Glial cell line derived neurotrophic factor, V-erb-b2 and Vascular endothelial growth factor A), cell growth and neuronal differentiation (CDK5, interleukin 3 and Notch gene homolog 2) and an APP-related protein (Amyloid beta precursor protein-binding-1).

Using the web-software STRING [http://string-db.org/], proteins derived from the genes of interest were connected in clusters according to their interactions and involvement in biological processes (Fig. 3 A). The software allows the net of interactions including other proteins closely linked to the one analysed to be extended, in order to obtain a better understanding of the possible pathways affected by the transgene effect in Tg2576 mice (Fig. 3 B). The analysis shows that almost all genes upregulated in Tg2576 NSCs interact with each other along different pathways, situating VEGF (vascular endothelial growth factor) as a node of the net.

DISCUSSION

At the present time, medical research relies on appropriate model systems to study disease mechanisms and to develop novel therapies. In particular, complex pathologies, such as AD, with no certainly ascertained causes and with no available cure, need new models for *in vitro* studies and for fast screening new molecules. Even if a number of animal models allow us to recreate diseases *in vivo*, *in vitro* systems are still needed to study pathologies at cellular and molecular level and to generate screening platforms for drug discovery.

The Tg2576 mice, which express the human APP695 transgene carrying the Swedish mutation, display several neuropathological features of AD, including plaque deposition[8] and early synaptic abnormalities[23, 24, 25, 26, 27]. This transgenic mouse is widely used as an animal model of AD, resembling the inherited form of the pathology.

In the present study NSCs isolated from Tg2565 mice were characterized as an *in vitro* model for AD due to their ability to preserve transgenic Aβ protein expression. The neurospheres generated from the primary culture of adult SVZ mice when cells are grown in the presence of mitogens (EGF and bFGF) are composed of a heterogeneous population of neural stem cells (NSCs), neural precursors and progenitor cells (NPCs). However, this mixed cell population is usually denoted as "neurostem cells". Neurosphere assay is an accepted model for the study of NSC self-renewal through the formation of different neurosphere generations (primary, secondary neurospheres) and also for the study of their capability to differentiate into neural cell phenotypes (neurons, astrocytes and oligodendrocytes)[18].

The complex pathology of AD is thought to include an impairment of neurotrophic signaling leading to alterations in the neurogenesis process[28]. Tg2576 derived cells show a significant decrease in cell doubling during the first stages of primary neurosphere development and a decreased yield of isolated neurons, thus resembling impairment of neurotrophic alterations in the neurogenesis process in Tg2576 mice[29] and in AD brains[30].

The ability of these cells to spontaneously differentiate when plated on a matrix mimicking the extracellular space allows us to study possible lineage and maturation differences between Tg2576- and wild-type-derived cells. A significant decrease was found in MAP2 positive cells in Tg2576 cultures, indicating a defect in neural lineage. Also neuron maturation is altered in Tg2576 compared to wild-type-derived animals. These cultures actually display a decrease in neurite extension of β-III-tubulin positive cells.

The role of APP in neurogenesis and neuron maturation processes has still to be fully elucidated, but a number of data suggest that the soluble APP (sAPP) alpha and the APP intracellular domain (AICD) affect proliferation, survival and migration of the NSC population[31], as well as neuronal maturation. The sAPP promotes gliogenesis, whereas AICD negatively modulates proliferation and maturation of the neural precursor[32]. This observation suggests that an APP overexpression could influence the cell fate of the NSCs, decreasing their number and impairing maturation.

The effect of the human APP transgene expression was thus analysed using neurogenesis-related PCR arrays, resulting in 11 genes overexpressed in the Tg2576 neural stem related to Wt cultures. Among those, APP- binding-protein-1 (APP-BP1) is more than two-fold up-regulated in Tg2576-derived neurospheres. Overexpression of the APP-BP1 gene has already been described in cortices and hippocampus of this animal model[33] and, in addition, overexpression of this gene in primary neurons is related to apoptosis induction and increase in DNA synthesis[34]. The expression of the APP-BP1 gene in Tg2576 NSCs seems also to resemble the up-regulated expression found in the lipid rafts in the hippocampi of AD brains[35].

Genes involved in the neurotransmission and differentiation processes are also affected by the presence of the transgene. Factors involved in driving neurogenesis in AD include the cholinergic system since acetylcholine acts as a growth-regulatory signal in the brain[36]. The overexpression of the acetylcholinesterase gene found in this *in vitro* model can partially represent an AD environment, where treatment involving acetylcholinesterase

inhibitors in clinical use for the symptomatic treatment of memory defects have shown a potential for stimulating neurogenesis[37]. Overexpression of neurotrophins and growth factors like GDNF[38] and VEGF[39] also mimic mechanisms acting to compensate the neurotrophic and differentiation deficits in these cells.

Other factors, such as interleukin-3[40], dopamine receptor D2[41], and cyclin-dependent kinase-5 (CDK5)[42], have already been shown to be involved in neurogenesis and AD models.

