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Research Article

Neurotrophic Features of Human Adipose Tissue-Derived Stromal Cells: *In Vitro* and *In Vivo* Studies

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Due to its abundance, easy retrieval, and plasticity characteristics, adipose-tissue-derived stromal cells (ATSCs) present unquestionable advantages over other adult-tissue-derived stem cells. Based on the *in silico* analysis of our previous data reporting the ATSC-specific expression profiles, the present study attempted to clarify and validate at the functional level the expression of the neurospecific genes expressed by ATSC both *in vitro* and *in vivo*. This allowed evidencing that ATSCs express neuro-specific trophins, metabolic genes, and neuroprotective molecules. They were in fact able to induce neurite outgrowth *in vitro*, along with tissue-specific commitment along the neural lineage and the expression of the TRKA neurotrophin receptor *in vivo*. Our observation adds useful information to recent evidence proposing these cells as a suitable tool for cell-based applications in neuroregenerative medicine.

1. Introduction

Adipose-tissue-derived adult pluripotent cells, commonly known as adipose tissue stromal cells (ATSCs) are mesenchymal stem cells (MSCs) residing in the connective stroma of adipose tissue. They represent a valuable source of adult stem cells, being easily isolated from an abundant and accessible tissue [1–3]. Their plasticity along with the ease of *in vitro* culturing and propagation makes them the most used cell type in a wide range of tissue regeneration applications [4–7].

We have previously shown the ATSC-specific molecular properties, by comparatively analyzing the geno-mewide expression profiles of MSCs from different adult tissues [3]. The study allowed indicating the main molecular features which regulate the stemness maintenance of MSCs and a more extensive plasticity of ATSC *in vitro*. The complete result dataset of this previous study (available at the Gene Expression Omnibus (GEO) database, http://www.ncbi.nlm.nih.gov/gds, accession number GSE8954) also indicated that ATSCs specifically express neurospecific genes.

The purpose of this study is to extract the biologically significant genes from this dataset and validate the functional relevance of the neurotrophic genes expressed by ATSC both *in vitro* and *in vivo*.

2. Materials and Methods

2.1. In Silico Biological Analysis of the Microarray Dataset. In order to identify the candidate genes involved in the neurotrophic properties of ATSCs, the gene list of ATSC-specific genes obtained through the microarray-based gene profiling of ATSC compared to bone-marrow-derived mesenchymal cells (BMSCs) and fibroblasts (http://www.ncbi.nlm.nih.gov/gds, accession number GSE8954) [3] underwent an *ad hoc* biological analysis, aimed at finding neurologically relevant genes. For this purpose, the list of 441 genes specifically upregulated in ATSC (P value <0.01), resulting from the statistical analysis (see [3] for statistical methods used in data analysis), were categorized according to the "biological function" annotations implemented from the Gene Ontology

Annotation (GOA) database (http://www.ebi.ac .uk/GOA/). Specific neuroprotective, neurodevelopmental, and/or neurotrophic functions were further studied using the "Gene Reference Into Function" tool in GenBank (http://www.ncbi .nlm.nih.gov/gene/about-generif).

2.2. Patients and Specimens. Adipose tissue (AT) specimens were obtained by lipoaspiration from healthy volunteers (mean age 40.2 ± 14.2 years) upon obtaining a written consent. A skin biopsy was obtained from the retroauricular region of an healthy male donor (aged 45) and served for the isolation of human dermal fibroblasts (HDF). Individuals data were handled confidentially and anonymously. All the procedures employed in this study were approved by the ethical committee of the Catholic University of Rome (Rome, Italy; number P552 (A.779)/CE2007).

2.3. Chemicals and Reagents. Cell culture media and supplements were purchased from Lonza (Basel, Switzerland). Enzymes, growth factors, and all other chemicals used in this study were purchased from Sigma (Sigma-Aldrich, St Louis, Mo,USA), unless otherwise specified.

