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The Study of Comparative Nsc, S And Bmsc, S Neurons Therapy For Treatment of Artificially Created Neuron Disorders In Sheep

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Abstract. The main problem with embryonic stem cell research is the tissue incompatibility. Millions of lines must be established in order to serve a significant percentage of potential patients. The use of autologous adult stem cells (cells from the patient) eliminates the problems with tumorogenesis, mutation, and tissue incompatibility.Multipotent stem cells can be found in the numerous adult tissues. The isolation and expansion of neural cell types has become increasingly relevant in restorative neurobiology. To examine the developmental potential of adult sheep brain cells, we applied conditions favoring the growth of neural stem cells and bone marrow stem cells to multiple cortical regions, resulting in the identification and selection of a population of adult sheep neural progenitors.. It has also been seen that BMSC can differentiate into neural cells and therefore can be used for restoration of brain damage. We tested the theory that BMSC could change into neural precursor cells by co culturing BMSC, identified by using β-galatosidase, with fetal primary neuronal cultures. We concluded that, our findings suggest an unprecedented developmental plasticity and proliferative potential are retained in CNS glia throughout life and the use of autologous adult stem cells eliminates the problems with tumor genesis, mutation, and tissue incompatibility in both NSC,s and ESC,s therapy.

Keywords: NSC,s;BMSC,s;Teratoma;ANHPs; β-galatosidase, sheep

1. INTRODUCTION

Recently, scientists demonstrated that human, mouse, and rat postnatal bone marrow (BM) contains primitive progenitors termed multipotent adult progenitor cells (MAPCs), (Reyes et al.,2001; Schwartz et al.,2002). In contrast to another class of adherent BM-derived stem cells, MSCs, MAPCs are CD44- and major histocompatibility complex class I (MHC I)-negative. MAPCs can be expanded under defined low-serum conditions for more than 100 (human) or 400 (rat) PDs without telomere shortening or karyotypic abnormalities. MAPCs not only differentiate into mesenchymal cell types (osteoblasts, chondrocytes, adipocytes, and smooth and skeletal myoblasts) , (Reyes et al.,2001)but also into cells with phenotypic and functional characteristics of endothelial cells (Reyes et al.,2001), hepatocytes (Schwartz et al.,2002), and neural cells (Jiang et al.,2003).

The discovery of neural stem cells (NSCs) in the adult mammalian central nervous system (CNS) has dramatically changed our view on the regenerative capacity of this organ(Temple ,*2001*; Lie et al.,2004) . We now realize that the adult brain, including humans, retains the ability to replenish its cellular constituents, neurons and glia, although the extent is very limited compared with lower vertebrates (*2001*; Lie et al.,2004)).Adult NSCs have also been shown to participate in neuronal cell replacement after injury, raising the possibility of stem cell-based therapy for neurological disorders (Goldman ,*2005).*

In this study, we demonstrate that an unprecedented developmental plasticity and proliferative potential are retained in CNS glia throughout life and the use of autologous adult

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stem cells eliminates the problems with tumorogenesis,mutation, and tissue incompatibility in both NSC,s and ESC,s therapy.

2. MATERIALS AND METHODS

2.1. Isolation and derivation of neural progenitors

Primary tissue was gathered from individuals undergoing surgery related to intractable temporal lobe epilepsy (*n*=10), data presented from 2-5 years old sheep.All procedures were performed in accordance with animal tissue handling and use guidelines. Primary tissue was stored overnight in ice-cold Dulbecco's modified eagle medium with F12 supplements (DMEM/F-12, Gibco, Grand Island, NY) medium containing 20 μg/ml penicillin, 20 μg/ml streptomycin and 25 ng/ml amphotericin B (collectively abx, Invitrogen, Carlsbad, CA). Surgical samples ranging from 0.5-1.5 cm^3 were used from hippocampus (containing hilus), temporal cortex and sub ventricular zone [SVZ, including anterior horn (*n*=3) and segmented lateral ventricle (*n*=1), see Table S1 in the supplementary material). Tissue samples were immersed in 1x phosphate-buffered saline (PBS, 25°C, pH 7.3) lacking $CaCl_2$ or $MgCl_2$, and were manually dissociated into 1 mm^3 pieces under sterile conditions. Dissociates were collected by centrifugation (400 *g*, 5 minutes) and resuspended in 0.25% trypsin (10^6 cells/ml, 15 minutes, 37°C, pH 7.3, Sigma). Dissociates were further triturated using restricted bore pipetting. Cells were collected by centrifugation (400 *g*, 5 minutes), resuspended in proliferative media (10^6 cells/ml) and seeded onto uncoated 75 cm^2 culture flasks (TPP, Switzerland) overnight (12 hours, 37°C, 5% humidified CO_2).

