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Brown vs. White Adipose Tissue: Brown Adipose Tissue is a Source of Multipotent Stem Cells
with Greater Propensity for Neurogenesis

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Physiological Science

by

Ashley Abigail Penton

2016

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ABSTRACT OF THE THESIS

Brown vs. White Adipose Tissue: Brown Adipose Tissue is a Source of Multipotent Stem Cells
with Greater Propensity for Neurogenesis

by

Ashley Abigail Penton

Master of Science in Physiological Science

University of California, Los Angeles, 2016

Dr. Kristina Bostrom, Co-Chair

Dr. Fernando Gomez-Pinilla, Co-Chair

Spinal cord injuries can lead to life long debilitations, in some cases, paralysis. Patients suffering with spinal cord injury, however, may find hope for improved functionality of debilitated limbs via cell transplantation. Because adipose tissue is an abundant cell source that can be harbored from a patient through liposuction, we investigated and compared the differential propensity of brown and white adipose tissue-derived stem cells to generate neuronal cells. Although previous studies have shown white adipose stromal cells (ASCs) and dedifferentiated fat (DFAT) cells contain neurogenic potential, there has not been studies focused on ASCs and DFAT cells derived from brown adipose tissue, a subtype functionally different from white adipose tissue. In

this study, we therefore focus on determining the differential potential of white and brown-derived ASCs and DFAT cells to generate neural cells.

To study the difference we cultured these cells in neural induction medium or “basic medium”, i.e 10% FBS in DMEM, and compared their expression of neural precursor genes, neuronal genes and glial genes. Our results indicate brown-derived adipose tissue as compared to white-derived adipose tissue contains a greater potential to become neuronal or glial-like cells; brown-derived ASCs cultured with 20% serum replacement in DMEM, showed significant increases in its neuroprogenitor while brown-derived DFAT cells in basic medium contained a higher potential to generate glial-like, s100B+ cells. Therefore, our results suggest brown adipose tissue-derived stem cells would be a more ideal source of neural stem cells than the previous white adipose tissue-derived stem cells.

The thesis of Ashley Abigail Penton is approved.

Xia Yang

Kristina Bostrom, Committee Co-Chair

Fernando Gomez-Pinilla, Committee Co-Chair

University of California, Los Angeles

2016

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INTRODUCTION

Spinal cord injuries can lead to life long debilitations, in some cases, paralysis. This is caused by extensive axonal damage, degeneration, and neuronal loss.²³ Patients suffering from spinal cord injury, however, may find hope for improved functionality of debilitated limbs via cell transplantation. Indeed, studies have shown that the most promising strategy to promote functional recovery in acute and subacute spinal cord injury is cell transplantation.^{7,23}

Recent studies have revealed that adipose tissue-derived stem cells may provide alternative stem cell sources for spinal cord injuries. Adipose-tissue derived stem cells include either adipose stromal cells (ASCs) from the stromal vascular fraction (SVF) of digested adipose tissue or dedifferentiated fat (DFAT) cells generated from “ceiling” or “floating” culture of lipid-rich, floating adipocytes.^{4,8,9} ASCs derived from white adipose tissue have been shown to generate early neural progenitor cells that can differentiate into oligodendrocytes, astrocytes and neurons.^{1,8,16} DFAT cells from the same source, on the other hand, were shown to increase functionality of a mouse’s spinal cord after injury.⁸

Interestingly, these two stem cell sources are differentially isolated. For example, the method used to gather ASCs dispenses a large amount of mature adipocytes from the cell suspension. Comparisons between ASCs and DFAT cells have revealed DFAT to be a more homogenous population of cells in regards to stem cell marker expression.²¹ Furthermore, in initial cultures, DFAT positively expressed CD90 (a neuronal surface marker) at higher levels than ASCs.^{8,21} Therefore, we hypothesized that DFAT cells would be a more optimal stem cell source for neural differentiation than ASCs.

Interestingly, an additional dimension of adipose tissue-derived stem cells is that not all adipose tissue is the same. Traditionally adipose tissue is classified as white or brown, with distinct cell lineages of white or brown adipocytes.¹⁸ White adipose tissue is characterized by a large single, spherical vacuole with a few mitochondria and is located in multiple discrete locations.^{4,18} Commonly, white adipose tissue is classified as either subcutaneous or visceral. It functions to serve as a site of nutrient homeostasis, behaving like a storage depot.^{8,18} However, it also functions as an endocrine organ, secreting growth factors, cytokines, hormones and proteins related to immunological and vascular function.^{8,18} Furthermore, white adipose tissue accessibility has made it a target tissue for the generation of ASCs and DFAT cells.

Brown adipose tissue (BAT), on the other hand, is characterized by its high vascularization, high number of mitochondria and its multiple, small, multilocular lipid droplets.⁴ It also contains highly specialized thermogenic activity, dissipating stored energy as heat.¹⁸ This thermogenic process is controlled by the central nervous system, specifically the sympathetic nervous system.¹³ When activated, uncoupling protein-1 (UCP1) within the mitochondria of BAT “uncouples” fuel oxidation from ATP synthesis, thereby dissipating heat.¹⁸ BAT is also known to vary between species and among age groups. For example, in rodents, classic brown adipocytes cluster in specific depots located in the interscapular and peritoneal regions.^{4,18} In humans, BAT was found significantly in infants and thought to be devoid in adults. However, recent findings have shown significant depots of genuine BAT in the supraclavicular and spinal regions of adult humans.¹⁸ Interestingly, to our knowledge, BAT derived DFAT cells have not been studied.

These highlighted differences between brown and white adipose tissue suggest a potential stem cell difference between brown and white derived ASCs and DFAT cells. In regard to

neuronal differentiation, brown adipose tissue's high innervation by the sympathetic nervous system and vascularity²⁰ suggest it would be a better source of neuronal precursor cells.

In this study we hypothesize that stem cells derived from white and brown adipose tissue, respectively, differ in propensity to undergo neurogenesis. We compare and characterize brown and white derived DFAT cells and ASCs using fluorescence-activated cell sorting (FACs), RNA analysis and immunofluorescence. Overall, the results revealed differential qualities between the two stem cell sources. First, we optimized the culture conditions allowing cells to undergo neurogenesis. The cells were cultured on 0.1% gelatin in basic induction medium, i.e 10% FBS in DMEM, or in a 2-step neural induction medium. Second, we used bright-field imaging, immunofluorescence and real-time quantitative polymerase chain reaction to study cell differentiation. The results revealed both brown and white derived ASCs and DFAT cells are able to generate neuronal cells and glial-like cells in 10% FBS/DMEM and in neural induction medium. The differences between each stem cell source in their propensity to undergo neurogenic differentiation was dependent on culture medium and induction day. Third, focusing on brown-derived ASCs, we unexpectedly found these cells to contain a greater potential of becoming neuronal cells in neurogenic medium while brown-derived DFAT cells exhibited a greater potential of becoming glial-like cells in basic medium. From these findings, we suggest brown adipose tissue to have a higher propensity for neurogenic differentiation and potentially be a better source of neural precursor cells.

MATERIALS AND METHODS

COLLECTION OF ADIPOSE TISSUE:

For collection of mouse adipose tissue for cell isolation, wild-type mice at 4-8 weeks of age were euthanized by inhalation of isoflurane (5-30%) followed by cervical dislocation. Adipose tissue was collected postmortem.

ISOLATION AND CULTURE OF DFAT CELLS AND ADIPOSE-DERIVED STEM CELLS:

DFAT Cell Isolation:

White adipose tissue was collected from subcutaneous tissue located on the back of the mice while brown adipose tissue was collected from the interscapular region.^{9,18} In order to prevent contamination, each tissue type was collected using separate autoclaved instruments and placed in their respective 50 mL test tube containing phosphate-buffer saline (PBS). Afterward, white and brown adipose tissue were separately washed three times in PBS, until clear, and then minced in 10 cm petri dishes with 15 mL of 0.2% collagenase, 2% bovine serum albumin (BSA) in Dulbecco's modified Eagle's medium (DMEM) (10-013-CV, Corning Life Sciences). White and brown minced adipose tissue in collagenase were then transferred to new 50 mL test tubes where they were further digested while agitated (85 RPM) for 45 minutes at 37°C. Next, the cell suspensions were filtered into 10 cm petri dishes and washed twice with 10 mL of culture medium (DMEM containing 20% fetal bovine serum, FBS, and 0.5% penicillin-streptomycin, antibiotic). Each suspension was then poured in a new 50 mL test tube. In order to isolate white and brown DFAT cells, the test tubes containing each respective adipose tissue were centrifuged

for 3.15 min at 12000 RPM. After centrifugation, the top creamy layer was collected and added to a new 50 mL test with 10 mL of culture medium. The test tubes were centrifuged for 1 minute. The adipocytes were then plated in culture medium.

Adipose Stromal Cell Isolation:

The same procedure used for DFAT cell isolation was used for ASC isolation except for the last step. To isolate white and brown ASCs, the test tubes containing each respective adipose tissue were centrifuged for 3.15 min at 12000 RPM. The stromal fraction of white or brown adipose tissue were then collected and added to culture medium. Each was then plated into 10 cm petri dishes.

FLOW CYTOMETRIC ANALYSIS:

For characterization of the phenotypes of DFAT cells and ASCs, the cells were cultured in culture medium until 100% confluent. Fluorescence-activated cell sorting (FACS) analysis was performed after the first passage using conjugated anti-mouse antibodies against Sca1 (PE, 553336; BD Biosciences), cKit (FITC, 553354; BD Biosciences), CD105 (PE/Cy7, 120409; BioLegend), CD11b (Alexa Fluor 488, 101219; eBioscience), CD90 (PE, 12-0900-81; eBioscience), SSEA-1 (Alex Fluor 488, 125609; BioLegend), CD31 (PE, 553373; BD Biosciences) and CD34 (FITC, 11-0341-85; eBioscience), all diluted 1:100 in 1% BSA.

OPTIMIZATION OF NEURAL DIFFERENTIATION:

Method 1:

After digestion and centrifugation of brown and white adipose tissue, the top creamy layer of adipocytes was collected and added to culture medium. The homogenate was dispersed among two 12-well petri dishes and a 10 cm petri dish. These cells were cultured in culture medium until DFAT cells emerged (day 5), after which floating cells and debris were removed. The DFAT cells were further cultured until the cells in the 12-well petri dishes were 70% confluent (approximately day 12). They were then treated with “pre-inducing medium”: DMEM supplemented with 20% KnockOut™ Serum Replacement (10828028; Gibco™) and 0.5% antibiotic for 7 days. Afterward, the medium was changed to “basic medium”: 10% FBS, 0.5% antibiotic, in DMEM, or to 1st stage - neural induction medium: Glasgow’s Minimal Essential Medium (GMEM, 11710-035; Gibco™) supplemented with 10% KnockOut™ Serum Replacement, 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, and 0.1 mM 2-Mercaptoethanol (2-ME). After 5 days, 1st stage - neural induction medium was changed to 2nd stage - medium: GMEM supplemented N-2 Supplement (100x, 17502-048; Gibco™), 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids and 0.1 mM 2-ME for up to 12 days. Cells were washed with PBS twice between each medium replacement. Cells were monitored every day to observe morphological changes. Medium was changed every 2-3 days. The neural induction protocol was developed by Ke, Y (2009).²⁹

Method 2:

After digestion and centrifugation of brown and white adipose tissue, the top creamy layer was collected and added to culture medium. The homogenate was dispersed among two 12-well petri dishes and a 24-well petri dish. These cells were cultured in culture medium until DFAT cells emerged (day 5), after which floating cells and debris were removed and medium replaced with basic medium. Conditions were changed from method 1 to determine effects of basic medium on newly isolated brown and white-derived DFAT cells. The cells remained in basic medium until the cells in each petri dish were approximately 70% confluent. Due to the lower concentration of serum, the isolated cells required a longer culture time before proper confluence was reached. Once 70% confluence was reached, the basic medium was removed, cells were washed twice with PBS, and then cultured in pre-inducing medium for 6 days. Subsequently, cells were washed twice with PBS and medium replaced with either basic medium or neural induction medium. In this method, neural induction medium consisted of 2nd stage medium only. Medium was changed every 2-3 days for up to 7 days.

Method 3:

DFAT Cells: After digestion and centrifugation, the top creamy layer of white and brown-derived adipose tissue were collected and added to culture medium in separate test tubes. The homogenates were dispersed among their respective 6-well petri dishes and 10 cm petri dishes. After reaching 100% confluence, white and brown-derived DFAT cells were passaged onto two 12-well petri dishes and 8-well glass chamber slides that were coated with 0.1% gelatin at a density of 2.5×10^4 cells/cm². After 24 hours, medium was replaced with pre-inducing medium or basic medium. Cells that were cultured in basic medium maintained in this medium for the

entirety of the experiment with medium changed every 3 days. Cells that were cultured in pre-inducing medium were washed twice with PBS on the seventh day and cultured in neural induction medium for another 10 days. “Neural induction medium” for method 3 included: 1:1 ratio of DMEM and Ham’s F12 Nutrient Mixture (F12) (Gibco™) with 1% nonessential amino acids, 2% B-27 Supplement (17504-044; Gibco™), 1% N-2 Supplement, 0.1 mM 2-ME, 20 ng/mL recombinant mouse fibroblast growth factor basic, bFGF (3139-FB; R&D Systems). Neural induction medium was changed every 3 days.¹

ASCs: After digestion and centrifugation, the stromal vascular fraction of white and brown-derived ASCs was collected and plated on 10 cm petri dishes in culture medium. After reaching 100% confluence, white and brown-derived ASCs were passaged onto two 12-well petri dishes and 8-well glass chamber slides that were coated with 0.1% gelatin at a density of 2.5×10^4 cells/cm². The previous protocol, method 3 for DFAT cells, was repeated using ASCs derived from the same mice used for DFAT cells.

RNA ANALYSIS:

Method 1:

RNA was extracted after DFAT cells were 70% confluent in culture medium, on the 7th day of culture in pre-inducing medium and after 3, 6, or 12 days in neural induction medium or basic medium (Figure 3A). RNA extraction, cDNA preparation and real-time polymerase chain reaction (RT-qPCR) were performed as previously described.²⁸ Primers and probes for mouse TUBB3, mouse CLDN11, mouse Nestin, mouse Sox1 and mouse GAPDH which were all supplied by Life Technologies as part of *TaqMan*[®] Gene Expression Assays.

Method 2:

RNA was extracted after DFAT cells were 70% confluent in basic medium, on the 6th day of culture in pre-inducing medium and after 3, 5, and 7 days in basic medium or neural induction medium (Figure 4A). The samples were processed and analyzed as outlined under *Method 1*.

Method 3:

RNA was extracted on the following days: day 0 (24 hours after seeding), 7, 10, 13, and 17 (Figure 6A). These days correlate to culture in culture medium, 7 days in pre-inducing medium or basic medium, 3 days cultured in neural induction medium or 10 days in basic medium, 6 days cultured in neural induction medium or 13 days in basic medium, 10 days in neural induction medium or 17 days in basic medium. The samples were processed and analyzed as outlined under *Method 1*. In addition, RT-qPCR was performed for mouse NeuroD1, mouse Sox2, mouse Vimentin, mouse S100b, mouse GFAP, and mouse Olig2.

IMMUNOFLUORESCENCE:

Method 2:

Immunofluorescence was performed on white and brown-derived DFAT cells cultured in 24-well dishes 6 days after treatment in neural induction or basic medium. The cells were washed three times in PBS and then fixed in 4% paraformaldehyde overnight at 4°C. The next day the cells were blocked with 1% chicken serum in PBS for 30 minutes and then incubated overnight at 4°C with the appropriate primary antibody. Rabbit anti-GFAP was diluted 1:1000 (RA22101; Neuromics) and mouse anti-Tuj1 (MAB1195; R&D Systems) was diluted 1:100 in 1% BSA in PBS. The next day, cells were incubated with either secondary antibody Alexa Fluor (AF) 488-

conjugated (green fluorescence) chicken anti-rabbit or AF 594-conjugated (red fluorescence) chicken anti-mouse. The cells were washed with PBS and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, catalog#: D9542). Afterward cells were washed repeatedly with PBS and then imaged.

Method 3:

Immunofluorescence was performed on cells cultured in 8-well glass chamber slides coated with 0.1% gelatin. White and brown-derived DFAT cells and ASCs were fixed overnight at 4°C 7 days after treatment in pre-inducing medium or basic medium and 6 days in neural induction medium or 13 days in basic medium using 4% paraformaldehyde. The next day, cells were washed three times with PBS and permeabilized with 0.2% Triton 100x for 20 minutes. Cells were washed once and blocked for 1 hour with 10% chicken serum in 1%BSA or 10% goat serum in 1% BSA depending on the host secondary antibody. Afterward, blocking buffer was removed and the cells were incubated overnight at 4°C with the appropriate antibody. Mouse anti-Tuj1 (1:100, MAB1195; R&D Systems), mouse anti-MAP2 (1:500, M 4403; Sigma-Aldrich), mouse anti-alpha internexin (1:500, MAB5224; Chemicon International), rabbit anti-S100b (1:100, ab52642; Abcam), rabbit anti-NG2 (1:500, AB5320; Millipore), rabbit anti-GFAP (1:1000, G9269; Dako Cytomation), rabbit anti-Nestin (1:100, sc-20978; Santa Cruz Biotechnology), hamster anti-CD31(1:200, MAB1398Z; Millipore). The next day, cells were washed repeatedly with PBS and incubated with the appropriate secondary antibody and DAPI for 1 hour. Chicken anti-mouse (1:1000, AF-594 conjugated), chicken anti-rabbit (1:1000, AF-488 conjugated) in 1% BSA, or goat anti-mouse (1:1000, AF-594 or AF-488 conjugated), goat anti-hamster (1:1000, AF-488),

goat anti-rabbit(1:1000, AF 488 or AF-594 conjugated) in 1% BSA. Afterward cells were washed repeatedly with PBS and then imaged.