2.4. ATSC Isolation and Culture. Mesenchymal stromal cells were isolated in primary culture from the lipoaspirates, as already described elsewhere [3]. Briefly, AT was extensively washed, mechanically fractionated, and digested using 0.1% collagenase type VIII. The lysed tissue was then filtered through a 100 μm mesh, and the cell suspension was centrifuged. The cell pellet was then plated in T75 tissue culture flasks using Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.2 ng/mL fibroblast growth factor beta (bFGF). Cells were subcultured as previously described [3] and then used for *in vitro* and *in vivo* experiments, as detailed in the following paragraphs. ATSCs growth kinetics up to fifteen culture passages and their immunophenotype were assessed as already described elsewhere [6].

2.5. HDF Isolation and Culture. Dermal fibroblast were isolated in primary culture from the skin biopsy and cultured as previously described [8]. These cells served as a mesoderm-derived differentiated controls to produce the conditioned medium (HDF-CM) used in the *in vitro* experiments (see following paragraphs).

3. *In Vitro* Experimental Procedures: Neural Cell Line Cultures and Treatments

In order to assess the functional significance of the neurotrophic genes specifically expressed by ATSCs, LAN5 and PC12 cells were used as neural undifferentiated cell lines for the *in vitro* experiments. These cell lines are commonly employed as valuable models to study the neuronal differentiation and degeneration processes *in vitro* [9–13].

3.1. Cell Lines and Treatments. The human LAN-5 dopaminergic cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM glutamine, 40 μg/mL gentamicin, and 10% heat-inactivated fetal calf serum (FCS), according to standard protocols [13]. Cells were plated at a $10^4/cm^2$ seeding density in 24-well plates. The day after plating, ATSCs were seeded in the same wells using a $10^4/cm^2$ seeding density. In addition, separate wells of LAN5 cells were cultured in presence of ATSC-conditioned medium (ATSC-CM), which was obtained by filtering through a 0.2 μm cellulose acetate filter the supernatant medium of subconfluent ATSC cultures. Thereafter, both LAN5-ATSC cocultures and ATSC-CM-treated cells were grown for three days without changing the culture medium.

The rat PC12 noradrenergic cell line was seeded at a density of 5000 cells/cm^2 in RPMI 1640 medium containing 5% fetal calf serum and 10% horse serum and grown till 80% confluence, according to standard protocols [12]. Between the third and the fourth culture passage, cells were plated in 24-well plates, using a $10^4/cm^2$ seeding density. The day after plating, the PC12 culture medium was replaced by either ATSC-CM or HDF-CM. LAN5 and PC12 cells in standard culture medium served as controls in the experiments. As PC12 cells are known to differentiate along a functional neuronal phenotype upon NGF treatment, cells primed with 50 ng/mL of NGFβ were used as positive control of differentiation [9]. Cellular morphology was evaluated by an invertoscope up to four days of culture.

4. *In Vivo* Experimental Procedures: Neonatal Rat Brain ATSC Inoculation

4.1. Adenoviral-Mediated Cell Transduction. In order to make ATSC recognizable in living tissues, cells were transfected using a defective adenoviral vector carrying the enhanced green fluorescent protein (AdEGFP) as a reporter gene. AdEGFP stocks were kindly provided by the Vector Core Facility of the University of Pittsburgh (Pa, USA). Cells were plated at a $10^4/cm^2$ seeding density and treated with AdEGFP using a multiplicity of infection (MOI) of 100 plaque-forming units (pfu)/cell. The efficiency of cell transduction was assessed observing fluorescent cells 48 hours later using an invertoscope equipped with a fluorescent lamp. EGFP-expressing cells were then inoculated in neonatal rats, as further described.