2.2. Neuroectoderm Differentiation.

ShMAPCs were plated at 3 x 10^3 cells per cm^2 on 10 ng/ml FN-coated coverslip placed inside six-well plates in MAPC medium overnight. The medium was then switched to MAPC basal medium with 100 ng/ml bFGF, 10 ng/ml Noggin, 20 μM retinoic acid for 28 days. After 14 days, 10 ng/ml BDNF and GDNF were also added. Half-changes of medium occurred every 7 days until day 28. Neural differentiation was evaluated via Q-RT-PCR for early neural transcript factors, Islet-1 transcription factor, orthodenticle homolog 2 (Otx-2), and paired box gene 6 (Pax-6), as well as neural cell adhesion molecule and the more mature neuronal marker MAP2, NF200, tau, and myelin basic protein (MBP) every 7 days. Cultures were also analyzed via immunofluorescence for NF200, MAP2, τ, and GFAP. A total of 34 differentiations were done from sMAPCs from three donors at 60–100 PDs.

2.3. Karyotyping

Cells, plated at 500 cells per cm^2 48 hours prior to harvesting, were subjected to 10 μl/ml colcemid incubation for 2–3 hours and collected with 0.25% Trypsin-EDTA followed by lysis with a hypotonic solution and fixation in alcohol. Metaphases were analyzed after Giemsa staining.

2.4. Neurosphere formation and generation of neuronal cell types

PD 3 progenitors (cultured under adherent conditions 3 days) from SVZ and temporal cortex were placed in neurosphere conditions as described (Kukekov et al., 1999). Briefly, cells were seeded at a density of 5x10^4 cells/ml in proliferative media containing 1% methylcellulose in anti-adhesive conditions. EGF and bFGF were supplemented bidaily, and neurosphere formation was visually tracked using light microscopy at 7, 14 and 21 days. Matched cultures of clonal seedings were prepared every fifth passage. To differentiate adherent progenitors, serum, EGF and bFGF were removed from the culture media and supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.5 mM 1-dibutyryl cAMP, and 25 ng/ml NGF (Ronnett et al., 1990). Media supplements were replaced every third day. FGF8 (100 ng/ml), sonic hedgehog peptide

(500 ng/ml), pleiotrophin (100 ng/ml) and retinoic acid (0.5 μM) were purchased from Sigma. Matched differentiating cells were co-cultured with 10 μM BrdU for 2 days following factor addition. Cells were immunocytochemically evaluated 2, 3, 5 and 7 days later, and were electrophysiologically evaluated 7 days after differentiation.

2.5. Electron microscopy

PD 5 SVZ cells were grown in defined proliferative media on LPO-coated aclar coverslips. Fixation and processing of thin sections was standard. Samples were visualized on a Leica EM10A transmission electron microscope at magnifications between 1 and 16,000x. Images were captured using a CCD digital camera (Finger Lakes Instrumentation, Lima, NY).

3. RESULTS

3.1. Characterization and expansion of primary cells as AHNPs

Sheep brain tissue (anterolateral temporal lobe) was derived from individuals undergoing resection associated with medically intractable epilepsy. Tissue was microdissected into regions containing hippocampus, SVZ or temporal cortex gray matter. Isolated dissociates were maintained as a monolayer on uncoated plastic dishes throughout culture in defined proliferative media, modified from a standard protocol for the culture of neural stem cells (Scheffler et al., 2005).