STATISTICAL ANALYSIS:

RT-qPCR was analyzed by comparing the differential expression of white (w)ASCs, brown (b)ASCs, white (w)DFAT and brown (b)DFAT cells on each day. Gene expression for each cell source was normalized against wASCs day 0. Data were analyzed for statistical significance by two-way ANOVA RM, Tukey's Test (when comparing cell sources) using the GraphPad Prism 6 software or two-way ANOVA RM, Sidak's Test (when comparing treatment types). *p* values less than 0.05 were considered significant and recorded in figure legends.

RESULTS

FACS CHARACTERIZATION: CELLULAR DIFFERENCES

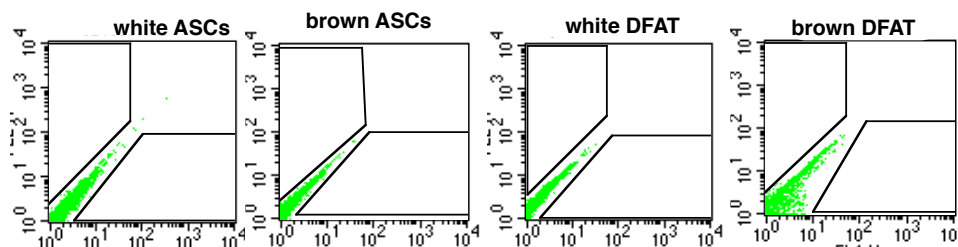
White and brown-derived DFAT cells and ASCs were characterized by their expression of multipotent or pluripotent stem cell surface markers [Figure 1] or endothelial related surface markers [Figure 2]. The markers chosen were Sca1, cKit, CD90, SSEA-1, CD31, CD34, CD105 and CD11b. Sca1, or stem cell antigen 1, plays a role in hematopoietic progenitor/stem cell lineage fate, i.e. a multipotent stem cell marker. cKit is a stem cell factor receptor which marks for pluripotency.²⁵ CD90 is a surface marker that marks hematopoietic stem cell and neuron differentiation as well as T-cell activation.²⁵ For this particular study, we used CD90 as a partial determinant for propensity to undergo neurogenesis. SSEA-1 is another marker for pluripotency as it is expressed in murine embryos and human germ cells.²⁵ CD31, or PECAM-1, is expressed in mouse ES cells and is classified as a platelet-endothelial adhesion molecule. CD34 is an antigen that is expressed on hematopoietic progenitor cells. CD105, also known as endoglin, is expressed on hematopoietic stem/progenitor cells.¹⁵ Lastly, CD11b is expressed on monocytes/macrophages.

When we analyzed the expression of multipotent or pluripotent cell markers in white and brown-derived ASCs and DFAT cells, we found Sca1 to be abundantly expressed in white-derived ASCs (wASCs) and DFAT cells (wDFAT) as well as brown derived ASCs (bASCs), 93.62%, 99.55% and 88.17% Sca1+ respectively [Figure 1B]. However, brown-derived DFAT cells (bDFAT) showed a 9 fold decrease in expression of Sca1 (9.48%) [Figure 1B]. cKit, on the other hand, was rarely expressed (0.00%-0.01%) by each of the stem cell sources [Figure 1B]. When we analyzed expression of CD90, we found it to be more highly expressed in wDFAT

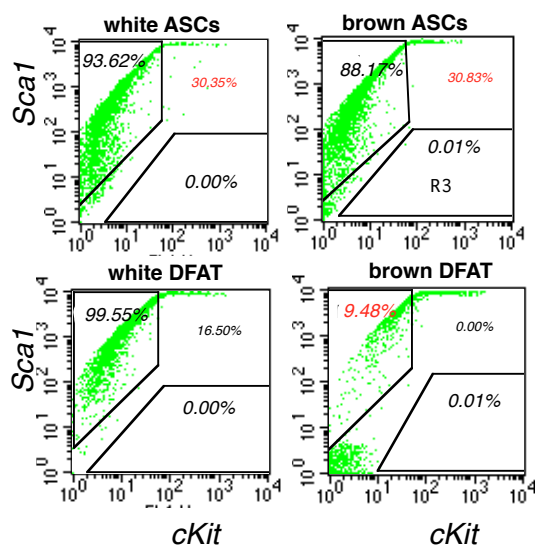
[Figure 1C]. In wDFAT, CD90 is expressed in 7.15% of the cell population, while in wASCs, bASCs, and bDFAT, the cell populations expressed 2.75%, 1.43% , 0.69% CD90+ cells, respectively [Figure 1C]. Like cKit, SSEA-1 was shown to be rarely expressed (0.00%-0.09%) by each stem cell source. From these results, it appears that mouse ASCs and DFAT cells contain greater multipotent rather than pluripotent characteristics. Interestingly, of these groups of cells, mouse ASCs appear to contain a higher population of multipotent stem cells [Figure 1B]. Although this may be the case, wDFAT appears to contain an initial higher population of cells with neuronal potential [Figure 1C].

Regarding endothelial associated surface markers, wASCs, wDFAT, and bASCs contained about 1% CD105+ cells while bDFAT contained 0.12% CD105+ cells [Figure 2B]. CD11, however, was poorly expressed (0.04%-0.02%) in all cell types [Figure 2B]. Interestingly, CD31 was only expressed by wDFAT and bDFAT, although the expression was low: 0.75% CD31+ cells and 0.03% CD31+ cells, respectively [Figure 2C]. However, bASCs, showed a significant increase in CD34 expression: 11.76% CD34+ cells compared to wASCs (0.45% +), wDFAT(5.08% +), and bDFAT cells (0.20% +) [Figure 2C]. In conclusion, the stem cell sources show differential expression of surface markers with bASCs significantly expressing more hematopoietic positive cells and wDFAT cells expressing more neuronal positive cells, i.e .highest CD90 expression.

A. Control



B. Sca1 / cKit:



C. CD90 / SSEA-1

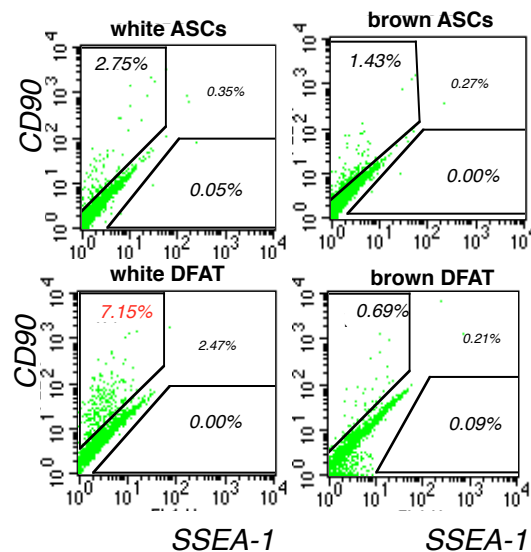
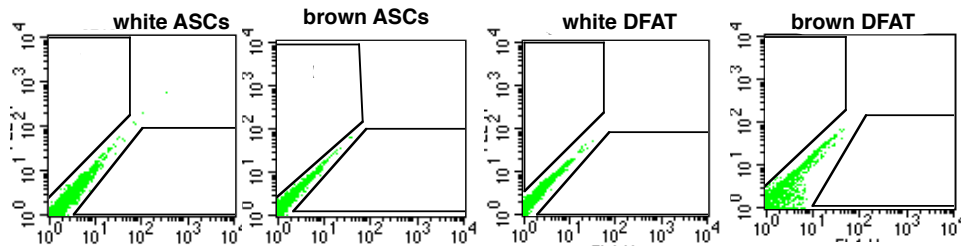
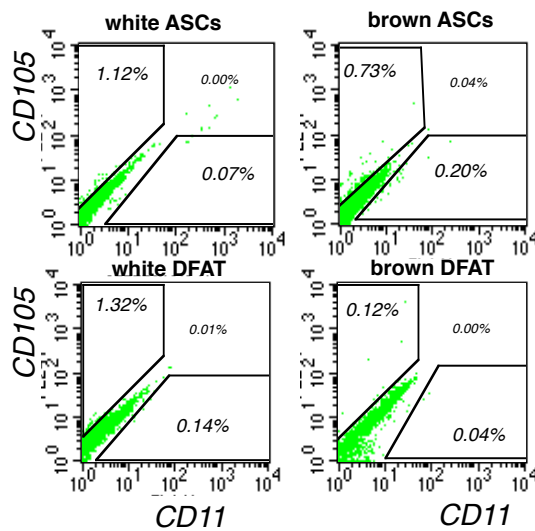


Figure 1: Expression of multipotent or pluripotent stem cell markers: Flow Cytometric Analysis of white and brown-derived ASCs and DFAT cells. **(A):** Negative control for each stem cell source. **(B):** Expression of Sca1, classifies hematopoietic progenitor cells, and cKit, classifies pluripotent stem cells. Brown-derived DFAT cells show lowest expression of Sca1 while white and brown-derived ASCs show highest coexpression of Sca1 and cKit. **(C):** Expression of CD90, classifies neuronal differentiation, and SSEA-1, surface marker of pluripotency. White-derived DFAT cells show highest CD90 expression while pluripotency is not made evident.

A. Control



B. CD105 / CD11



C. CD31 / CD34

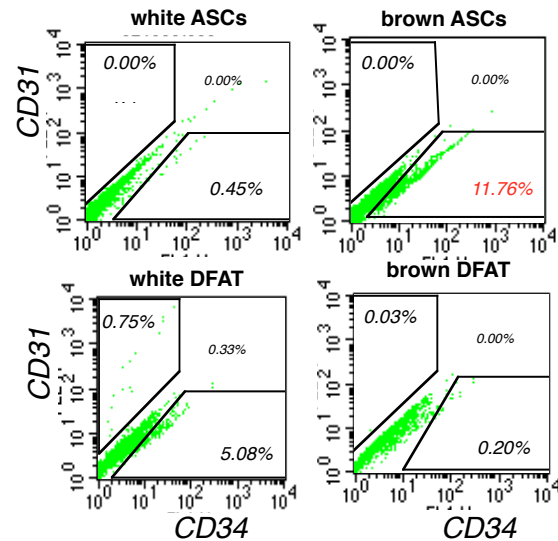


Figure 2: Expression of endothelial related markers: Flow Cytometric Analysis of white and brown-derived ASCs and DFAT cells. **(A):** Negative control for each stem cell source. **(B):** Expression of CD105, classifies hematopoietic progenitor cells, and CD11b, classifies monocytes/macrophages. Brown-derived DFAT cells show lowest expression of CD105 while white DFAT and white and brown-derived ASCs show similar expression of CD105. **(C):** Expression of CD31, classifies murine ES cells and platelet-endothelial adhesion molecules, and CD34, surface marker for hematopoietic progenitor cells. Brown-derived ASCs shows the highest expression of CD34.

OPTIMIZATION OF NEURAL INDUCTION:

Method 1:

Initially, our goal was determine whether wDFAT and bDFAT can generate neuronal-like cells. From our previous, unpublished studies, we found human brown and white-ASCs to form ball-like structures when treated with 20% serum-replacement. Because the morphology of the cells were reminiscent of embryoid bodies, we decided to consider this phase a “pre-inducing” step. Feng et al.¹⁷ confirm this in their 2014 study and our later study provides further evidence that 20% serum replacement in DMEM acts as a “pre-inducing” step by increasing the expression of Sox1, a transcription factor that maintains progenitor multipotency and renewal [Figure 9B]. To ensure mouse DFAT cells would behave in a similar manner, we tested whether this “pre-inducing step” would cause morphological changes [see Figure 3A for induction scheme]. After 7 days of treatment, we cultured the cells with a neuronal induction protocol designed for embryonic stem cells. Our “control” cells were treated with basic medium, i.e 10%FBS/DMEM. The neuronal induction protocol was 2-staged. The first stage was designed to stimulate embryoid body formation, while the second stage was designed to differentiate the embryoid bodies into neurons. Treatment in the first stage medium did not result in typical embryoid body formation [Figure 3D, 3E]. However, after a total of 6 days in neuronal induction medium (5 days in first stage, 1 day in neuronal maturation medium), brown and white-derived DFAT cells showed some cells sprouting processes [Figure 3H, 3I]. Whereas, in basic medium, the cells began to differentiate into other cell types [Figure 3F, 3G].

During this experiment, RNA was collected on days 0, 7, 10, 14, and 19 (day 10 referring to 3 days in neural induction). However, when reversed transcribed, RNA concentration was

shown to decrease. On day 0, wDFAT contained 41 ng/uL while bDFAT contained 34 ng/uL. After 7 days in “pre-inducing” medium wDFAT and bDFAT contained 33 ng/uL and 20 ng/uL, respectively, and after 6 days (i.e. 14 days) in neural induction there was even less RNA expressed by each cell type. Thus, the protocol poorly supported the differentiating cells. Because the cell density was very low, yet the bright-field results indicated neuronal-like cells [Figure 3], we chose not to further analyze the results but continue modifying the neural induction protocol.

A. Method 1 Schematic

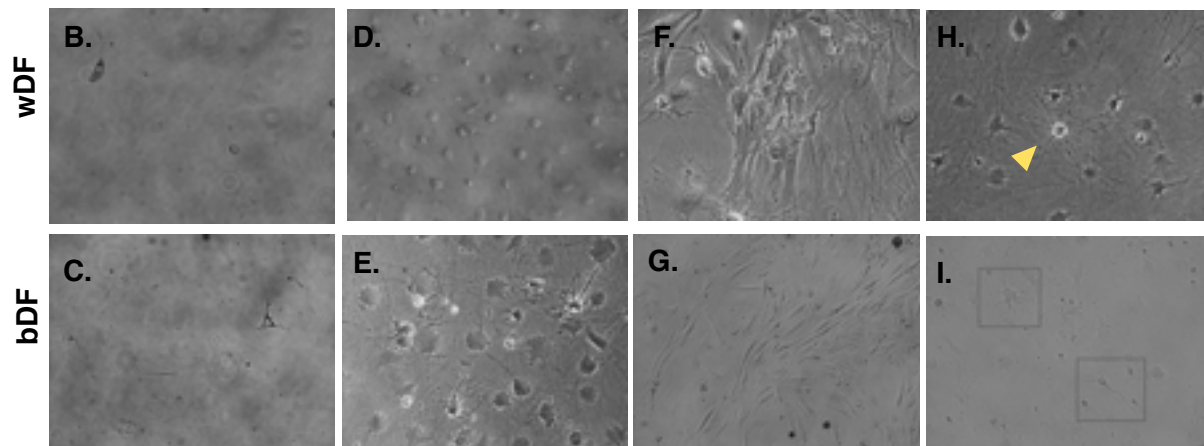
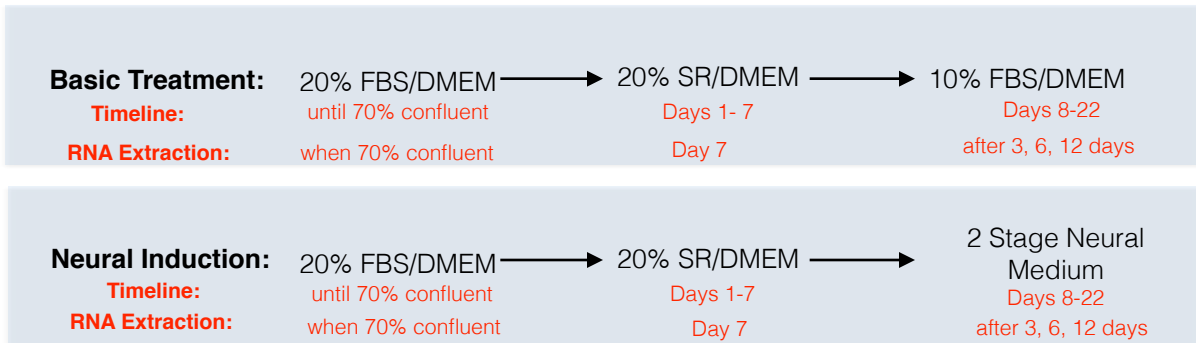


Figure 3: Method 1 Induction Schematic and Bright-Field Images from concurrent days. **(A):** Induction schematic. White and brown-derived DFAT cells were plated on 12-well petri dishes with 20%FBS/DMEM. Once the cells reached 70% confluence **(B&C, 10x magnification)**, the cells were washed with PBS and medium was changed to 20%SR/DMEM, i.e pre-inducing medium. After 7 days **(D&E, 20x magnification)**, white and brown-derived DFAT cells were split into two groups, basic treatment, i.e 10%FBS/DMEM, or a 2-stage neural treatment. Pictured is white and brown-derived DFAT cells after 6 days in 10%FBS/DMEM **(F&G, respectively, 20x magnification)** and 6 days in the neural treatment protocol, as described above **(H & I, 20x magnification)**. Here we see serum replacement (D, E) causing the cells to obtain a spherical morphology. Basic treatment (F,G) causes the cells to lose their ball-like morphology while neural treatment maintains this morphology of the cells (H, I). Neural treatment appears to progress the budding of processes from the ball-like cells.

Method 2:

In the previous protocol, we determined 1st stage neural induction protocol did not help support the cells, but rather enhanced their deterioration. This result could have been influenced by a lack of proper nutrients required by DFAT cells. In our second protocol [Figure 4A], we sought to determine whether basic medium used prior to the “pre-inducing” step would affect neuronal culture of wDFAT and bDFAT. Our reverse-transcribed data shows basic medium, used during initial conditions, was associated with low RNA concentration; day 0 wDFAT contain 32 ng/uL, bDFAT contained 18 ng/uL. This was made evident when the “pre-inducing” step further decreased the RNA concentrations. Figure 4E demonstrates the decrease in cell density. Because cell density was low, we removed the cells from the pre-inducing medium after 6 days and proceeded with neuronal induction [Figure 4A].