4.2. Cell Transplantation. Human ATSCs were transduced with Ad.eGFP 48 hours prior to *in vivo* transplantation. The surgery was performed on neonatal rats at postnatal day 1 (P1), after the induction of deep anesthesia by hypothermia. A small parietal hole was made into the skull above the frontal cortex, and cells were slowly injected into the lateral ventricle (1 mm posterior to the bregma, 1 mm lateral to the midline, and 2–2.5 mm ventral to the pial surface) using a glass micropipette coupled to a Hamilton microsyringe. For each animal treated, 5×10^4 ATSCs suspended in 1 μL of Puck's saline A (Invitrogen, Carlsbad, Ca) were used. Sham-operated animals were injected with the same volume of

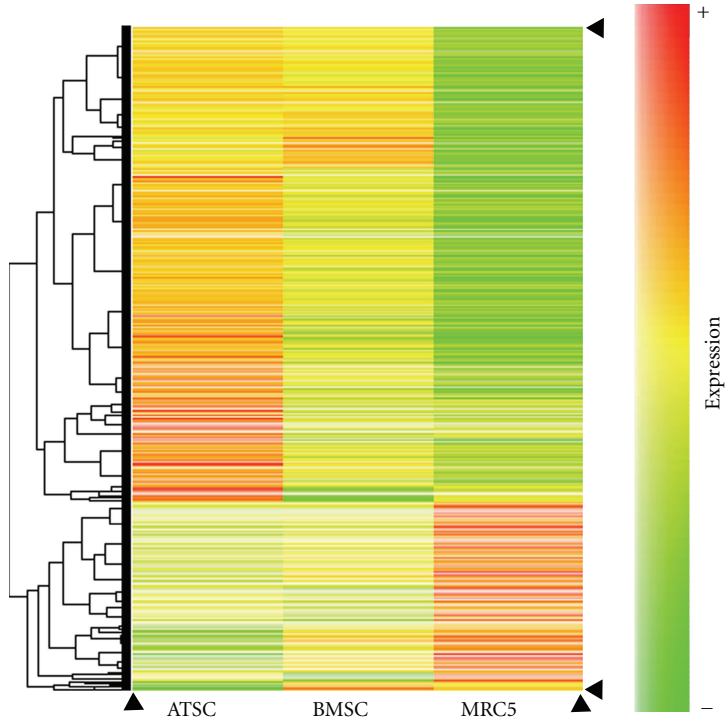

Figure 1: Hierarchical clustering of microarray data. The dendrogram shows all the 441 genes differentially expressed in ATSC (selected by *t*-test, *P* value 0.01) resulting from the statistical analysis [3]. Each row represents a single gene, while cell types are grouped in columns. The colored representation of gene expression is shown according to the scale on the right side of the figure. BMSC: bone-marrow-derived stromal cells; MRC5: human lung fibroblast cell line. See [3] for details.

saline solution. Following treatment, the skin was rapidly sutured, the pups were warmed under a lamp and returned to the dame. All animal protocols used have been approved by the Animal Experimentation Committee of the Catholic University of Rome.

4.3. Tissue Processing. The animals were sacrificed 7 and 15 days after injection ($n = 6$ for each group of ATSC treated rats, and $n = 3$ for each group of sham-treated animals). Under deep anaesthesia (ketamine/diazepam 1 : 1 i.p.), they were perfused through the aorta with 100 mL of saline solution, followed by 100 mL of 0.01 M, pH 7.4 PBS, and 4% paraformaldehyde. Thirty minutes after perfusion, the brains were removed from the skull, postfixed in 4% PBS paraformaldehyde for 2 h and immersed in 30% sucrose. Serial 40 μm thick coronal sections were cut on a freezing microtome. The first series of sections was mounted in Vectashield (Vector, UK) for fluorescent evaluation of eGFP-expressing cells. Other series of adjacent sections were processed for immunohistochemistry.

4.4. Immunohistochemistry. Anti-GFAP (polyclonal, Dako, Glostrup, Denmark, 1 : 1000 overnight at 4°C), -Doublecortin (policlonal, Chemicon, Temecula, Ca, 1 : 3000, overnight at 4°C), -NeuN (monoclonal, Chemicon, Temecula, Ca, 1 : 500, 48 h at 4°C), -O4 (monoclonal, Chemicon, Temecula, CA, 1 : 500, overnight at 4°C), and -TrKA (Santa Cruz Biotechnology, Heidelberg, Germany, 1 : 1000 overnight at 4°C) were revealed using cyanine fluorochromes-labeled secondary antibodies (donkey anti-mouse Cy3 or donkey anti-rabbit Cy3, Jackson Immunoresearch Laboratories, West Grove, Pa, 1: 400) following incubation for 1 hour at RT. Sections were mounted in Vectashield for fluorescent visualisation of labeled cells. Controls were prepared by omitting the primary antibodies.