To identify cultured cell types, primary cells were examined for expression of phenotypic markers. Following dissociation, immunocytochemistry on primary cells 4days in vitro (DIV) revealed a heterogeneous population containing predominantly astrocytic ($GFAP^+$) cells, but included rare neuronal ($NeuN^+$, PSA-$NCAM^+$) and oligodendrocyte ($CNPase^+$, $O4^+$) phenotypes. Following expansion in defined growth medium, a population of progenitors is established as the sole proliferating population by 12 DIV. These cells are defined by the conserved expression of nestin (Fig. 1A), with the retention of the morphological and antigenic properties ascribed to type I protoplasmic astrocytes (Cammer and Tansey, 1988). Cells present 12 DIV frequently co-express both immature and astrotypic markers (Fig. 1A-D,G), and do not express mature neuronal (NeuN) or oligodendroglial (O4) markers. To further characterize these cells, we performed single cell patch clamp recordings for highly expanded PDs ells (*n*=4). Recorded cells exhibited ubiquitous gliotypic membrane potentials (Sontheimer, 1994), with a RMP of -28.3±4.2 mV, a C_m of 277.2±189.7 pF, a R_m of 214.5±156.1 MΩ, and a R_a of 14.9±3.1 MΩ. Recorded cells displayed prominent Na^+ channel activity and K^+ channel activity (Fig. 1E). To determine the composition and dynamics of proliferating populations, cells undergoing 10and 20 PDs were cultured in the presence of the thymidine analog BrdU. In our culture conditions, only $nestin^+$ cells appear to re-enter the cell cycle, as shown by their rapid increase in prevalence (55.2±17.2% at 4days in culture versus 99.7±0.2% at 20days) and near-ubiquitous incorporation of BrdU throughout culture [average 98.9±0.8% ($nestin^+$/$BrdU^+$)/$BrdU^+$ for 10 and 20PD populations following 48 hours BrdU administration]. This finding was confirmed through appreciation of extensive intermediate filaments in cells subjected to ultrastructural examination (data not shown). Furthermore, the rate at which BrdU increases in culture is contiguous with the known doubling rate of expandable populations derived from multiple forebrain regions (Fig. 1H). In the absence of defined growth factors, BrdU incorporation is rapidly attenuated in $nestin^+$ cells, which accompanies a cessation of growth (Fig. 1H). Neurons (PSA-$NCAM^+$) and oligodendrocytes ($CNPase^+$) were not appreciated in proliferating culture conditions after 12DIV.cellsdisplaying a stellate or reactive morphology were rarely detected in culture.

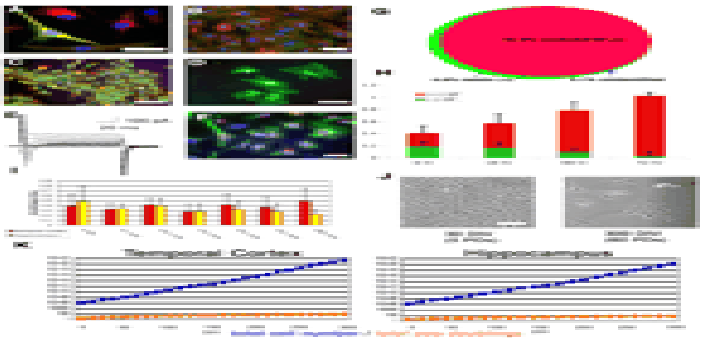

Figure 1. Expansion of primary neural cells as a homogenous population of AHNPs.