Interestingly, after 6 days in neural induction medium, the cells appeared more neuronal-like in nature via their extending processes [Figure 4H, 4I]. In basic medium, on the other hand, we observed a combination of differentiation into other cell types [Figure 4F, 4G]. Thus, basic medium appeared to create a heterogenous population of cells, while neural induction medium helped maintain a homogenous population of neuronal-like cells.

To determine whether wDFAT and bDFAT could generate neuronal cells, we stained the cells with DAPI, Tuj1, and GFAP after 6 days in neural induction medium or basic medium (i.e. day 12). Tuj1, an intermediate filament commonly expressed in early and differentiated neurons,^{10,19} was more highly expressed in wDFAT and bDFAT cultured in neural induction medium [Figure 5]. WDFAT cultured in neural induction medium showed more mature neurons [Figure 5C], while bDFAT cells were observed to contain a high population of neuronal progenitor cells,

evident by Tuj1 expressed in mitotic cells [Figure 5D]. GFAP, glial fibrillary acidic protein, is expressed by cells of astroglial lineage in the CNS and by neural stem cells in the adult brain.¹⁹ In Figure 5A and 5B, we observe wDFAT to contain a higher population of GFAP-positive cells. Observed coexpression of Tuj1 and GFAP [Figure 5A] is indicative of progenitor cells beginning to differentiate into a neuronal lineage. The results suggest that brown and white-derived DFAT cells can generate neuronal cells and progenitor-like cells using either basic medium or neural induction medium. Neural induction medium, however, provides a seemingly more homogenous and greater population of neuronal cells.

A. Method 2 Schematic:

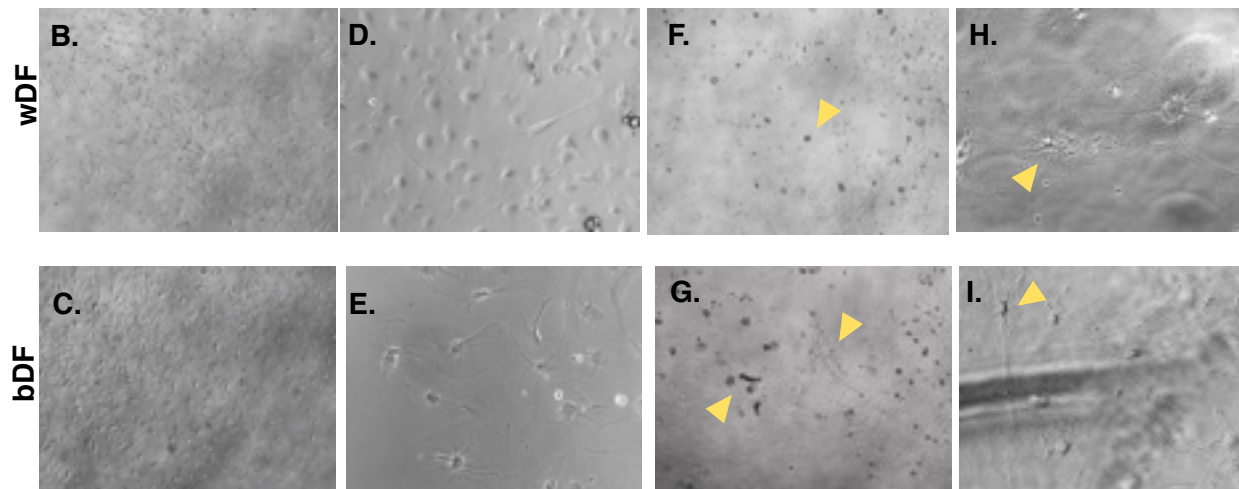
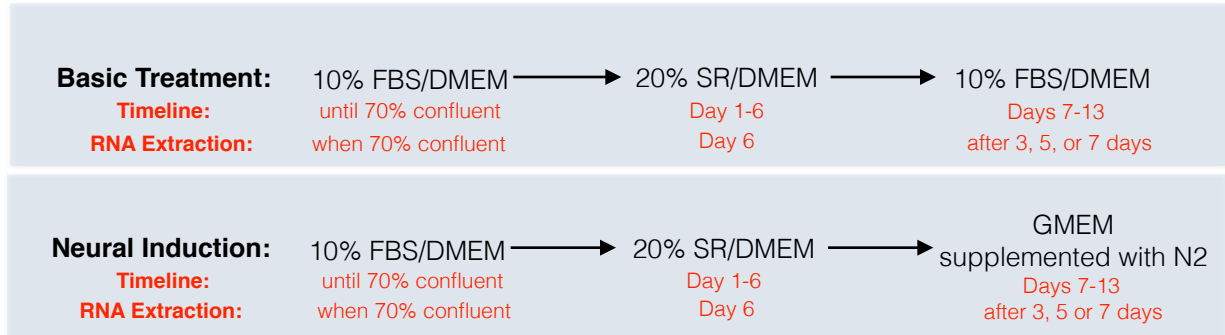


Figure 4: Method 2 Induction Schematic and Bright-Field Images from concurrent days. **(A):** Induction schematic. White and brown-derived DFAT cells were cultured on 12-well petri dishes with 10%FBS/DMEM until 70% confluence was reached. Once the cells reached 70% confluence (**B&C**, 10x magnification), the cells were washed with PBS and medium was changed to 20%SR/DMEM, i.e pre-inducing medium. After 6 days (**D&E**, 20x magnification), white and brown-derived DFAT cells were split into two groups, basic treatment, i.e 10%FBS/DMEM, or neural treatment. Pictured is white and brown-derived DFAT cells after 6 days in 10%FBS/DMEM (**F&G**, 20x magnification) and 6 days in the neural treatment protocol, as described above (**H & I**, 20x magnification). Basic treatment (F,G) caused the cells to differentiate into other cell types, as shown by the arrows, while in neural medium, the cells began to sprout processes (H, I).

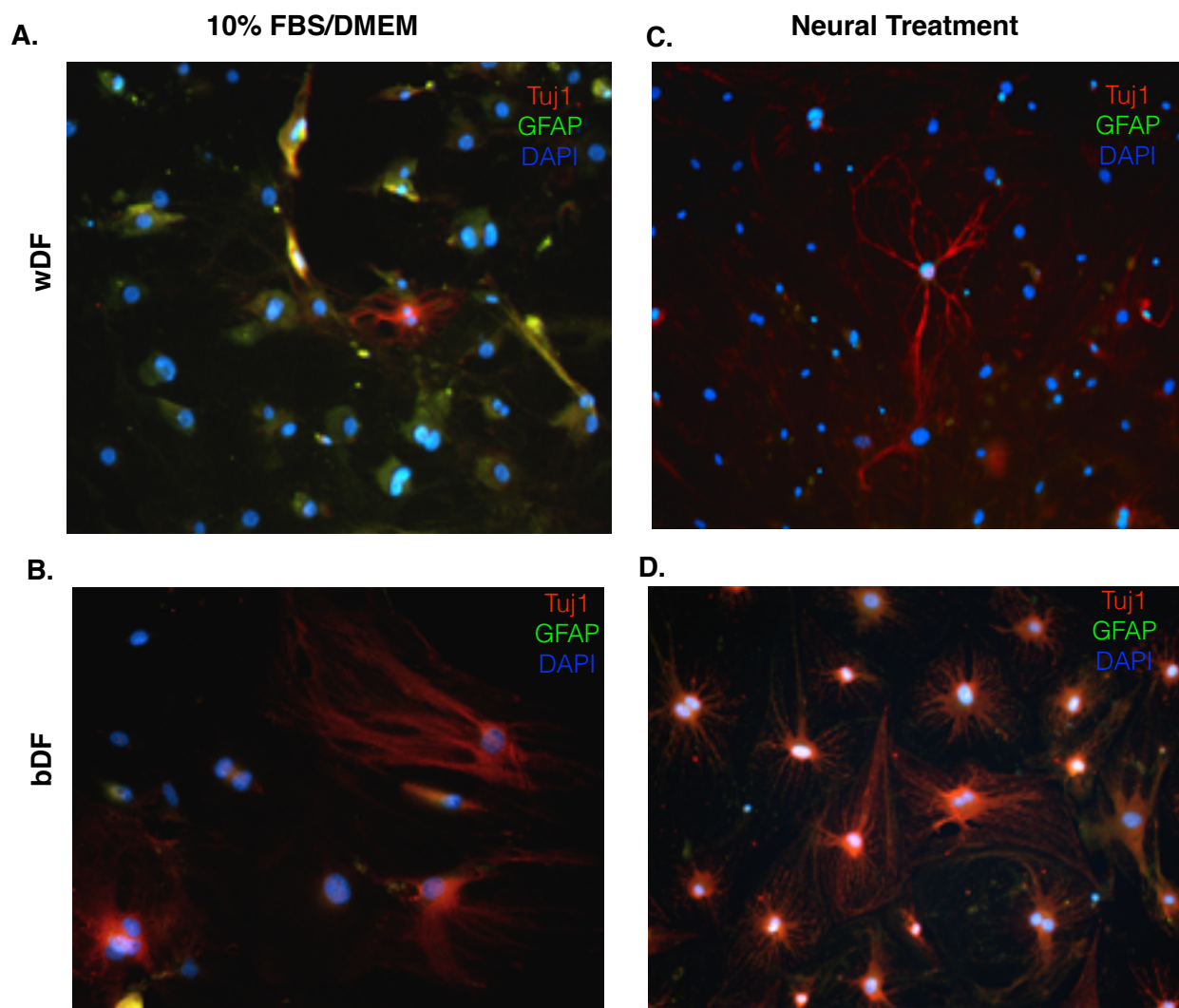


Figure 5: White and Brown-Derived DFAT Cells are Tuj1 Positive. 10x magnification. White and brown-derived DFAT cells were fixed on day 6 of neural treatment or basic treatment. GFAP, expressed in astroglial lineages, was more highly expressed in cells cultured in basic medium (**A, B**), specifically in white-derived DFAT. Although some Tuj1+ cells were found in basic treatment (**A, B**). There was a higher population of Tuj1+ cells after culture in neural induction medium (**C, D**). White-derived DFAT cells (**C**) are able to produce more mature neuronal cells. This is shown by the morphology of the cell imaged in figure 5C. Brown-derived DFAT, on the other hand, has a high population of neuronal progenitor cells (**D**). Together, these images show the ability of white and brown-derived DFAT cells to generate neuronal-like progenitor cells and neuron-like cells.

Method 3:

After qualitatively observing morphological changes of wDFAT and bDFAT to neuronal-like cells, method 3 [Figure 6A] aimed to improve induction protocol and sequentially provide quantitative data measuring the differences between wASCs, bASCs, wDFAT and bDFAT cells. Because previous methodology was unable to maintain a proper cell density for induction, we waited for the cells to obtain 100% confluence.¹ Afterward, we passaged each cell type onto two 12-well petri dishes and four 8-well glass chamber slides at a high density of 2.5×10^4 cells/cm² [Figure 6A]. Prior to seeding, the plates were incubated with 0.1% gelatin for 30 minutes to further ensure cell attachment. In method 3 of experimentation [Figure 6A], basic treatment is defined as basic medium (10% FBS/DMEM) maintained throughout the entirety of the experiment, i.e 17 days. The “pre-inducing” step was removed from basic treatment (*method 1 & 2*) to determine whether white and brown-derived DFAT cells and ASCs have the ability to “spontaneously” differentiate into neurons. Neural induction medium, as previously described, was also modified to include factors previously used on wASCs undergoing neuronal differentiation.¹

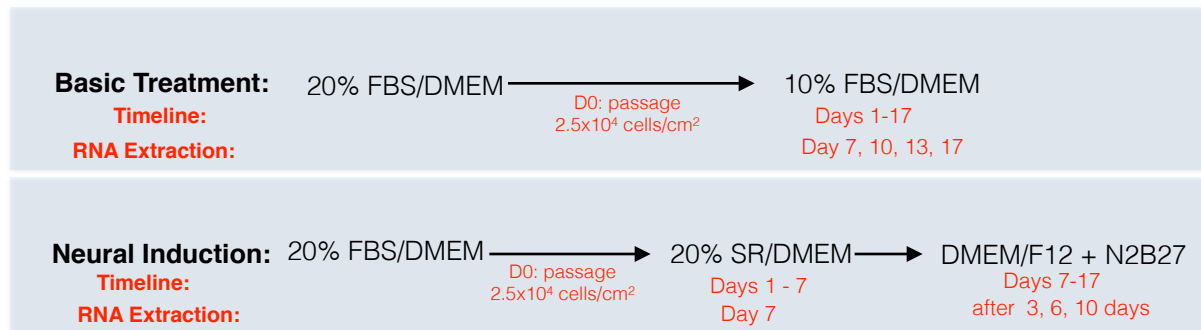
The modifications made to the protocol resulted in a significant increase in RNA concentration and cell density in comparison to the previous protocols. The only exception was in bDFAT, which maintained a low density, potentially from error in cell counting during seeding (Figure 6B). This can be visualized in figure 7H.

In Figure 7 and 8, bright-field 10x magnified images of wDFAT and bDFAT were taken on days RNA was collected. Figures 7A-7D illustrate cells on day 0, i.e 24 hours after plating and before medium was replaced to basic or neural treatment. Like previously shown, “pre-

inducing” medium causes morphologically changes, some of which contain extending processes [Figure 7E-H]. Differences between the cell types exist between differentiation capabilities. In Figure 7F, i.e bASCs in pre-inducing medium, we observe endothelial-like tube-structures. For bASCs to differentiate into endothelial cells is not surprising as FACS analysis shows a higher population of CD34+ cells [Figure 2C]. However, differences can be detected on day 7 of basic treatment. In these images [Figure 7I-7L], we detect heterogenous populations of cell differentiation in bASCs and wASCs. Both of which are shown to contain differentiating immature adipocytes. bASCs also show a differentiated, early neuronal-like cell [Figure 7J]. Although the cell types do not appear remarkably different, neural induction, i.e “pre-inducing medium”, causes the cells to obtain a homogenous population of round cells while basic treatment allows for uncontrolled differentiation of a multiplicity of cell types.

Bright-field 10x magnified images of wDFAT, wASCs, bDFAT and bASCs were taken on day 13 of treatment in basic medium or neural induction medium, i.e 6 days in N2B12 supplemented DMEM/F12, as previously described. Cells in neural treatment maintained a near homogenous population of round cells with extending processes [Figure 8A-D]. Cells in basic treatment continued to differentiate into fibrous structures and appeared 100% confluent [Figure 8E-8H]. Interestingly, we observe bASCs in pre-inducing medium to not only obtain ball-like structures, but differentiate into what may be cardiomyocytes or endothelial cells [Figure 8F].

A. Method 3 Schematic:



B. RNA Concentration:

ng/uL	Day 0	Day 7: Basic	Day 13: Basic	Day 7: Neural	Day 13: Neural
wASC	95.5	176.7	256.7	122.8	126.9
bASC	134	154.9	264.71	82.7	72.6
wDF	81.9	107.1	143.45	77	69.9
bDF	20.35	32.5	37.3	23.1	36.4

Figure 6: Method 3 Induction Schematic. **(A):** Induction schematic. White and brown-derived DFAT cells and ASCs were cultured in 20% FBS/DMEM until 100% confluence. Afterward, cells were passaged onto 0.1% gelatin coated plates: two 12-well petri dishes and 8-well chamber slides. 24 hours after seeding, medium was removed and replaced with either 10%FBS/DMEM (basic treatment) or pre-inducing medium (20% SR/DMEM). Cells cultured in basic treatment maintained in this medium for 17 days. Medium was changed every 3 days. Cells cultured in pre-inducing medium were washed with PBS 2x after 7 days and medium was replaced with DMEM/F12 with N2B27, as previously described. **(B)** RNA concentration as determined by reverse transcription. RNA concentration related to cell density on each specified day.