The colocalization of eGFP with the above-mentioned markers was examined with a Zeiss LSM 510 confocal laser scanning microscopy system.

5. Results

5.1. ATSCs Express Neurospecific Genes. Data extracted from previously published microarray data showed the selective upregulation of 441 genes ($P < 0.01$) in ATSC compared to BMSC and human fibroblast MRC5 cells (Figure 1). The *in silico* biological analysis of the microarray data (GEO dataset number GSE8954) allowed to identify a short list of biologically relevant genes, involved in neuroprotection, neural developmental processes, and neurotrophic functions (see Table 1). In particular, this 12-transcript list included genes, namely, nerve growth factor beta (NGFB), neuropilin 1

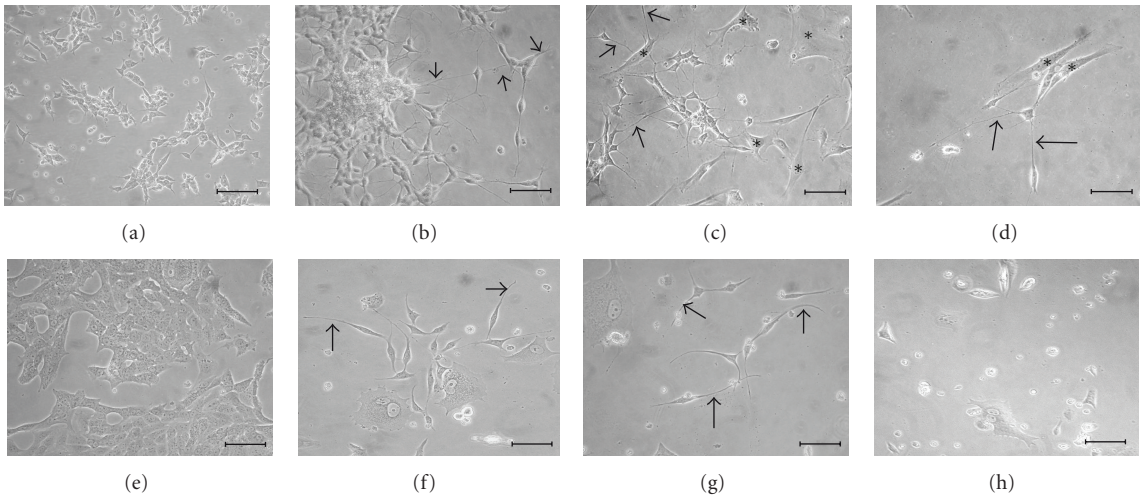

Figure 2: *In vitro* neurotrophic effects of ATSC. LAN-5 human neuroblasts and PC12 rat cells were cultured either ATSC-conditioned medium or co-cultured with ATSC and morphological modifications were monitored over time: (a) LAN5 in standard culture medium; (b) LAN5 cultured in ATSC-CM for 72 hours; (c) and (d) LAN-5 co-cultured with ATSC using a cell density of 10^4 cell/cm^2 for both cell populations; (e) PC12 in standard culture medium; (f) PC12 cultured in ATSC-CM for 4 days; (g) PC12 cultured in βNGF 100 ng/mL for 4 days; (h) PC12 cultured in HDF-conditioned medium for 4 days. Arrows show evidence of neurite outgrowth; asterisk ($*$) indicate ATSC in culture. Scale bar 100 μm in all panels except for panel $d = 10\,\mu$m.