3.2. AHNPs demonstrate phenotypic plasticity, and are a transplantable, modifiable cell source

To assess the ability of AHNPs to survive, integrate and assume a committed phenotype in vivo, AHNPs were injected into the right lateral ventricle of early postnatal sheeps. Engrafted cells were assessed for patterns of incorporation and immunophenotype 7 days later using human ribonuclear protein (HNA) to identify engrafted cells. Moderate reactive gliosis was appreciated in transplanted animals, which increased with animal age. Immunosuppression of young animals with cyclosporin A substantially reduced reactive gliosis and increased survival and engrafted cell distribution. Engrafted cells were primarily detected within the ependymal wall of the injected ventricle, with increasingly frequent distribution immediately adjacent to the injection site (Fig. 2A,B). HNA^+ cells were also frequently detected in the choroid plexus, adjacent to the third ventricle, cerebral aqueduct and (rarely) in the cerebellum. Immunocytochemistry revealed integrating cells infrequently adopt mature neuronal morphologies. Though many ventricularly engrafting cells did not express mature phenotype markers, engrafted cells were frequently found to co-express GFAP (Fig. 2C-F).

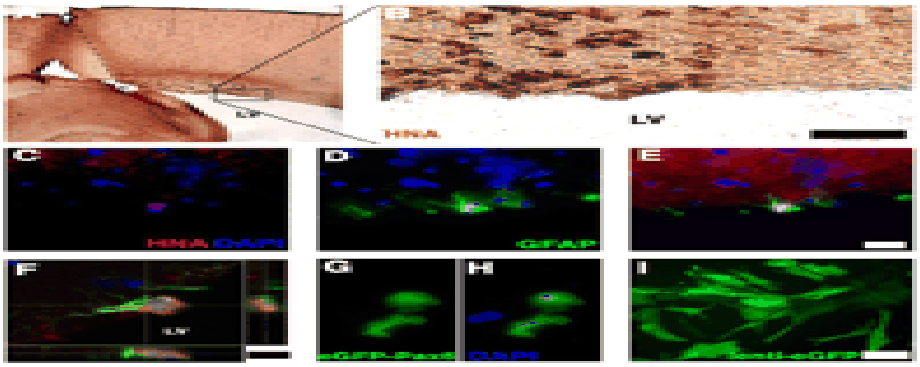

Figure 2. AHNPs maintain viability and assume glial phenotypes upon ventricular transplantation.

AHNPs are highly expandable, suggesting a potential role as a substrate for a transplantable cell source for delivery of gene products (Svendsen, 2004) or alteration of fate choice.

To examine survival and integration of AHNPs in the adult CNS, AHNPs (30 PDs) were transplanted into the cortex of adult (P90) NOD-SCID immunocompromised sheep.Transplanted AHNPs (HNA^+) were detectable following a 30-day engraftment period. In contrast to ventricularly engrafted cells, cortically implanted cells were found to express ß-III-tubulin and adopt neuronal morphologies with significant process extension (Fig. 3A,C). Astrocytic ($GFAP^+$, Fig. 3B) or oligodendrocytic ($CNPase^+$, data not shown) phenotypes were rarely detected in cortically integrating HNA^+ cells. Transplanted AHNPs were largely concentrated around the injection site, with limited migration along the dorsoventral axis.

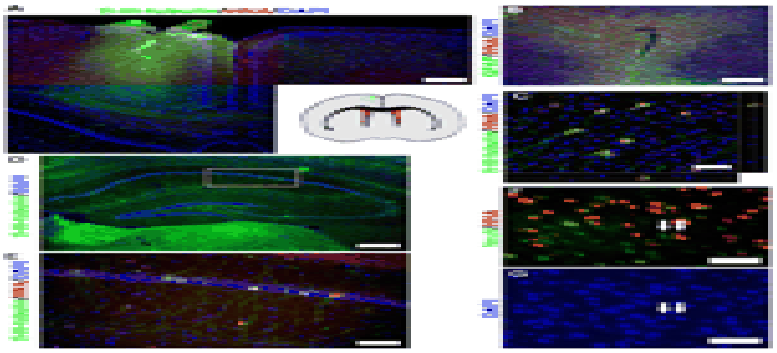

Figure 3. Cortically implanted AHNPs adopt predominantly neuronal fates.