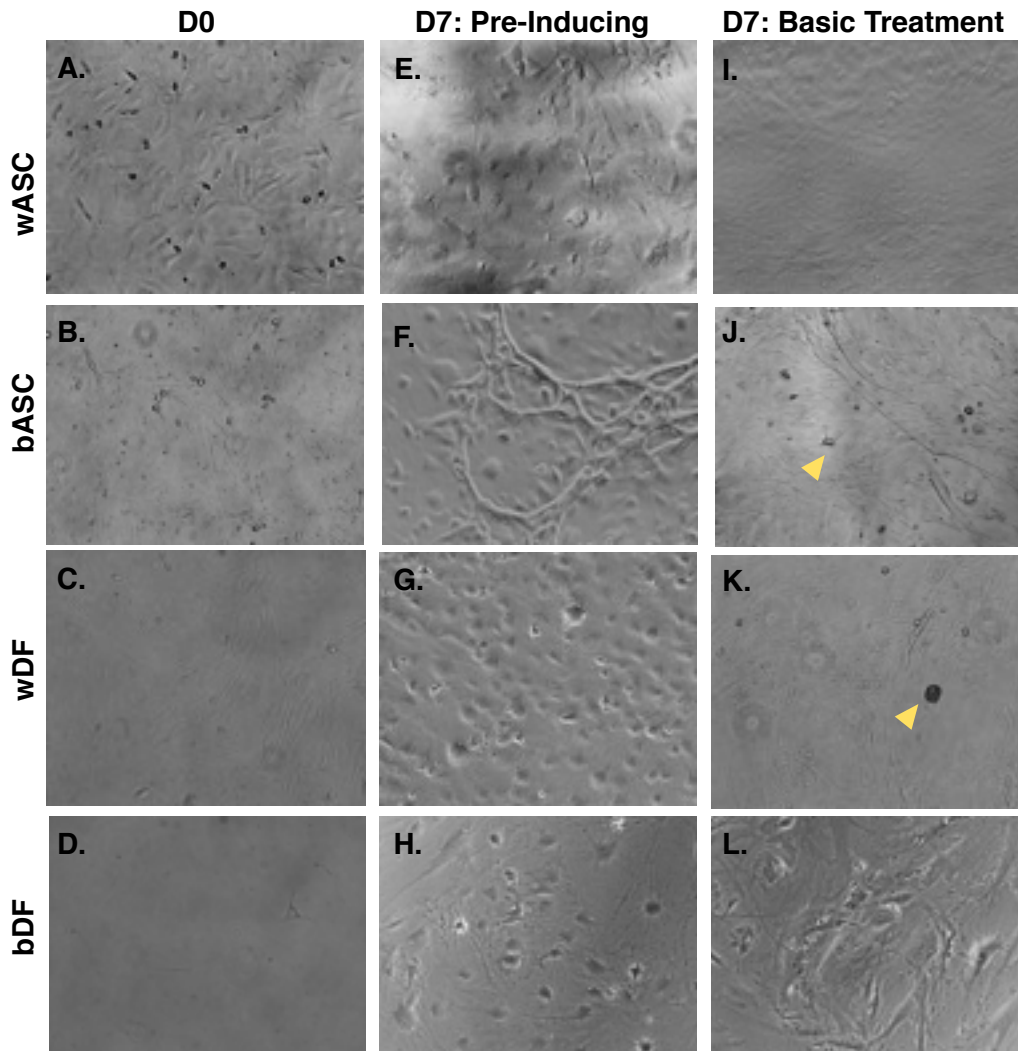


Figure 7: Method 3 Bright-Field Images from days 0 and 7 of treatment. **(A-D)** wASCs, bASCs, wDF and bDF cells 24 hours after seeding, cultured in 20% FBS/DMEM. **(E-H)** wASCs, bASCs, wDF and bDF cells after culture in pre-inducing medium(20% SR/DMEM) for 7 days. **(I-L)** wASCs, bASCs, wDF and bDF cells after culture in basic medium (10%FBS/DMEM) for 7 days. Pre-inducing medium influences the cells to obtain a ball-like morphology. Some of these cells begin to extend processes. Interestingly, bASCs (7F) show differentiation of endothelial-like cells as well. Brown-derived DFAT cells appear to contain a lower concentration of cells. By day 7, cells in basic medium begin to differentiate into different cell types. Figure 7J, the arrow points to a neuronal-like cell adjacent to a fibrous structure. Figure 7K, the arrow points to a differentiated adipocyte.

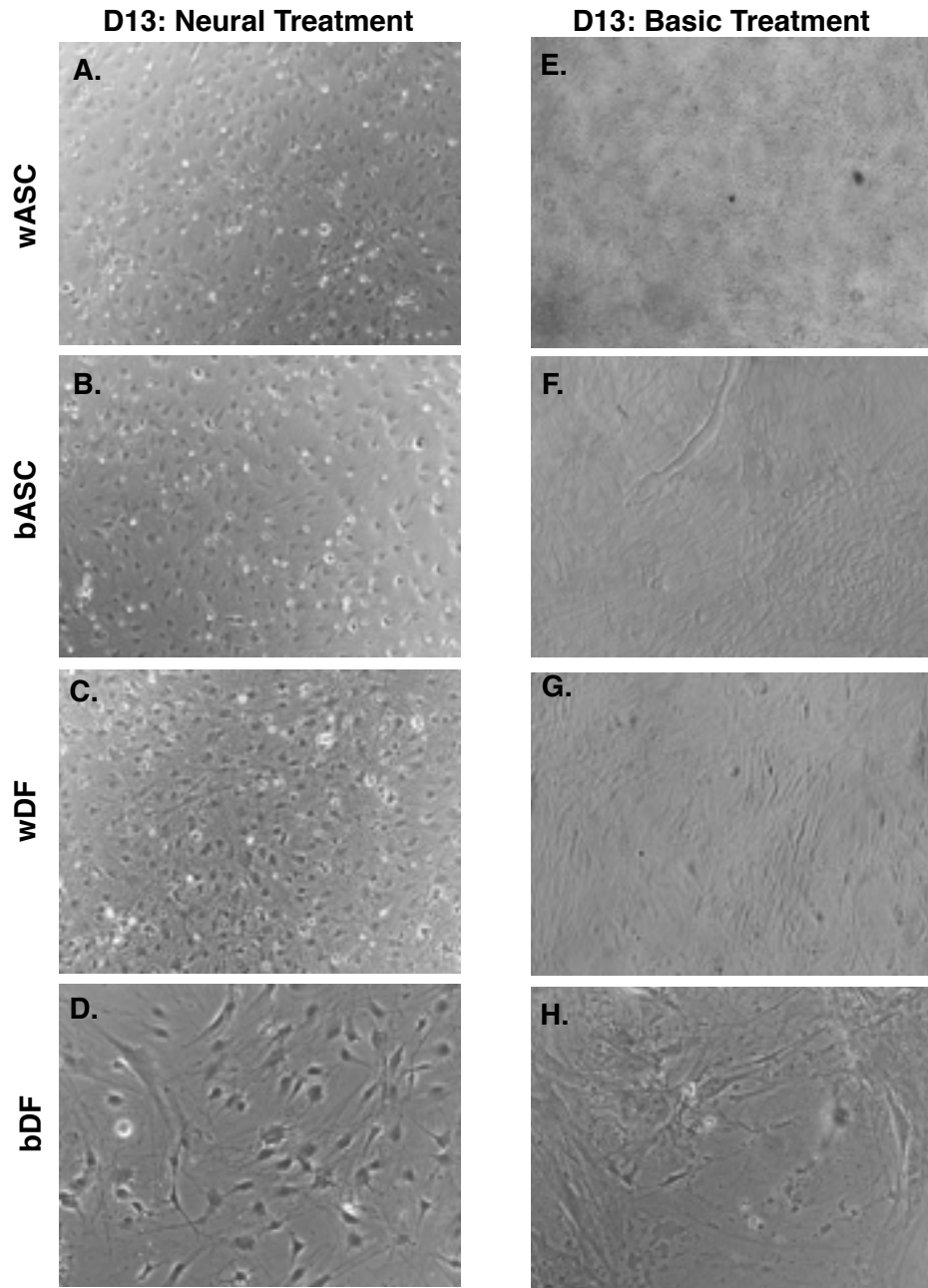


Figure 8: Method 3 Bright-Field Images on day 13 of treatment. 10x magnification. **(A-D)** wASCs, bASCs, wDFAT and bDFAT cells after culture in neural treatment for 13 days. **(E-H)** wASCs, bASCs, wDF and bDF cells after culture in basic medium (10% FBS/DMEM) for 13 days. In neural treatment, cells maintain a round morphology. In basic medium, cells achieve 100% confluence and show differentiation of various cell lineages.

GENE EXPRESSION OF NEURAL PRECURSORS:

Gene expression for the neural stem cell markers Sox1, Sox2, Nestin and Vimentin were examined and compared between wASCs, bASCs, wDFAT and bDFAT cultured using *Method 3* on days 0, 7, 10, 13 and 17 [Figure 6A] .¹

Expression of Sox1:

Our previous studies with “pre-inducing” medium suggested 20% serum replacement in DMEM had the capability of causing human ASCs to obtain an undifferentiated, pluripotent state. Sox1, as previously described, is a transcription factor that is broadly expressed in ectodermal cells committed to a neural fate.^{1,17} In Figure 9A, we show “pre-inducing” medium in comparison to basic medium causes a statistically significant increase of Sox1 expression in wDFAT. This implies “pre-inducing” medium positively affects the progenitor nature of these cells. Interestingly, on day 6 and 10 of neural treatment [i.e day 13 and 17, Figure 9C], there is significantly increased expression of Sox1. This suggests that neural induction increases the number of cells committed to a neuronal fate, especially within bASCs. In basic treatment [Figure 9B], on the other hand, we see variation of Sox1 expression with significant increases on day 13 in wASCs compared to bDFAT, wDFAT compared to bASC and bDFAT. Interestingly, bDFAT maintained a low plateau of Sox1 expression. From the results we conclude that there are differences in progenitor potential between each cell type. However, by “pre-inducing” the cells, we observe bASCs to contain a higher neuroprogenitor potential.

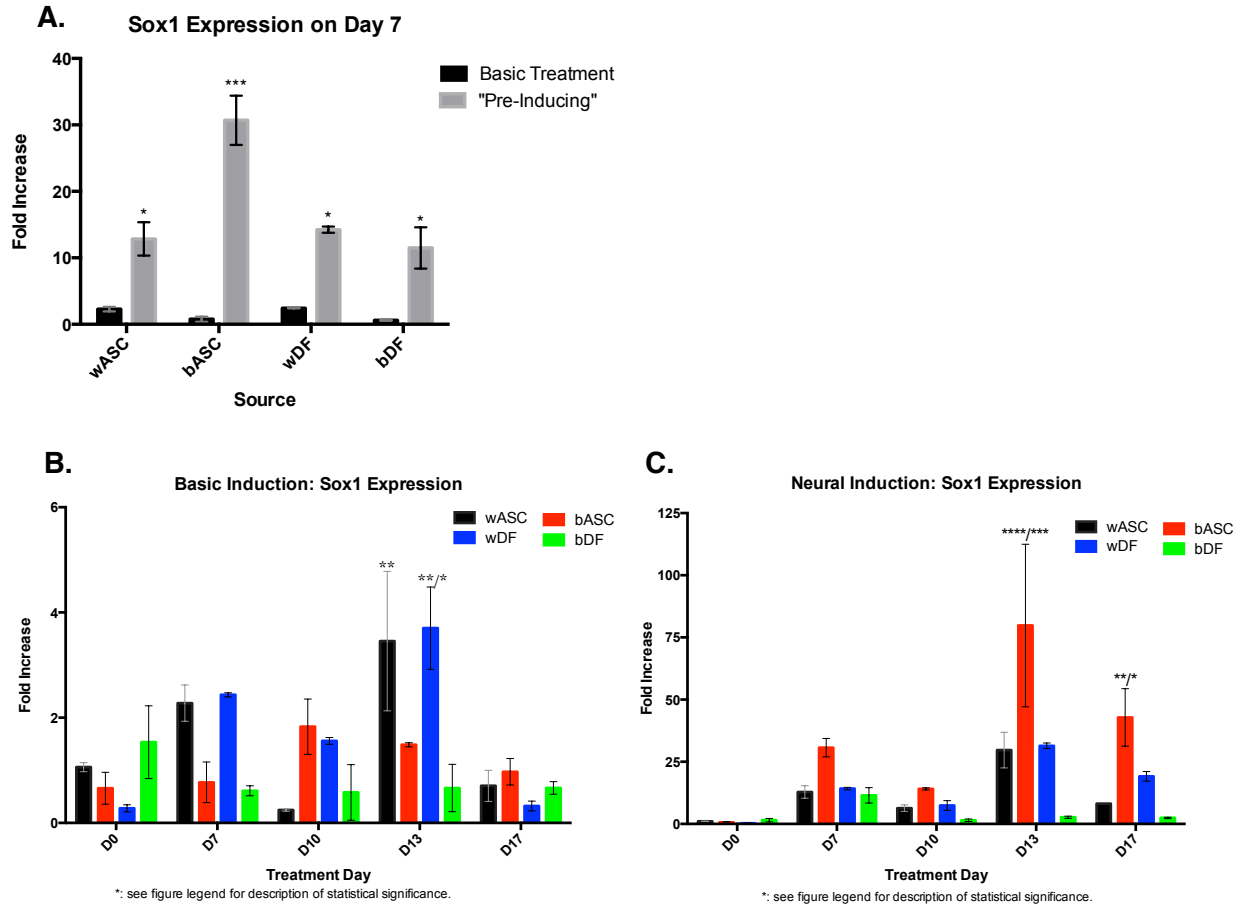


Figure 9: Evidence of Neuronal Precursors. Expression of Sox1 in white derived ASCs (wASC) or DFAT cells(bDF) and brown derived ASCs(bASC) or DFAT cells(bDFAT) on separate days after treatment. D0 on neural and basic treatment are derived from the same samples. **(A):** Sox1 expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or “pre-inducing” neural induction medium on day 7. Statistically significant differences were obtained using Sidak’s test. **(B):** Sox1 expression as determined by real-time polymerase chain reaction when cells were treated in basic induction medium, i.e 10% FBS in DMEM. Statistical significance as determined by Tukey’s multiple comparison test. **(C):** Sox1 expression as determined by real-time polymerase chain reaction when cells were treated in neural induction medium. Statistical significance as determined by Tukey’s multiple comparison test. For Sidak’s test (n=2), asterisks indicate statistically significant differences between basic treatment and pre-inducing treatment. For Tukey’s test (n=2), asterisks indicate statistically significant differences between wASC, bASC, wDF, or bDF within each respective day. *<0.05, **<0.01, ***<0.001.

Expression of Sox2:

Together with Sox1, Sox2 is broadly expressed in neuroepithelial cells. It acts to predetermine cells for neuronal differentiation while hindering precocious maturation by ensuring an undifferentiated state.¹⁷ Interestingly, we find “pre-inducing” medium enhances increased expression of Sox2 [Figure 10A], especially in bASCs and wASCs. Once “pre-inducing” medium was removed and replaced with neural treatment [Figure 10C], we find Sox2 expression from each cell source to decrease, suggesting differentiation. This is of no surprise as the number of mature neurons, NeuroD1+ cells, increases after removal of serum replacement [Figure 15]. Interestingly, we find that these decreases in Sox2 expression reaches levels similar to the expression of Sox2 in basic treatment [Figure 10B]. However, we find NeuroD1 to be expressed 10-40 fold more in the neural treated group of cells (Figure 15). This further suggests that “pre-inducing” medium behaves as a pre-inducing condition; it facilitates neural precursor proliferation.

It is also interesting to note bASCs and wASCs do not significantly differ from one another in either condition, although they both significantly express more Sox2 in basic treatment than wDFAT or bDFAT. However, in neural treatment, we find bASCs rather than wASCs to significantly differ from wDFAT and bDFAT [Figure 10B]. This provides further evidence that pre-inducing medium not only facilitates the proliferation of precursor cells but significantly enhances bASCs’ propensity to generate neuronal precursor cells.

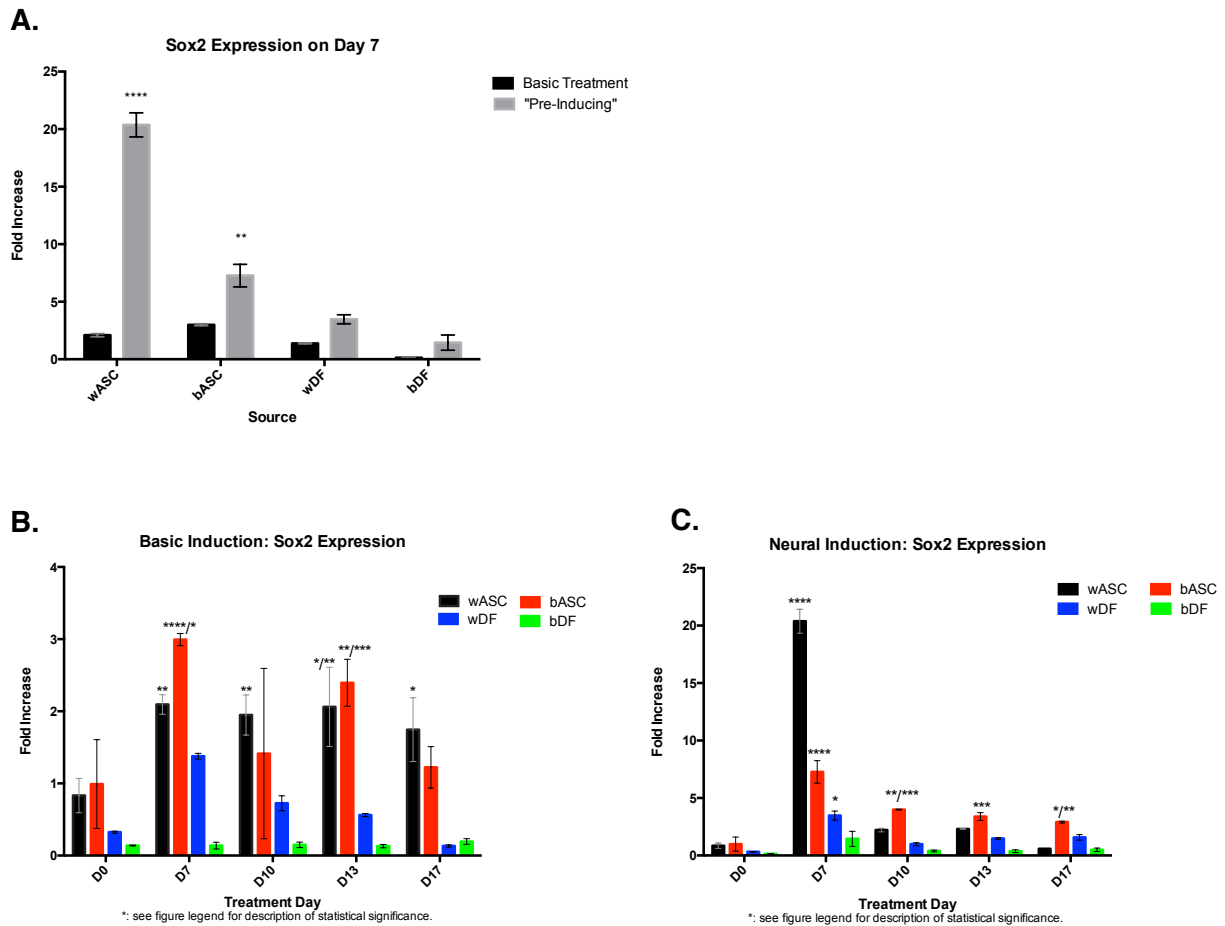


Figure 10: Evidence of Neuronal Precursors. Expression of Sox2 in white derived ASCs (wASC) or DFAT(wDF) and brown derived ASCs(bASC) or DFAT(bDF) on separate days after treatment. **(A):** Sox1 expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or “pre-inducing” neural induction medium on day 7. Statistically significant differences were obtained using Sidak’s test. **(B):** Sox2 expression as determined by real-time polymerase chain reaction when cells were treated in basic induction medium, i.e 10% FBS in DMEM. Statistical significance as determined by Tukey’s multiple comparison test. **(C):** Sox2 expression as determined by real-time polymerase chain reaction when cells were treated in neural induction medium. Statistical significance as determined by Tukey’s multiple comparison test. Sidak’s test (n=2), asterisks indicate statistically significant differences between basic treatment and pre-inducing treatment. For Tukey’s test (n=2), asterisks indicate statistically significant differences between wASC, bASC, wDF, or bDF within each respective day. *<0.05, **<0.01, ***<0.001.