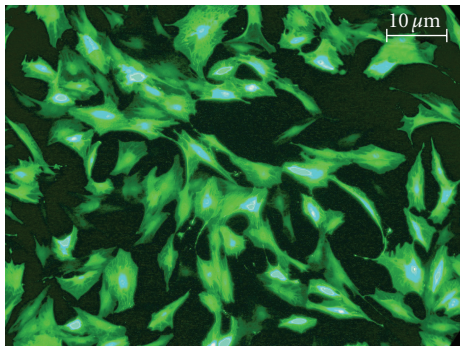

Figure 3: Efficient adenoviral-mediated transduction of ATSC. ATSCs were transfected with 100 pfu/cell of AdEGFP and fluorescent cells were observed after 48 hours: nearly 80% cells were EGFP-positive as shown in the figure.

(NRP1), and GTP cyclohydrolase 1 (GCH1), encoding soluble neurotrophins which are known to mediate neuronal growth, differentiation, migration, and neuroprotection [9, 14, 15]. The neuronal cadherin CDH2 belongs to the major transmembranar signalling complex cadherin/catenin that plays a key role in neuronal processes during early development. It is activated during neural circuit formation and maturation to mediate axonal outgrowth and arborisation [16, 17]. Moreover, nearly all genes in the list are implicated in developmental processes within the nervous system, such as neurogenesis, neuron differentiation, axonogenesis, axon guidance, nerve growth, and glia differentiation and migration (Table 1). The phosphoribosyl pyrophosphate synthetase 1 (PRPS1) and the phosphoglycerate mutase 1 (PGAM1) genes are implicated in metabolic pathways which are essential in neuronal function and maintenance (see function details and references in Table 1).

5.2. ATSCs Induce Neurite Outgrowth in PC12 and LAN5 Cells. In order to evaluate the effects of the supposed neurotrophic properties of ATSC, the capability of inducing visible changes in cell morphology of neural cells was first assessed *in vitro*. For this purpose, LAN5 cells were either cultured in ATSC-CM or cocultured with human ATSC for three days. Both cells cultured in ATSC-CM (Figure 2(b)) and those in coculture (Figures 2(c)-2(d)) displayed evident changes in shape and morphology, compared to those grown in standard culture medium (Figure 2(a)). The morphological changes consisted in the formation and elongation of neurite-like processes observed in discrete loci of the culture plate. The outgrown neurites seemed to establish contacts with both neural cells and ATSC in culture (Figure 2(d)).

In addition, the adrenergic PC12 cell line was cultured in presence of ATSC-conditioned medium (ATSC-CM) for four days. PC12 primed with βNGF and PC12 cultured in HDF-CM was used as positive and negative neuro-differentiation controls, respectively. The morphological analysis showed the extensive outgrowth and extension of neurite-like structures in both βNGF- and ATSC-CM-treated cells exhibiting essentially overlapping features (Figure 2(f)-2(g)), compared to cells cultured in standard medium (Figure 2(e)). Cells grown in HDF-CM showed clear morphological signs of distress, becoming small-rounded vacuolized cells, with a marked tendency to detach (Figure 2(h)).

Table 1: Selected ATSC-specific upregulated genes involved in neurospecific functions ($P < 0.01$).