3.3. Characterization of sMAPCs

All other clones were cryopreserved and not evaluated further. For the four clones, one each from three fetal and one postnatal sheep BM isolations that were maintained in culture, levels of Oct3a were between 5% and 20% of those identified in hESCs (Fig. 4E) and remained stable for >90 PDs. The expression of Oct3a in sMAPCs was confirmed by immunocytochemistry (Fig. 4D). It should be noted that Oct3a mRNA could not be detected in sheepMSCs (Fig. 4E). For all clonal MAPC populations, cell doubling time was 24 hours for the initial 30–40 PDs and 36–48 hours when cultures reached >40 PDs (Fig. 4A). As shown in Figure 4B and 4C, sMAPCs are round or triangular, lightly adherent, less than 10 μm in diameter, and exhibit a very high nucleus-to-cytoplasm ratio. sMAPCs have an instinctive ability to separate from each other following cell divisions, even when cells reach higher density (~4,000 cells per cm^2, just before they are passed). When cultures were allowed to grow to very high densities (>6,000 cells per cm^2), proliferation slowed down, demonstrating contact inhibition. Phenotypic analysis after 50 PDs indicated a homogenous population of cells that is negative for CD44, CD45, MHC class I, and MHC class II (Fig. 4J), whereas cells analyzed prior to 50 PDs were negative for CD45 and MHC class II but were mixed for expression of MHC class I and CD44 (Fig. 4I).

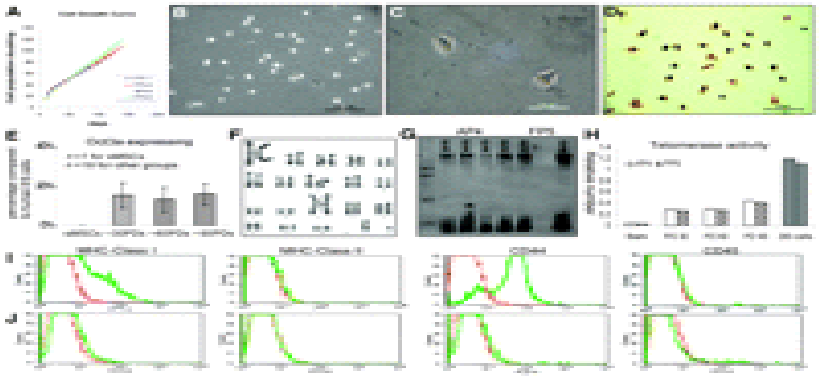

Figure 4. Sheep multipotent adult progenitor cells (sMAPCs) morphology and phenotype.

Telomere lengths were evaluated at ~30, ~60, and ~90 PDs in the three fetal sMAPC lines and one postnatal sMAPC line. Telomere lengths did not differ between MAPCs isolated from fetal or postnatal sheep, and telomeres did not shorten following extensive passaging (Fig. 4G). Significant levels of telomerase activity could be measured in the two sMAPCs populations tested, again irrespective of the age of the donor animal (Fig. 4H).

When established populations of sMAPCs were replated at high density (>2,000 cells per cm^2), and passaged every 4 days when they reached cell densities of >8,000 cells per cm^2 for 3–6

passages, they became morphologically larger (Fig. 5A). This was associated with an acquisition of CD44 and MHC class I antigens on the cell membrane (Fig. 5C), and a loss of Oct3a expression determined by Q-RT-PCR and immunohistochemistry (data not shown). When cultures were subsequently replated at low cell densities (100–500 cells per cm^2) for >20 PDs, they did not reacquire the typical small MAPC morphology, and they remained CD44- and MHC class I-positive and Oct3a-low (<0.001% of hESCs). Moreover, we found that telomeres shortened in cells allowed to grow at high density and replated for 20 and 40 PDs at low density (Fig. 5B).