Expression of Nestin:

Nestin, an intermediate filament, was initially identified as a marker of neural stem and progenitor cells. However, studies have reported nestin to be expressed in a variety of adult tissues.¹⁹ Because of this, nestin is more generally accepted to be associated with stem/progenitor cell populations that contain multipotent properties and regenerative potential.²⁴ During development, nestin is regulated by the upstream genes, Pax6 and Sox1.¹⁴ Therefore, it is of no surprise that cells with high expression of Sox1 would show low expression of nestin, which is what we find between figure 11C and figure 9C. Interestingly without sox1 induction via “pre-inducing” medium i.e basic treatment [Figure 9A], we find higher expression of nestin throughout each cell type, especially in bASCs. This is further highlighted in figure 9B where bASCs significantly express more nestin in the first 13 days of treatment in basic medium. On day 17, bASCs significantly differ from wDFAT and bDFAT, although there is increased expression of nestin in bASCs compared to wASCs. From these results, we conclude basic treatment maintains a variety of progenitor populations capable of regenerative potential while the “pre-inducing” step of neural treatment primes these cells into a neuronal lineage by increasing the expression of the early neuronal transcription factor Sox1.

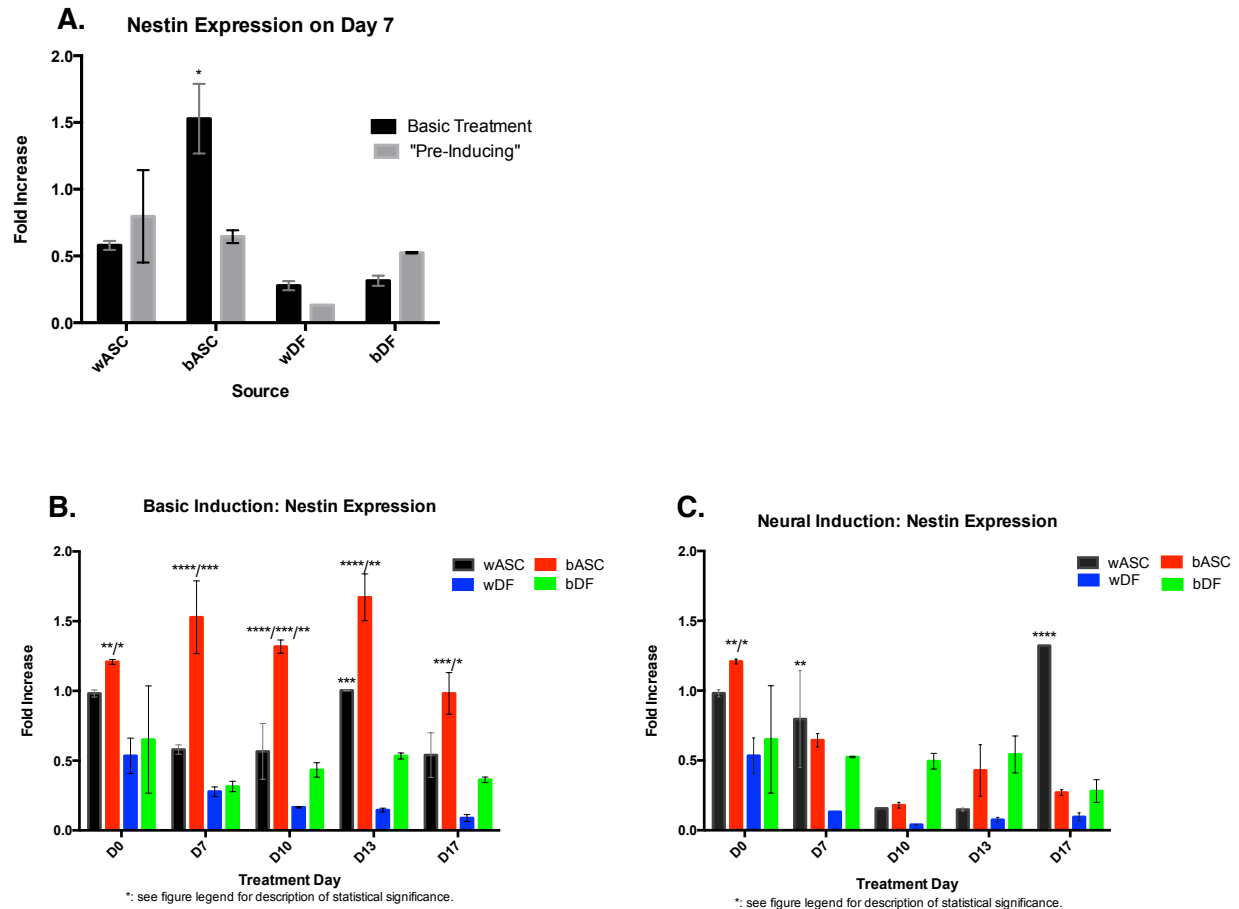


Figure 11: Evidence of Neuronal Precursors. Expression of Nestin, in white derived ASCs (wASC) or DFAT(bDF) and brown derived ASCs(bASC) or DFAT(bDFAT) on separate days after treatment. **(A):** Nestin expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or “pre-inducing” neural induction medium on day 7. Statistically significant differences were obtained using Sidak’s test and observed. **(B):** Nestin expression as determined by real-time polymerase chain reaction when cells were treated in basic induction medium, i.e 10% FBS in DMEM. Statistical significance as determined by Tukey’s multiple comparison test. **(C):** Nestin expression as determined by real-time polymerase chain reaction when cells were treated in neural induction medium. Statistical significance as determined by Tukey’s multiple comparison test. Sidak’s test (n=2), asterisks indicate statistically significant differences between basic treatment and pre-inducing treatment. For Tukey’s test (n=2), asterisks indicate statistically significant differences between wASC, bASC, wDF, or bDF within each respective day. *<0.05, **<0.01, ***<0.001.

Expression of GFAP:

Glial fibrillary acidic protein is an intermediate filament expressed by cells of astroglial lineage in the central nervous system.¹⁹ Neural stem cells in the adult brain have also been shown to express GFAP,¹¹ indicating this protein as a potential marker for neural progenitor cells. From our previous data, we would therefore expect to find increased expression of GFAP in cells treated with “pre-inducing medium,” and indeed, we find significant increases between “pre-inducing” treatment and basic treatment in wASCs, bASCs and wDFAT [Figure 12A]. Interestingly, GFAP expression follows closely to Sox2 expression: bASCs expression of GFAP is significantly higher in “pre-inducing” medium and each cell type decreases their expression of GFAP after 7 days in neural treatment [Figure 12C]. Furthermore, like Sox2, GFAP expression increases expression on day 7 in basic treatment [Figure 12B]. These results provide further evidence that pre-inducing medium not only facilitates the proliferation of precursor cells but significantly enhances bASC’s propensity to generate neuronal precursor cells. Once “pre-inducing” medium is removed, neural induction medium decreases GFAP+ progenitor cells, indicating neural differentiation, while basic treatment maintains the multipotent characteristics of these cells.

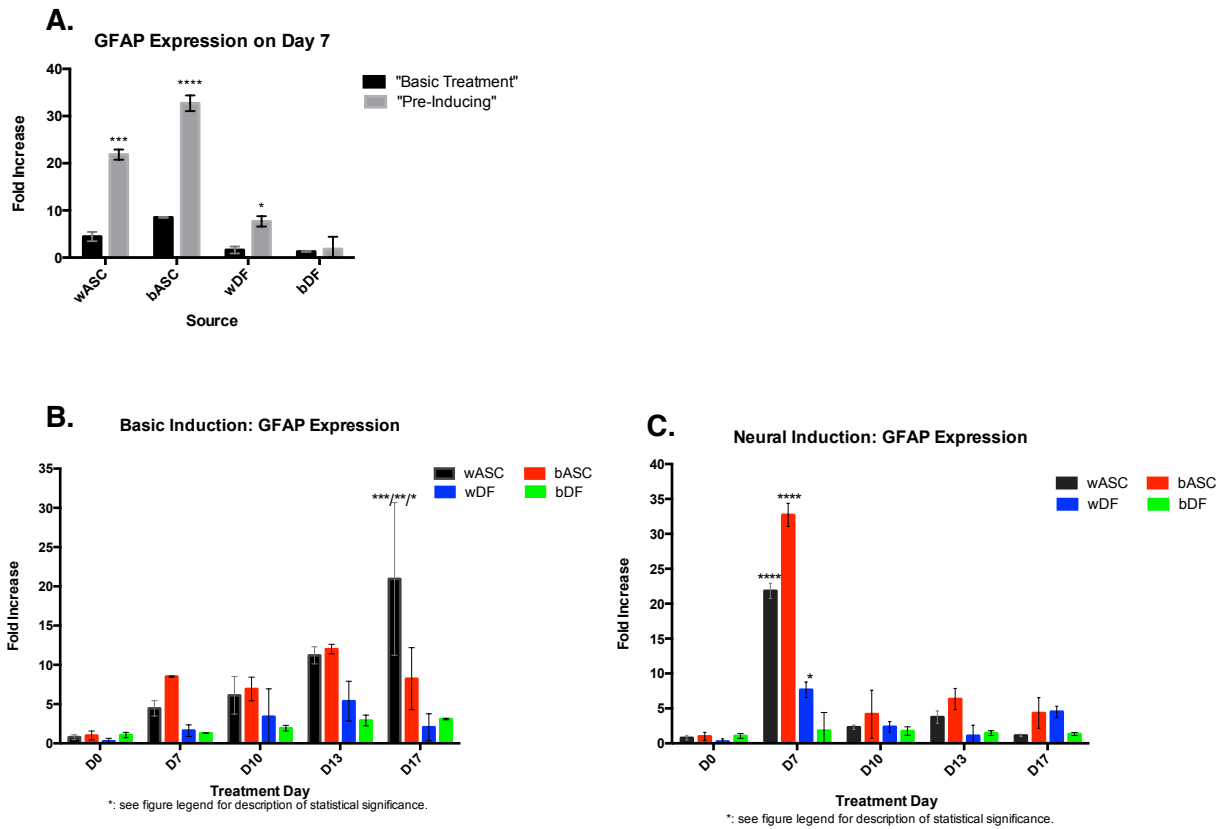


Figure 12: Evidence of Neuronal Precursors, GFAP+. Expression of GFAP, in white derived ASCs (wASC) or DFAT(bDF) and brown derived ASCs(bASC) or DFAT(bDFAT) on separate days after treatment. **(A):** GFAP expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or “pre-inducing” neural induction medium on day 7. Statistically significant differences were obtained using Sidak’s test. **(B):** GFAP expression as determined by real-time polymerase chain reaction when cells were treated in basic induction medium, i.e 10% FBS in DMEM. Statistical significance as determined by Tukey’s multiple comparison test. **(C):** GFAP expression as determined by real-time polymerase chain reaction when cells were treated in neural induction medium. Statistical significance as determined by Tukey’s multiple comparison test. Sidak’s test (n=2), asterisks indicate statistically significant differences between basic treatment and pre-inducing treatment. For Tukey’s test (n=2), asterisks indicate statistically significant differences between wASC, bASC, wDF, or bDF within each respective day. *<0.05, **<0.01, ***<0.001.

GENERATION OF NEURONAL CELLS:

Expression of Tuj1:

Tuj1, as previously described, is expressed in early and differentiated neurons. From our qPCR results, we find Tuj1 expression in “pre-inducing” medium to be greater than that in basic medium within wASCs [Figure 13A]. However, when we fixed and stained these cells on day 7, we find wASCs in basic medium to express 12.92% Tuj1+ cells while wASCs in neural treatment expressed 4.26% Tuj1+ cells [Figures 14A1, A2, E1, E2, I]. This difference could stem from culture conditions; RNA was extracted from a 12-well gelatin coated plate while cells for immunofluorescence were cultured in 8-well gelatin coated glass chamber slides. Differing material could have affect abundance of neuronal cells or simply there is a repression of RNA translation. Figure 14’s table reveals further inconsistency between qPCR results and immunofluorescence. We find, from counting nuclei and Tuj1+ cells in our stainings, that bDFAT express the most Tuj1+ cells, even though our qPCR reveals the contrary. This could be due to the low sample size of bDFAT, i.e smaller population of cells observed [Figure 6B]. Although bDFAT was measured to contain the greatest number of Tuj1+ cells in “pre-inducing” and basic treatment, these cells appear more progenitor like in nature [Figure 14D1, D2, H1, H2] compared to wASCs, bASCs, and wDFAT cells where majority of the cells consist of a bipolar morphology [Figures 14A1-C2, E1-G2].¹⁰ Furthermore, although quantitative data shows bDFAT to contain more Tuj1+ cells, wASCs and bASCs appear to contain a large amount of more mature neuronal cells in basic medium [Figures 14E1-F2] that have the capability of interacting with one another.