Gene symbol	Gene bank	Gene name	Neurospecific functions				Process	References
			protection	development	trophism	metabolism		
SLC1A1	NM_004170.5	Solute carrier family 1, member 1	+				Protection against glutamate neurotoxicity	[18]
CDH2	NM_001792.3	Cadherin 2, type 1, N-cadherin (neuronal)		+			Pre-to-postsynaptic adhesion neuronal migration Axonogenesis synapse assembly	[19]
CELF2	NM_001025077.2	CUG triplet repeat, RNA binding protein 2	+	+			Motor neuron survival splicing control during development	[7]
VLDLR	NM_003383.3	Very low density lipoprotein receptor	+	+		+	Protection against hypoxia and glucose starvation Nervous system development lipid uptake in neurons and astrocytes	[20]
NRP1	NM_003873	Neuropilin 1	+	+	+		Cell survival axon guidance Migration and invasion	[15]
NGFB	NM_002506.2	Nerve growth factor, beta polypeptide		+			Neuron differentiation nerve growth	[9]
ENC1	NM_003633.2	Ectodermal-neural cortex	+	+			Anti-apoptotic nervous system development	[21]
GCH1	NM_000161.2	GTP cyclohydrolase 1	+		+		Protection from brain damaging events secreted by astrocyte	[14]
FGF2	NG_012449.1	Fibroblast growth factor 2		+	+		Neurogenesis migration	[22]
NDN	NM_002487.2	Necdin homolog (mouse)	+	+			Protects neuron from oxidant stress Neuron development glial cell migration	[23]
PRPS1	NM_002764.3	Phosphoribosyl pyrophosphate synthetase 1		+		+	Purine synthesis nervous system development	[24, 25]
PGAM1	NM_002629.2	Phosphoglycerate mutase 1 (brain)	+			+	Regulation of energy metabolism neuroprotection against Aß-toxicity	[26]

5.3. In Vivo Analysis of ATSC-Specific Neurotrophic Features. The functional significance of the ATSC-specific upregulation of genes involved in the neural lineage has been further investigated *in vivo* after transplantation of ATSCs in the neonatal rat brain. ATSCs were efficiently transduced with Ad.eGFP prior to *in vivo* transplantation (Figure 3).

Histological examination of ATSC-transplanted young rats sacrificed 7 days after transplantation showed clusters of eGFP-positive ATSCs, characterized by rounded morphology, localized in the wall of the lateral ventricle, near the needle tract, surrounded by GFAP positive astroglial endings (Figure 4(a) A–C). In particular, based on the results