In conclusion, we propose that NSCs derived from animal models carrying human mutations possibly represent a novel and useful tool for drug discovery and drug screening in AD.

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COMMENTS

1Background

Alzheimer's disease is the most common form of dementia. Genetic studies indicate a possible role of the amyloid precursor protein in the disease mechanisms, thus indicating this protein and derived amyloid-β fragments as potential pharmacological targets. However, the lack of effective therapies leads to the need of new models for drug discovery and for the study of pathological processes.

2Researchfrontiers

Stem and re-programmed cells are looked as robust platforms and promising tools to create *in vitro* models suitable to study disease mechanisms, for pharmacological target identification and drug screening.

3 Innovations and breakthroughs

Neural stem cells isolated from transgenic animal models of diseases carrying human gene mutations could be used also to mimic in vitro neural ageing. Neural stem cells isolated from Tg2576 mice recapitulate aspects of in vivo patology, e.g. APP processing alterations, population doubling and neuronal differentiation process.

4Applications

The study results suggest that neural stem cells derived from a mouse model of Alzheimer's disease could be used as a platform for drug screening and to study disease mechanisms.

5Terminology

Neural stem cells are multipotent stem cells capable to generate the main central nervous system phenotypes (neurons, astrocytes and oligodendrocytes).

Adult neurogenesis: new neurons are formed and integrated in specific brain areas during adulthood.

Subventricular zone: this is one of the neurogenic areas of the adult central nervous system.

6Peer review

This is a scientific paper that illustrates an *in vitro* model based on neural stem cells derived from transgenic animals of interest in the study of pathological mechanisms of Alzheimer's disease (AD) and for testing new molecules for therapeutic purposes.

Table 2. Genotype-dependent cell yield per animal

	Wild Type	Tg2576
Primary neurospheres	$1.7 * 10^5 \pm 5*10^4$	$1.7 * 10^5 \pm 1*10^4$
Secondary neurospheres	$1.7*10^5 \pm 1.4*10^4$	$1.3*10^5 \pm 9.7*10^3$ (*)

Cells obtained from 4 animals were counted in primary and secondary neurosphere development. A decrease in the total number of cells per animal in Tg2576- compared to Wt culture was observed. Statistical studies performed by Student's *t*-test, * $p<0,05$.

Table 3. PCR array analysis

Gene	Regulation
Acetylcholinesterase	+ 3.41
Amyloid beta (A4) precursor protein-binding, family B, member 1	+ 2.33
CDK5 regulatory subunit associated protein 1	+ 2.07
Dopamine receptor D2	+ 2.62
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	+ 2.17
Glial cell line derived neurotrophic factor	+ 3.13
Interleukin 3	+ 2.69
Notch gene homolog 2 (Drosophila)	+ 2.29
Odd Oz/ten-m homolog 1 (Drosophila)	+ 3.81
Par-3 (partitioning defective 3) homolog (C. elegans)	+ 2.39
Vascular endothelial growth factor A	+ 3.01

Table represent the 11 genes resulted differentially expressed in Tg2576 NSCs related to Wt cells. Regulation is indicated as folds of increase. All the included differentially expressed genes resulted overexpressed in Tg2576 cells.

FIGURE LEGENDS

Figure 1. Transgenic protein expression and population doubling

Micrograph shows the positive 6E10 staining of Tg2576 plated cells derived from secondary neurospheres (A). A decrease in population doubling seems to occur in the first few days of primary neurosphere development (B), and is not repeated in secondary neurosphere formation (C).

Statistical analysis: Student's *t*-test. Graphs represent mean ± SE and asterisks represent significant differences between Tg2576 and Wt at the same DIV (* $p<0.05$). Bar: 50μm.

Figure 2. Morphological analysis

A – D. Micrographs showing immunostaining for β-III-tubulin (TuJ-1) and MAP2 in Wt and Tg2576 cells. Hoechst 33258 nuclear staining was used to determine the total cell number. E. The analyses of cell lineage indicate an increase in GFAP- and a decrease in MAP2-positive cells in Tg2576 compared to Wt. The number of CNPase-positive cells does not vary between the two genotypes. F, G. neural maturation analysis shows both a decrease in TuJ-1 positive branching of the third order and in neurite extension in cells derived from Tg2576 animals.

Statistical analysis: Student's *t*-test. Graphs represent mean ± SE and asterisks represent significant differences between Tg2576 and Wt (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Bars: A, C: 100μm; B, D, 50μm.

Figure 3. Functional protein net

The list of the neurogenesis-involved genes altered in Tg2576 versus Wt cells is reported in Table 2. The related proteins were clustered using STRING web software. Figure shows the 11 genes (A) and the extended net (B).

This is to certify that the English language of the attached text was revised and where necessary corrected by Ian McGilvray, British Language Services Sas, via A. Boito 44. 00199, Rome.

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