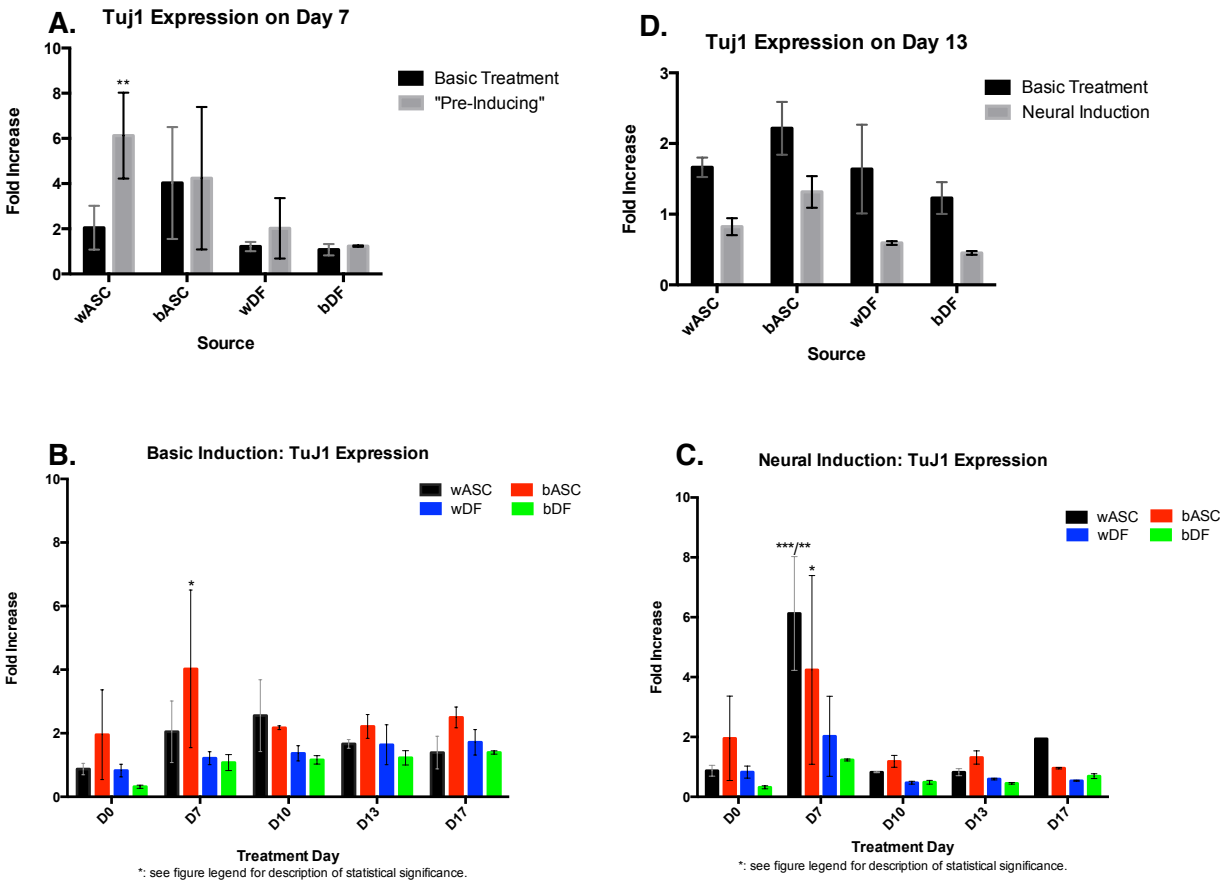
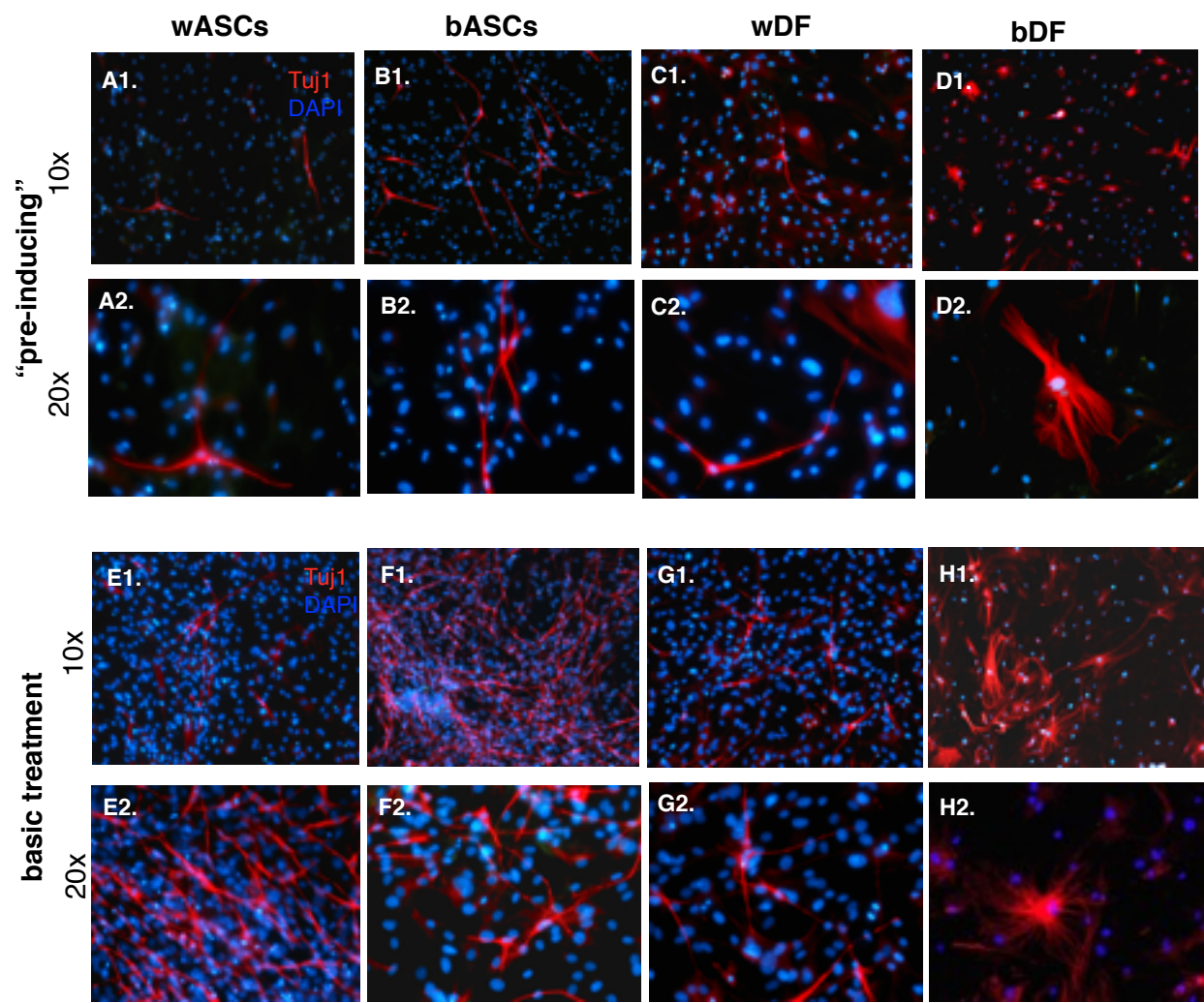


Figure 13: Evidence of Immature or Early Differentiated Neurons. Expression of Tuj1, i.e beta-Tubulin III (Tubb3), in white derived ASCs (wASC) or DFAT(bDF) and brown derived ASCs(bASC) or DFAT(bDFAT) on separate days after treatment. **(A):** Tuj1 expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or “pre-inducing” neural induction medium on day 7. Statistically significant differences were obtained using Sidak’s test. **(B):** Tuj1 expression as determined by real-time polymerase chain reaction when cells were treated in basic induction medium, i.e 10% FBS in DMEM. Statistical significance as determined by Tukey’s multiple comparison test. **(C):** Tuj1 expression as determined by real-time polymerase chain reaction when cells were treated in neural induction medium. Statistical significance as determined by Tukey’s multiple comparison test. **(D):** Tuj1 expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or neural induction medium on day 13. Statistically significant differences were obtained using Sidak’s test. Sidak’s test (n=2), asterisks indicate statistically significant differences between basic treatment and pre-inducing treatment. For Tukey’s test (n=2), asterisks indicate statistically significant differences between wASC, bASC, wDF, or bDF within each respective day. *<0.05, **<0.01, ***<0.001.



	“Pre-Inducing”	Basic Treatment
wASCs	4.26%	9.58%
bASCs	5.06%	12.92%
wDF	8.05%	2.06%
bDF	15.38%	30.62%

Figure 14: Expression of neuronal Tuj1+ cells on day 7. Cells were fixed with 4% paraformaldehyde after 7 days in “pre-inducing” medium or basic medium and stored overnight in 4°C. The next morning, cells were stained with mouse anti-Tuj1 and DAPI. Figures **A1-C2** are images taken at 10x magnification, figure **D1** is an image taken at 4x, after 7 days in “pre-inducing” medium. Figures **A2-C2** are images taken at 20x magnification, figure **D2** at 10x, after 7 days in “pre-inducing” medium. Figures **E1-G2** (10x magnification), **H1** (4x magnification) are images taken after 7 days in basic treatment while figures **E2-G2** (20x magnification), **H2** (10x magnification) are images taken after 7 days in basic treatment. The table lists the percentage of Tuj1+ cells for each cell source.

Expression of NeuroD1:

Interestingly, after day 7, qPCR results reveal Tuj1 expression in neural treatment decreases to levels similar to cells treated in basic medium [Figure 13D]. During this time period, however, we also observe neural treatment to correlate with significant increases of NeuroD1 expression in each cell type [Figure 15B], significantly in bASCs and wDFAT. NeuroD1 expression in basic treatment [Figure 15A], on the other hand, increases on day 7 and then decreases afterward. Interestingly, wDFAT in basic medium is shown to express a significantly higher amount of NeuroD1 from days 7 to 13 compared to bDFAT and wASCs. This suggest, neural treatment affects the neurogenic potential of each cell type; wDFAT in basic medium has a higher potential than bDFAT and wASCs, while bASCs in neural treatment has a greater potential.

When comparing treatments, and keeping in mind NeuroD1 is required for the survival and maturation of adult-born neurons,³ we conclude that cells in neural induction medium become more mature over time, causing the loss of their progenitor-like nature, i.e decreased expression of Tuj1. Significant increases of NeuroD1 expression on day 13 [Figure 15D] in wASCs, bASCs, and wDFAT treated with neural induction medium compared to treatment with basic medium provides further evidence that cells that were Tuj1+ in “pre-inducing” medium began to mature in neural treatment. Interestingly, cells in “pre-inducing” medium do not express more NeuroD1 than those in basic medium [Figure 15C]. Therefore, significant differences obtained in NeuroD1 expression after 6 days of treatment with neural induction medium provides further evidence that neural treatment maintains the survival of the immature neuronal cells developed during initial conditions.

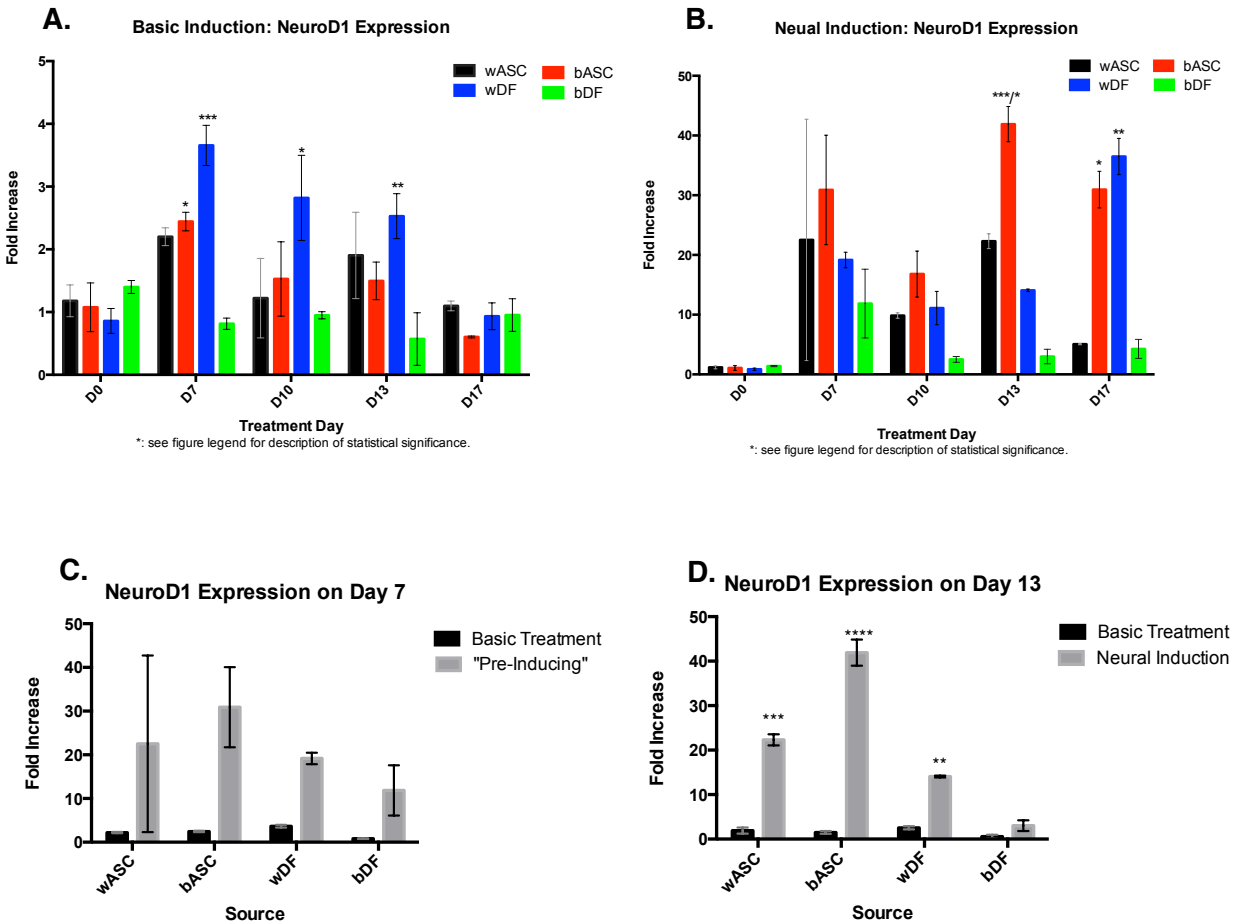


Figure 15: Evidence of Maturing Neurons, NeuroD1+ cells. Expression of NeuroD1, in white derived ASCs (wASC) or DFAT(bDF) and brown derived ASCs(bASC) or DFAT(bDFAT) on separate days after treatment. **(A):** NeuroD1 expression as determined by real-time polymerase chain reaction when cells were treated in basic induction medium, i.e 10% FBS in DMEM. Statistical significance as determined by Tukey’s multiple comparison test. **(B):** NeuroD1 expression as determined by real-time polymerase chain reaction when cells were treated in neural induction medium. Statistical significance as determined by Tukey’s multiple comparison test. **(C):** NeuroD1 expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or “pre-inducing” neural induction medium on day 7. Statistically significant differences were obtained using Sidak’s test. **(D):** NeuroD1 expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or neural induction medium on day 13. Statistically significant differences were obtained using Sidak’s test. Sidak’s test (n=2), asterisks indicate statistically significant differences between basic treatment and pre-inducing treatment. For Tukey’s test (n=2), asterisks indicate statistically significant differences between wASC, bASC, wDF, or bDF within each respective day. *<0.05, **<0.01, ***<0.001.

Immunofluorescence of Tuj1+ cells after 13 days of induction:

During this experiment, we attempted to fix and stain the cells after 17 days of induction, however, after 17 days in the glass chamber slides, the cells were loosely attached. To obtain results from neural induction, we repeated the experiment and fixed the cells after 6 days in neural induction medium [day 13, Figure 6A] or 13 days in basic medium. Afterward, cells were stained with mouse anti-Tuj1 and rabbit anti-Nestin antibodies. Although cells were counted prior to plating, the density of each cell type was less than the previous experiment [Figure 16I-L], which appeared to cause the cells to generate neuronal cells at a slower rate [Figure 16A-H]. However, neuronal cells were still generated. Interestingly, wASCs and bASCs in basic medium [Figure 16E, F] were the only cell type to generate a high density of bipolar neuronal cells. This is most likely a result of cell to cell interactions as wASCs and bASCs proliferated at a higher rate in basic medium [Figure 16E, F DAPI amount, figure 16I,J RNA concentration]. In neural induction medium, wASCs, bASCs and wDFAT were observed to contain a low quantity of bipolar neuronal cells. While wASCs and bASCs showed more Tuj1+ cells, wDFAT and bDFAT showed a higher expression of nestin+ cells in neural treatment [Figure 16C,D]. Coexpression of Tuj1 and nestin were also observed in wDFAT and bDFAT in basic and neural treatment (figure 16G, H, D), indicating a transitional period from neural progenitor cells to early neuronal cells.

From these results we can conclude there are differences between each cell type, bASCs, bDFAT, wASCs and wDFAT; ASCs generate more mature neuronal cells while DFAT cells are more prone to maintain progenitor-like characteristics.

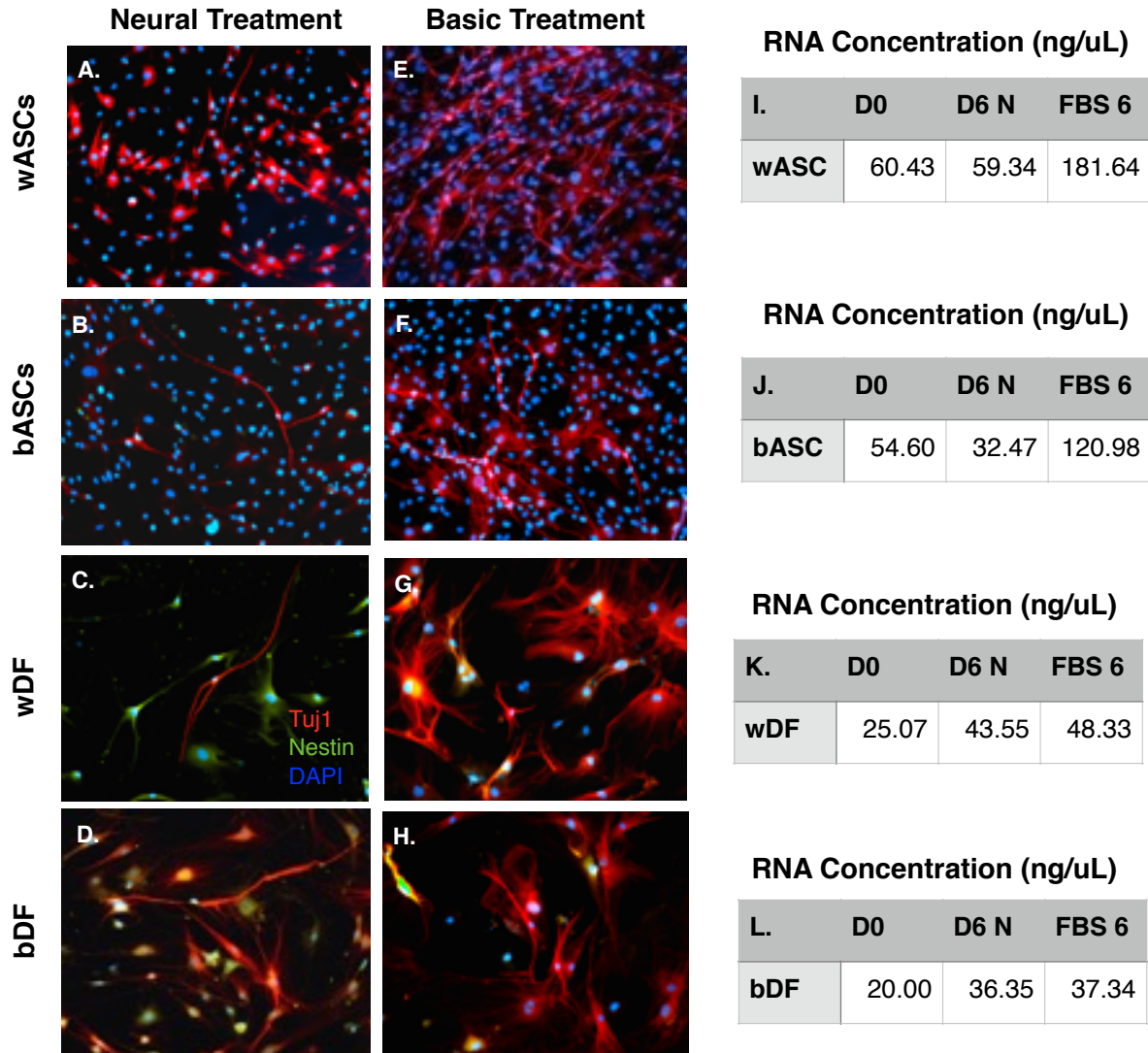


Figure 16: Expression of neuronal Tuj1+ cells on day 13 of a fourth experiment. Cells were stained with mouse anti-Tuj1 and rabbit anti-Nestin after 13 days of treatment in neural induction medium or basic medium. Figure 15F was taken with 4x magnification while the others were taken with 10x. As observed, all cell types in either basic medium or neural induction medium are Tuj1+. However, we find nestin to be highly expressed in wDF (C) and bDF (D) in neural treatment while it is expressed in low quantities in wDF and bDF (G,H) in basic treatment. Co-staining of nestin and Tuj1 are observed in these figures, suggesting progenitor cells entering a post-mitotic neuronal state. Although each cell type expresses Tuj1+, they are observed to be in different states of maturation. wASCs in neural induction medium (A) is in an early neuronal progenitor state, as show by the morphology. wASCs in basic medium (E), however, have entered a more mature state, as shown by their bipolar morphology. Looking closely at bASCs, we find a more progenitor like cells in neural treatment (B) and more mature neuronal cells in basic medium (F). bDF and wDF are shown to maintain more progenitor like qualities in both treatment types (C,D,G,H), although in neural treatment, we find some mature like neuronal cells (C, D), i.e cells with bipolar morphology. (I.-L.) RNA concentration of these cells on day 0 of collection and day13.