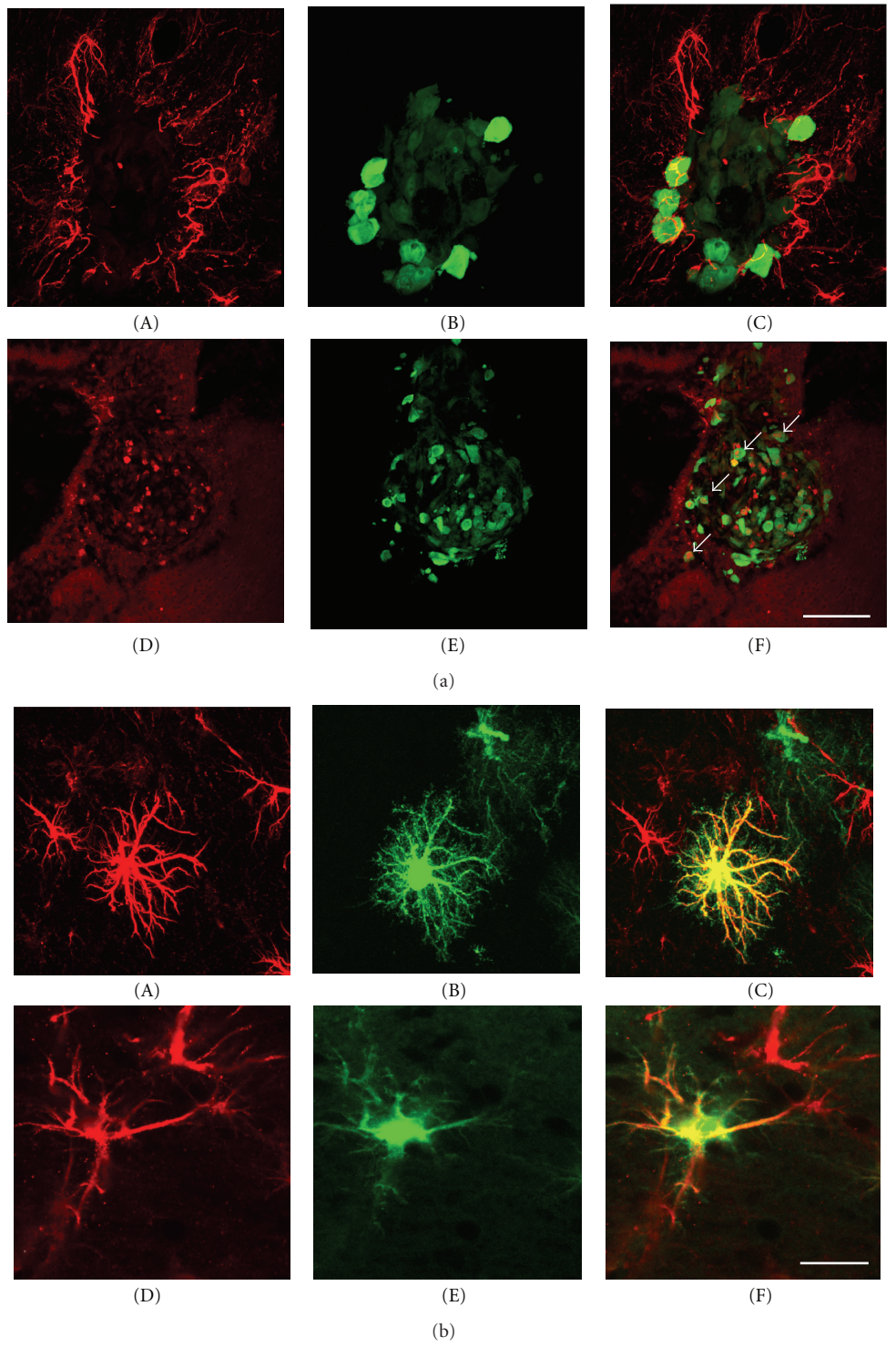

FIGURE 4: Engraftment of human ATSCs within newborn rat brain. (a) Confocal microscopy micrographs showing the engraftment of eGFP-positive (green; A, D) ATSCs within newborn rat brain 1 week after cell infusion. ATSCs exhibit a round morphology (A, C), are surrounded by GFAP-positive astrocytes (red; B, D), and express TRKA (red, B, D, arrows). (b) Engraftment and *in vivo* differentiation of human ATSCs within newborn rat hippocampus 2 weeks after implantation. Confocal images of GFAP (red; A) or TRKA (red; D) immunolabeled eGFP (green; B, E) expressing ATSCs. Engrafted cells express the astrocytic marker GFAP (yellow, C) and the TRKA receptor (yellow, F). Scale bars: (a) A–C 120 μm, (a) D–F 420 μm, (b) A–C 80 μm, and (b) D–F 60 μm.

observed *in vitro*, we assessed the expression of the anti-NGF-β receptor, as to further investigate the significance of the NGF/TRKA signaling pathway. ATSCs exhibited immunopositivity for the TRKA antibody 7 days after transplantation (Figure 4(a) D–F). At this time point, no co-localization with neuronal (Doublecortin, NeuN), astroglial (GFAP), or oligodendroglial (O4) markers were observed (not shown).

Histological examination of young rats sacrificed 15 days after transplantation confirmed the survival of ATSCs in the brain of injected animals. Grafted cells examined at this time point were mainly localized within the brain parenchyma, near the ventricular system and frequently in the hippocampus. They showed a bipolar or multipolar morphology with processes extending in various directions. Interestingly, confocal microscopy examination revealed that many of these eGFP- positive ATSCs coexpressed also the astroglial marker GFAP (Figure 4(b) A–C), while no colocalization between eGFAP and Doublecortin, NeuN, or O4 was found (not shown). Virtually all engrafted ATSCs expressed immunopositivity for anti-TRKA antibody (Figure 4(b) D–F)). Sham-operated animals exhibited only a mild GFAP-stained glial reaction around the needle tract (not shown).

6. Discussion

Different evidences indicated that transplanted MSCs promote endogenous repair of neurologically damaged areas and neural differentiation, via the release of soluble trophic factors and cytokines [27].