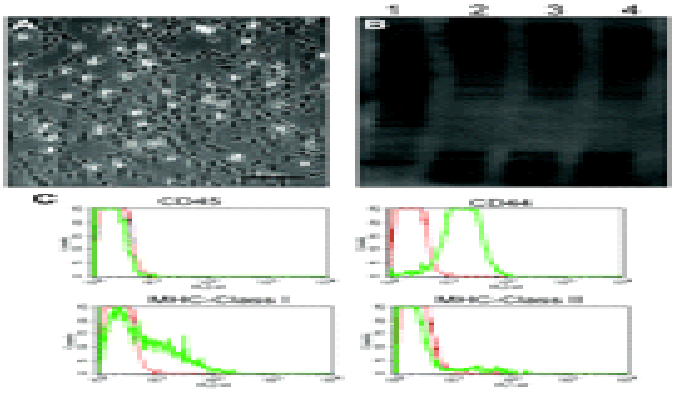

Figure 5. Loss of multipotent adult progenitor cell (MAPC) phenotype upon maintenance of MAPCs at higher density.

3.4. In Vitro Differentiation of sMAPCs

The in vitro differentiation capabilities of sMAPCs to neuroectoderm was evaluated by the addition of cytokines on the basis of previous differentiation studies of ESCs (Li et al.,2005)and MAPC differentiation (Reyes et al.,2001; Jiang et al.,2003).Studies were done using two fetal MAPC lines and one postnatal MAPC line, and differentiation was performed multiple times at population doublings 60–100. sMAPCs could also be induced down a neuroectodermal pathway, as for endothelium and hepatocyte, we demonstrate sequential activation of early neural commitment transcripts by d14 and more mature transcripts, including NF200 and MBP, by d28 following culture of sMAPCs with noggin, RA, and bFGF, followed by BDNF and GDNF. Again we confirmed neuroectoderm-like differentiation by IF with antibodies against NF200 (91% ± 3%), τ(81% ± 8%), GFAP (5% ± 2%), and MAP2 (86% ± 7%) (Fig. 6).

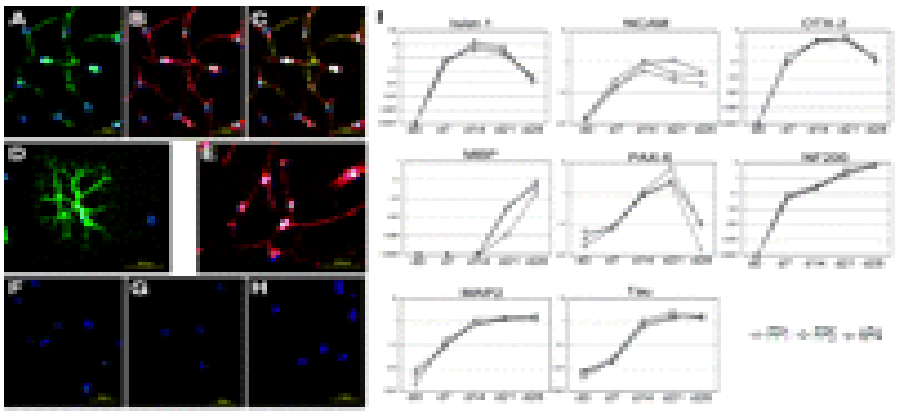

Figure 6. In vitro differentiation of sheep multipotent adult progenitor cells (sMAPCs)

4. DISCUSSION

Bone marrow stromal cells (BMSC) are universal and come to the damaged tissue by the influence of different signal substances they are changed into the necessary specialized cells to replace the dead ones. Studies have shown that when BMSC were introduced into an experimental animal with neural damage, that the occurrence of neuron disorders was almost fully removed. It has also been seen that BMSC can differentiate into neural cells and therefore can be used for restoration of brain damage. We tested the theory that BMSC could change into neural precursor cells by co culturing BMSC, identified by using β-galatosidase, with fetal primary neuronal cultures.

We concluded that, our findings suggest an unprecedented developmental plasticity and proliferative potential are retained in CNS glia throughout life and the use of autologous adult stem cells eliminates the problems with tumorogenesis, mutation, and tissue incompatibility in both NSC,s and ESC,s sheep therapy.

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