GENERATION OF GLIAL-LIKE CELLS

Expression of Vimentin:

Vimentin is an important structural protein expressed in less mature glial cells, especially radial glia, cells that generate neuronal and glial cells in the developing brain.^{6,12} Vimentin, therefore, acts as an identifier of glial precursor cells. Because of this, we would expect a higher quantity of vimentin expression in the pre-inducing step of neural treatment. Indeed, we find increased expression of vimentin [Figure 17C], however a significant difference is only identified within wASCs. In previous figures, we show neural treatment after day 7, i.e step 2 of neural treatment, restricts the progenitor potential [Figure 10C]. As expected, vimentin, an early glial marker, is significantly decreased by day 13 in neural treated wASCs, bASCs and wDFAT [Figure 17B, D]. In basic treatment, vimentin expression remains relatively at basal levels [Figure 17A], further providing evidence that basic treatment may maintain these cells in a progenitor state.

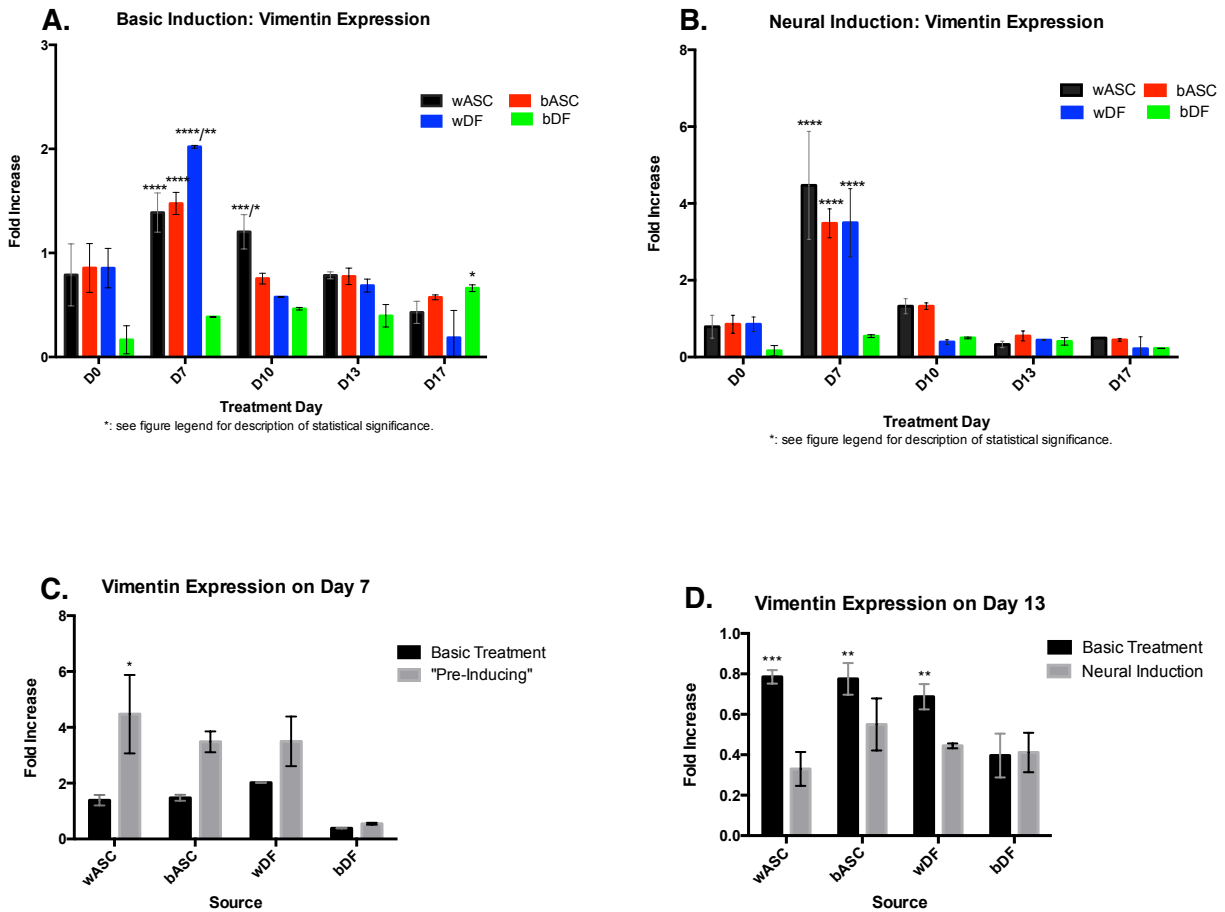


Figure 17: Evidence of Glial Precursors. Expression of Vimentin, in white derived ASCs (wASC) or DFAT(bDF) and brown derived ASCs(bASC) or DFAT(bDFAT) on separate days after treatment. **(A):** Vimentin expression as determined by real-time polymerase chain reaction when cells were treated in basic induction medium, i.e 10% FBS in DMEM. Statistical significance as determined by Tukey's multiple comparison test. **(B):** Vimentin expression as determined by real-time polymerase chain reaction when cells were treated in neural induction medium. Statistical significance as determined by Tukey's multiple comparison test. **(C):** Vimentin expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or "pre-inducing" neural induction medium on day 7. Statistically significant differences were obtained using Sidak's test. **(D):** Vimentin expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or "pre-inducing" neural induction medium on day 7. Statistically significant differences were obtained using Sidak's test. Sidak's test (n=2), asterisks indicate statistically significant differences between basic treatment and pre-inducing treatment. For Tukey's test (n=2), asterisks indicate statistically significant differences between wASC, bASC, wDF, or bDF within each respective day. *<0.05, **<0.01, ***<0.001.

Expression of s100B:

Interestingly, we see a similar trend between expression of vimentin and s100B, an astroglial marker expressed in satellite cells and astrocytes of the CNS [Figure 18A, D].¹⁹ However, after 7 days in basic treatment, expression of s100B remains significantly high in bDFAT cells compared to wASCs and bASCs [Figure 18A]. Expression of s100B remains high in wDFAT as well, but decreases by day 17. A similar trend is noted in bASCs. Interestingly, wASCs remains relatively consistent throughout the time course. After 7 days in neural treatment, we see an opposite affect; s100B expression in neural treatment decreases in all cell types significantly more than those in basic treatment [Figure 18B, D]. From these results, we can conclude “pre-inducing” medium, step 1 of neural induction, causes the cells to enter a glial-like stage that acts like a neural stem cell, i.e radial-glia like.^{11,12,26} Once cells are introduced to another medium, i.e neural induction, they decrease their progenitor potential and mature into neuronal cells [Figure 15]. Basic treatment, on the other hand, maintains progenitor potential and produces glial-like cells as well as neuronal cells.

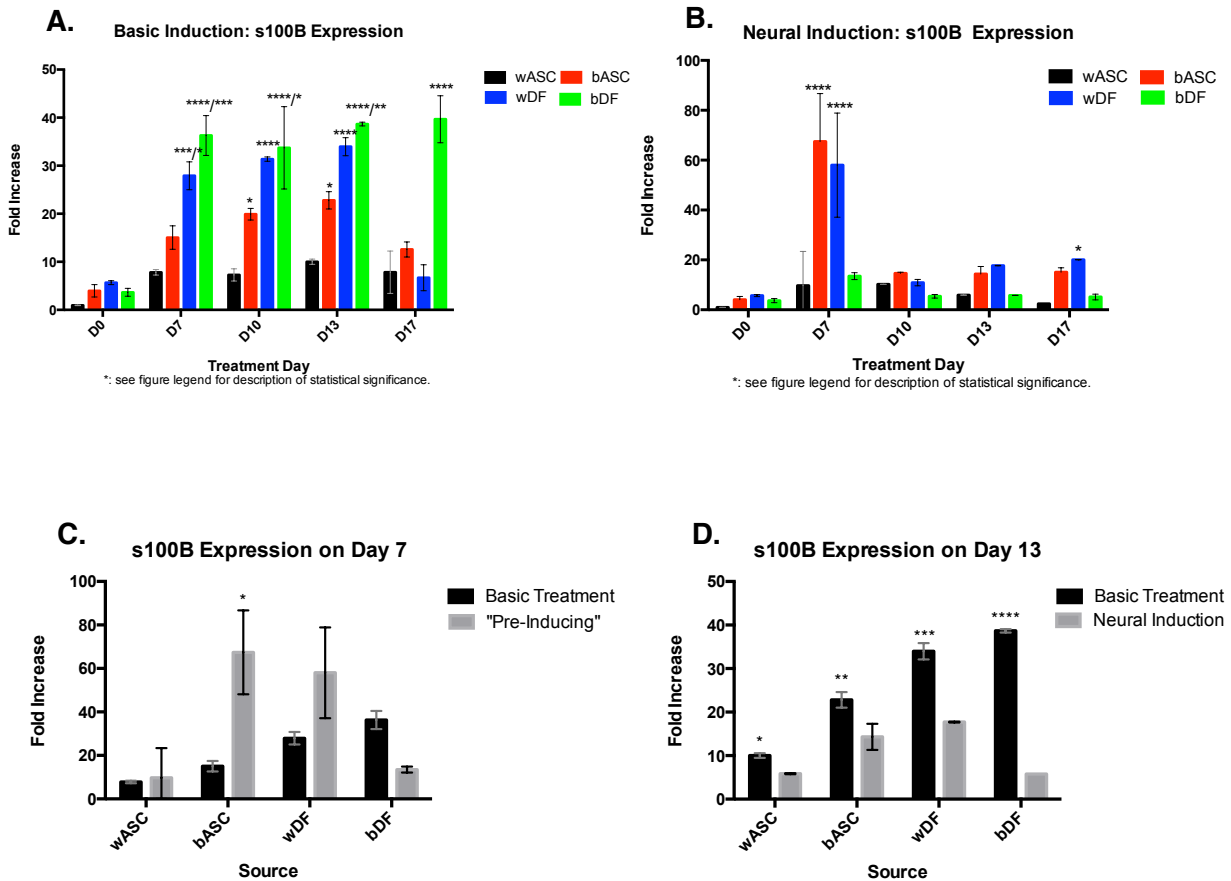


Figure 18: Evidence of Glial-like Cells, S100B+. Expression of S100B, in white derived ASCs (wASC) or DFAT(bDF) and brown derived ASCs(bASC) or DFAT(bDFAT) on separate days after treatment. **(A):** S100B expression as determined by real-time polymerase chain reaction when cells were treated in basic induction medium, i.e 10% FBS in DMEM. Statistical significance as determined by Tukey's multiple comparison test. **(B):** S100B expression as determined by real-time polymerase chain reaction when cells were treated in neural induction medium. Statistical significance as determined by Tukey's multiple comparison test. **(C):** S100B expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or "pre-inducing" neural induction medium on day 7. Statistically significant differences were obtained using Sidak's test. **(D):** S100B expression as determined by real-time polymerase chain reaction when cells were treated with basic treatment or neural induction medium on day 13. Statistically significant differences were obtained using Sidak's test. Sidak's test (n=2), asterisks indicate statistically significant differences between basic treatment and pre-inducing treatment. For Tukey's test (n=2), asterisks indicate statistically significant differences between wASC, bASC, wDF, or bDF within each respective day. *<0.05, **<0.01, ***<0.001.

Immunofluorescence of s100B+ cells after induction for 7 or 13 days:

Expression of s100B was determined from cells cultured during experiment 4 [Figure 16I-L]. As shown in our qPCR results, “pre-inducing” medium caused an increase in s100B expression. From our immunofluorescent results, we find wASCs and bASCs treated with pre-inducing medium to express a higher number of s100B+ cells that morphologically appear astrocytic [Figure 19A,B,C,D]. From our qPCR results, statistical analysis revealed bASCs to express higher levels of s100B+ cells in pre-inducing medium vs. basic medium. Our immunofluorescence mirrors this; bASCs in pre-inducing medium appear to contain a higher number of s100B cells compared to the other cell types and treatments. In addition there is high expression of s100B in wASCs during basic and pre-inducing treatment, even though qPCR results do not suggest it [Figure 18C]. wASCs appear to express the greatest s100B+ cells in basic medium [Figure 19E]. Interestingly, bDFAT and wDFAT s100B+ cells do not contain morphological features similar to astrocytes, therefore, a possible reason for these differences is differential maturation of cells.

The previous explanation is strengthened by s100B expression on day 13 of induction [Figure 20]. In these immunofluorescent images we find s100B to be significantly higher in wDFAT and bDFAT cells in basic treatment [Figure 18A, 18D, 20G, 20H]. Once again, RNA analysis and immunofluorescence may differ due to the differing plating materials or RNA translation of the respective genes. One must also take into account that these immunofluorescent results are semi-quantitative, at best, whereas the qPCR results are more precise for comparisons. Overall, our results suggest that in basic treatment, bDFAT cells and wDFAT cells have a higher

potential to become glial cells than brown or white-derived ASCs. Once cells are introduced to other treatments, i.e neural treatment, glial potential changes.

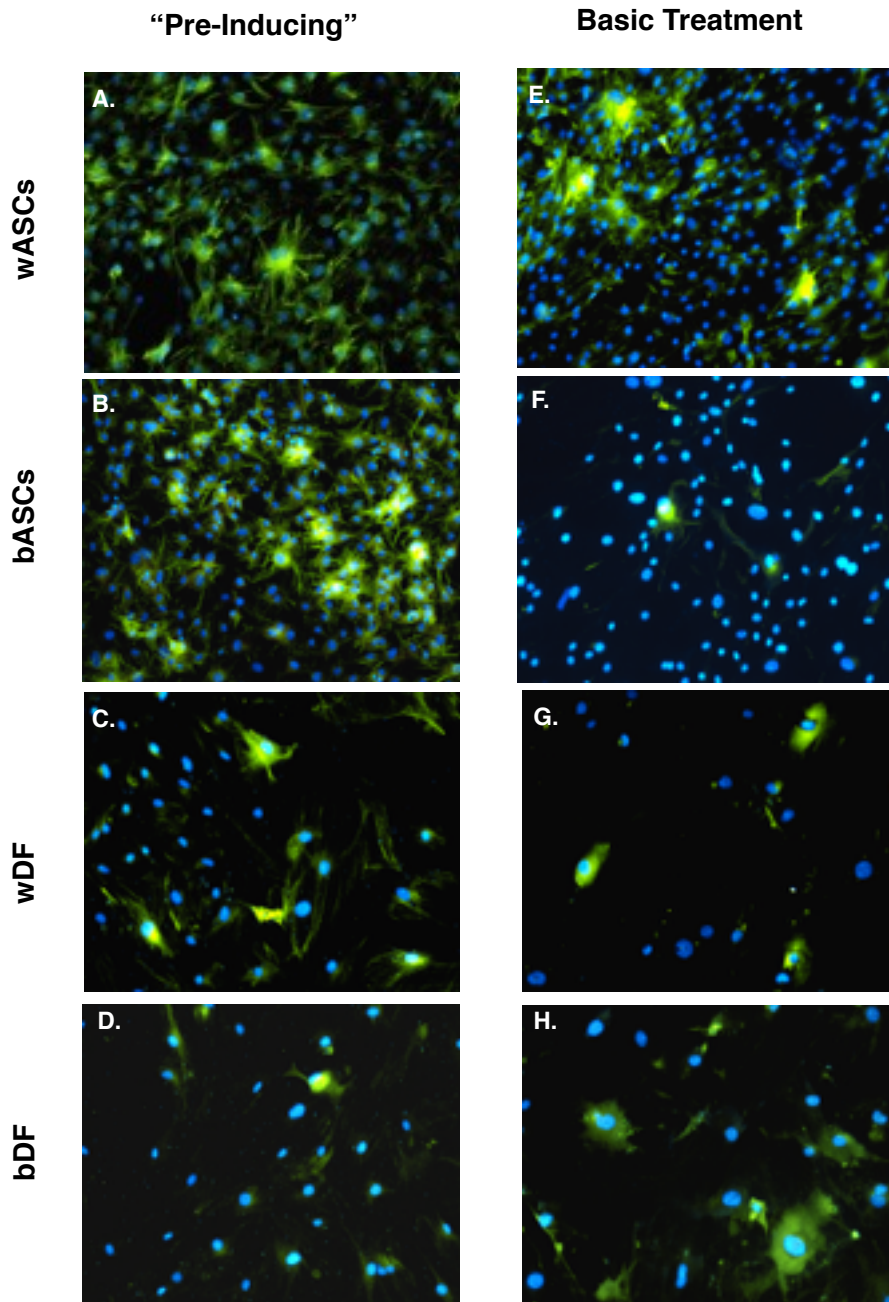


Figure 19: Expression of s100B+ cells on day 7. 10x magnification. Interestingly, on day 7 we find wASCs and bASCs in pre-inducing medium to contain a high quantity of s100B (**A, B**) with lower quantities of s100B+ cells in basic medium (**E, F**). wASCs in basic medium (**E**), however, were observed to contain a high quantity of s100b+ cells. wDF and bDF cells in pre-inducing medium (**C, D**) contained a low quantity of s100B+ cells. These cells, however, contained astroglial structures while wDF and bDF cells in basic medium (**G,H**) contained a low population of s100B cells that did not contain the same morphological structures.