In particular, recent studies indicated that ATSC culture medium should contain neurotrophic factors, which were able to induce neuritogenesis in PC12 cells *in vitro* and protect brain from both hypoxic damage and glutamate neurotoxicity [28–30]. Nonetheless, only selected molecules have been dosed in ATSCs as possible neurotrophic candidates [28–31], while the expression of a wider panel of neuro-specific molecules has not been assessed in ATSCs so far.

The possible complete list of neurotrophic/neuroprotective factors specifically expressed by ATSC is proposed in this study, as a result of the *in silico* analysis of differentially expressed genes in MSC isolated from different adult tissues [3]. This revealed that ATSCs strongly and specifically express at least three neurotrophins: NFGB, NRP1, and FGF2. These secreted molecules reasonably represent the molecular background of ATSC-neurotrophic features. The *in vitro* assays in this study demonstrated that ATSCs could in fact induce neurite outgrowth not only in PC12, but also in human neuroblasts (LAN5 cell line). The induction of neuronal differentiation should be the result of the demonstrated presence of soluble secreted factors in ATSC culture medium [28] along with cell-to-cell contacts with neural cells *in vitro*. Thus, this event could be reasonably mediated by both NGFB, which promotes neuronal differentiation [9], and NRP1 that guides axon growth [15]. Also the nonneurospecific growth factor FGF2 could play a role in this event, being able to promote neurogenesis [22]. In addition, the adhesion molecule CDH2 that is expressed on the plasma membrane and is involved in axonogenesis and synapse assembly [19] could play a role in ATSC-mediated neuronal differentiation of LAN-5 cells. Although, the possibility that other factors participate in mediating this effects cannot be excluded.

Our data could also suggest that ATSCs neurotrophic function resides in a sort of astrocyte-like phenotype, as they specifically express genes belonging to the glial phenotype, including VLDR, FGF2, and NDN, according to GOA annotations. To this end, the necdin homolog (NDN) gene, involved in the NGFB signalling pathway, is particularly relevant, as it drives glial migration during nervous system development and is expressed in the cell projections [23]. Although the neural transdifferentiation capacity of MSCs has been largely debated, many recent studies emphasise the possibility of both bone marrow- and adipose tissue derived-undifferentiated stromal cells to differentiate along the neuroectodermal lineage to neuronal-like cells of the ectodermal lineage, mainly *in vitro* [32–42].

Recent data indeed assess the importance of cell-cell interactions along with the release of growth factors from the host tissue in ATSCs neural transdifferentiation [43]. In line with these observations, the results obtained *in vivo,* following cell implantation in the neonatal rat brain, indicate that ATSCs survive, migrate, and essentially differentiate toward an astroglial fate. Taken together, our observations suggest that ATSCs show a predisposition to the neural fate as they express a molecular phenotype resembling neural commitment *in vitro* and transdifferentiate along the neural lineage *in vivo*.

Recent reports evidence the successful implantation and migration of ATSCs *in vivo* using experimental models of rat brain ischemia, where they were able to promote functional recovery [44–47]. In addition, different groups reported the neural transdifferentiation of ATSCs transplanted in the injured spinal cord [48, 49], evidencing that, when detached from the physiological niche, they express ectoderm neural markers [50]. We may speculate that secreted soluble factors from neighbouring cells and physical reciprocal contacts with neural cells may cause/facilitate transdifferentiation processes, as also indicated by the expression of the NGF receptor TRKA by transplanted ATSCs. This evidence, reported in *in vitro* studies [51], could suggest a possible autocrine mechanism on ATSC, as they express NGFB *in vitro*, although the functional significance of this observation deserves further studies.

Taken together, the results obtained in this study seemed to indicate that ATSC neurotrophic features reside in their specific capability of expressing not only secreted neurotrophins/neuroprotective molecules, but also structural protein-coding genes, mimicking the astrocyte function in sustaining neurons metabolism and function in the central nervous system and being able to differentiate into astrocytes. These properties, along with their reported capacity to migrate in injured tissues, could suggest possible future applications of ATSCs in many diverse neurological contexts.

Author Contribution

Wanda Lattanzi and Maria Concetta Geloso contributed equally to this work.

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