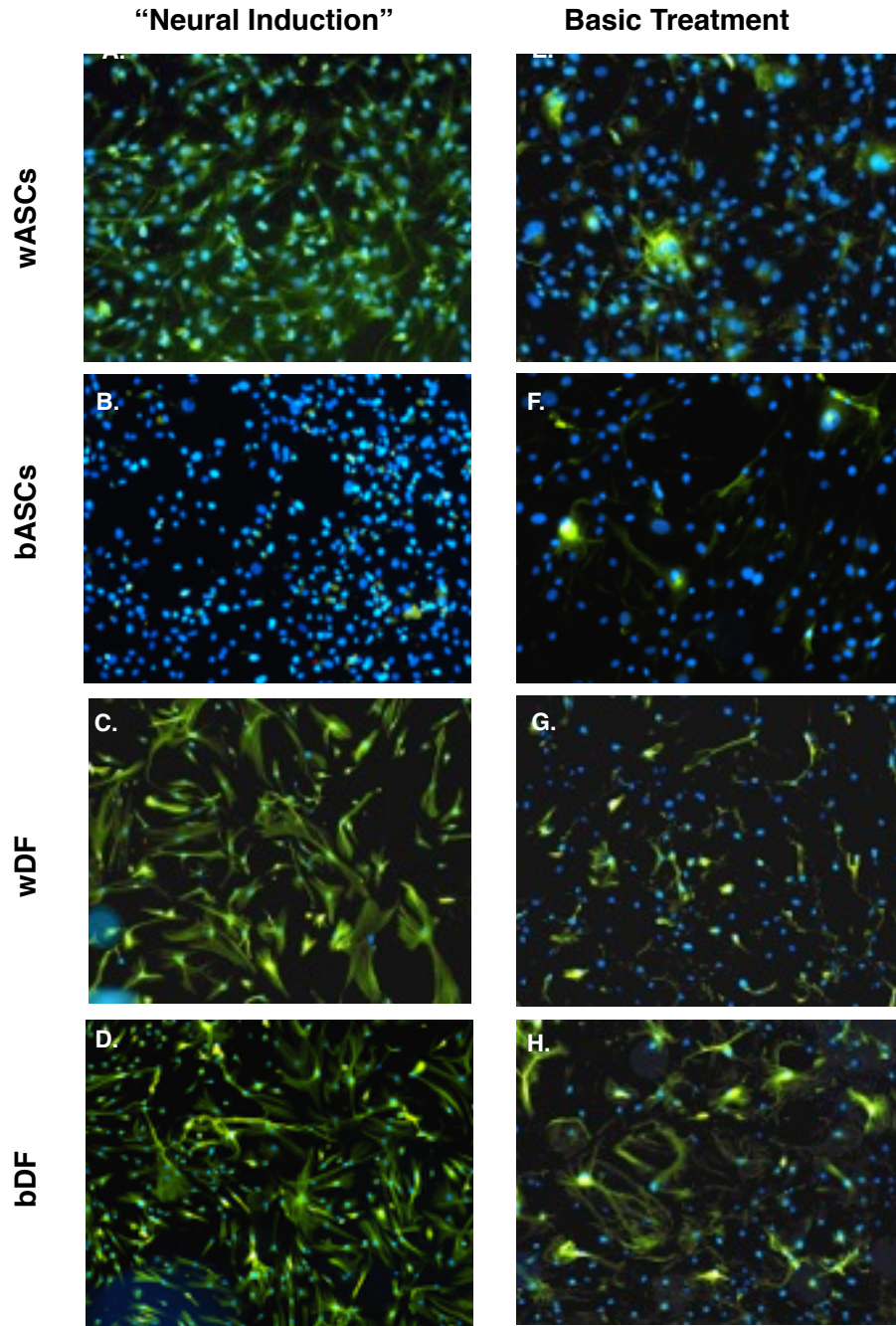


Figure 20: Expression of s100B+ cells on day 13. wASCs (10x magnification) and bASCs(4x magnification) in neural induction medium (**A, B**) and basic medium (**E,F**). wDFAT (4x magnification) and bDFAT (4x magnification) cells in neural induction medium (**C,D**). These cells contain morphological features of astroglial cells. wDFAT and bDFAT cells (4x magnification) in basic medium (**G, H**).

Interaction of s100B+ and Tuj1+ Cells

As previously mentioned, after 17 days of treatment, neural induced cells eventually decrease their attachment to the wells and detach during the washing phase of immunofluorescence. Because of this, we were unable to image these cells but were able to perform immunofluorescence on wASCs, bASCs, wDFAT, and bDFAT treated with basic medium [Figure 21]. These cells were cultured in the 12-well petri dishes for 17 days and then stained with mouse anti-Tuj1 and rabbit anti-S100B. From these images, we can delineate the differences between cell types. wASCs [Figure 21A] expressed a high density of Tuj1+ bipolar cells. Coexpression of s100B+ cells with Tuj1 was also detected, but s100B+ cell fluorescence was less intense, suggesting wASCs are able to generate neuronal cells [Figure 21C]. bASCs also contain Tuj1+ and s100B+ cells, supporting that they too can generate neuronal cells [Figure 21B]. In bDFAT cells, on the other hand [Figure 21C], we observe a large population of high intensity s100B+ cells with some coexpression of Tuj1 suggesting they are more glial-like in nature. Interestingly, wDFAT cells contain a higher population of Tuj1+ bipolar cells [Figure 21D]. Overall, immunofluorescent results support the presence of glial and neuronal cells by indicating the presence of corresponding proteins. Semi-quantitatively, white-derived cells appear to generate a more restricted population of neuronal cells.

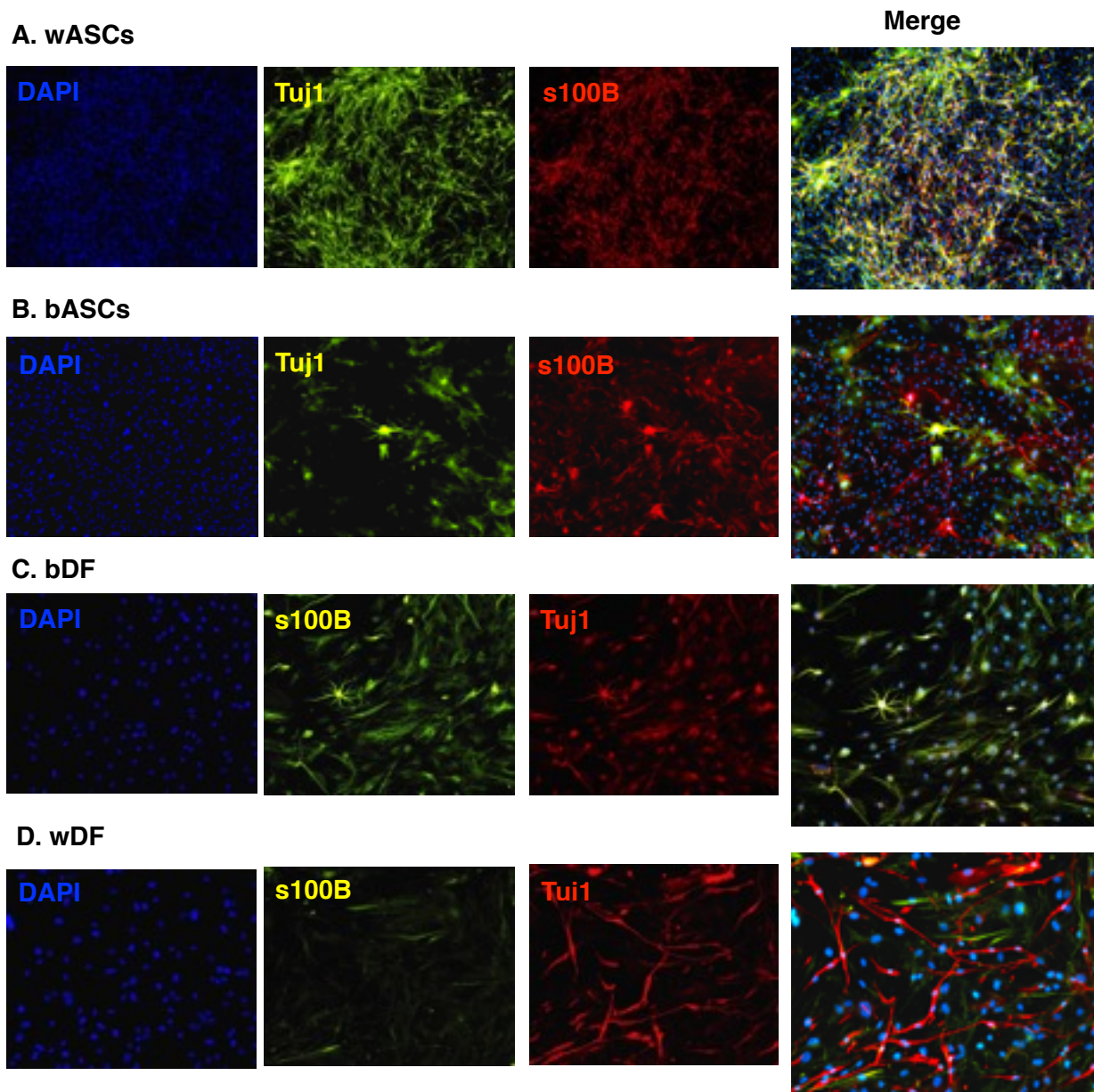


Figure 21: Interaction of S100B+ cells and Tuj1+ cells on day 17; differences between cell types. 4x magnification. wASCs, bASCs, wDF and bDF cells were fixed with 4% paraformaldehyde on the last day of treatment in basic medium. Cells in neural induction medium could not be imaged on day 17 due to low attachment. In basic medium, we observe stark differences between the cell types. wASCs (**A**) show a high density of Tuj1+ cells with coexpression of S100B, suggesting these cells may be progenitor in nature as radial glial cells, which are s100B+, are astroglial cells that are precursors to glia and neurons. (**B**) wASCs show an even distribution of s100B and Tuj1+ cells with extending processes. (**C**) bDF cells show a high density and intensity of s100B+ cells. Although there are Tuj1+ cells, intensity is decreased. (**D**) wDF cells show Tuj1+ cells with a high intensity of expression. Glial nature of wDF is less than the other cell types, as shown by the low expression of s100B.

DISCUSSION

Past studies have revealed white adipose tissue-derived ASCs and DFAT cells have the capability of generating neuronal and glial-like cells.^{1,16} In this study, we sought to illustrate the differing characteristics of ASCs and DFAT cells derived from brown and white adipose tissue, respectively, in order to support our hypothesis that brown adipose tissue contains multipotent stem cells with higher propensity to undergo neural differentiation, i.e generate neurons and glia.

12,26

Adult Neural Stem Cells: Neurons and Glia

As previously described, adult neural stem cells give rise to neurons and glia. In the adult mammalian brain, this process is facilitated by a restricted population of radial glia cells that are capable of generating astrocytes, ependymal cells, neurons, and oligodendrocytes.^{11,12,26} In past studies, however, radial glia were classically believed to be immature glial cells that guide neuronal migration and function as scaffolding.^{6,11} To identify potential astroglial-like neural stem cells within our population of multipotent adipose-derived stem cells, we used GFAP to mark neural stem cells and vimentin to determine radial-glia like properties.^{6,11} S100B, was used to determine the presence of astrocytes, a glial cell that provides essential services to the neurons they support, including synapse formation and regulation of blood flow.²⁷ Because radial-glia like neural stem cells have the ability to generate oligodendrocytes in the adult brain, we used Olig2 to determine the presence of differentiating oligodendrocytes. To determine the presence of early, post-mitotic neurons we assessed for the presence of Tuj1, while NeuroD1 assessed the presence of mature neurons.^{3,10}

First step: Are wASCs, bASCs, wDFAT and bDFAT cells different?

Before we began to assess the neural stem cell progeny of wASCs, bASCs, wDFAT and bDFAT cells, we assessed whether ASCs and DFAT cells derived from white or brown adipose tissue exhibited differing characteristics. From our analysis, we found bASCs to contain more hematopoietic positive cells [Figure 2C]. Interestingly, endothelial cells have been suggested to stimulate self-renewal and expand neurogenesis of neural stem cells.²⁰ This supports a potential explanation for our observation that bASCs in pre-inducing medium have a higher proportion of neural-like progenitor cells, i.e they significantly expressed Sox1 to a higher degree [Figure 9A]. Furthermore, these cells were capable of forming endothelial-like cells in the pre-inducing medium [Figure 7F], suggesting the heterogenous cell population within bASCs may have contributed to their higher neuroprogenitor potential. Interestingly, our FACS data supported previous studies showing wDFAT cells to express more CD90+ cells [Figure 1C].⁸ This is of interest because it indicates that pre-inducing medium alters the neural potential of these cells. For instance, wDFAT expresses more NeuroD1 in basic treatment [Figure 15A] compared to the other cell types, yet in pre-inducing medium, bASCs for the first 13 days of induction exhibit the highest expression of NeuroD1. So even though wDFAT contains a higher population of CD90+ cells during initial conditions, bASCs, when cultured in “pre-inducing” medium, is able to generate more neuronal cells than the other cell types.

Our results indicate that each cell type is different and their stem cell population is malleable; different treatments affect the cell-lineage they differentiate into. For example, “pre-inducing” medium, i.e 20% serum replacement in DMEM, increases the neuroprogenitor potential of each cell type [Figure 9A, 10A, 12A].¹ Furthermore, the ability of our multipotent

stem cells to generate neural precursor cells was dependent on seeding density and environment, i.e coated plate.⁵ Without plating at a high density and with a coat (*Method 1 & 2*), our cells decreased in cell number and maintained a more progenitor-like state.

Neuronal and Glial Gene Expression:

After determining a suitable methodology for induction, we determined the presence of neural precursor cells, neuronal cells and one type of glial cell - astrocytes. Thus, we observed each cell type to generate astroglial-like cells as well as neurons. In regards to the neural precursor cells, the progeny of the cells were dependent on treatment. When cells were introduced to “pre-inducing medium” we observed significant increases in the expression of neural precursor genes, including Sox1, Sox2, and GFAP. Nestin expression did not increase during pre-inducing treatment, which could be due to high Sox1, a transcription factor that acts upstream of Nestin.¹⁴ Our results also suggest that pre-inducing medium may enhance differences between the cell types; bASCs and wASCs take on a higher propensity to generate neural precursor cells and mature neurons by the end of the treatment.

While both white and brown ASCs demonstrated a higher propensity to become neuroprogenitor cells and neuronal cells, we found the corresponding DFAT cells, especially bDFAT cells, in basic medium, i.e “spontaneously”, to express a significantly highly number of glial, s100B+, cells. Together, it suggest that brown-derived adipose tissue are a better source of neural precursor cells as it is able to generate enriched populations of neurons and glia, depending on whether DFAT or ASCs are cultured.

In Vitro Expression of Tuj1+ and s100B+ Cells:

Immunofluorescent results revealed a high expression of neuronal markers in both brown and white-derived ASCs treated with basic or neural induction medium. In the basic medium, wASCs generate clusters of bipolar Tuj1+ cells that appear to interact with one another. bASCs and wDFAT also generate bipolar Tuj1+ cells, although, bASCs contain s100B+ cells interacting with the Tuj1+ cells while wDFAT cells lacked s100B+ cells. In regards to wDFAT cells in neural treatment, it appears as though pre-inducing medium alters the potential of these cells, causing them to become more glial-like in nature. While wASCs, wDFAT and bASCs contain a high density of neuronal cells, bDFAT cells are almost exclusively glial, s100B+, cells by the end of treatment. Overall, our immunofluorescent results support the presence of neuronal and glial markers expressed in our qPCR results and further supports that bDFAT has an enriched potential to become glial-like cells.

Conclusion:

Our results suggest that neural treatment causes both white and brown ASCs to generate a population enriched of neuronal cells while the corresponding DFAT cells generate a population enriched of astroglial cells. If DFAT cells are restricted to a glial fate, these cells still have a high potential to be used in therapeutics. For example, many neurodegenerative diseases are influenced by damaged or mutated proteins within glia.²⁷ Therefore, DFAT cells, specifically s100B + bDFAT cells, could be injected in order to dilute the toxin action of mutant glia.²⁷ Basic treatment, on the other hand, appeared to alter the balance between neuronal cells and glial cells; brown-derived ASCs and DFAT contained a mixture of glial cells and neuronal cells while white-derived ASCs and DFAT cells appeared to be more neuronal in nature.

Although qPCR and immunofluorescent results exhibited varied neuronal or glial-potential within cell types, our qPCR results gave a precise quantitative measure of neuronal and glial expression, whereas our immunofluorescent results were semi-quantitative at best. Therefore, the results derived from immunofluorescence lend support to our qPCR results because they provide evidence of the presence of corresponding protein expression. To provide a more precise measure of the types and numbers of neural stem cell progeny, future studies could include FACS or Western Blot, depending on the concentration of cell protein.

In conclusion, wASCs, bASCs, wDFAT and bDFAT cells contain differential potential to become neurons and glia. From our results, brown adipose-derived multipotent stem cells appear to be a better heterogenous source of glia and neurons.

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