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A Novel Serum Free Primary Astrocyte Culture Method that Mimic Quiescent Astrocyte Phenotype

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Abstract

Background—Primary astrocyte cultures have been used for decades to study astrocyte functions in health and disease. The current primary astrocyte cultures are mostly maintained in serum-containing medium which produces astrocytes with a reactive phenotype as compared to *in vivo* quiescent astrocytes. The aim of this study was to establish a serum-free astrocyte culture medium that maintains primary astrocytes in a quiescent state.

New Method—Serum free astrocyte base medium (ABM) supplemented with basic fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) (ABM-FGF2-EGF) or serum supplemented DMEM (MD-10%FBS) was used to culture primary astrocytes isolated from cerebral cortex of postnatal day 1 C57BL/6 mice.

Results—Compared to astrocytes cultured in MD-10%FBS medium, astrocytes in ABM-FGF2-EGF had higher process bearing morphologies similar to *in vivo* astrocytes. Western blot, immunostaining, quantitative polymerase chain reaction and metabolic assays revealed that astrocytes maintained in ABM-FGF2-EGF had enhanced glycolytic metabolism, higher glycogen content, lower GFAP expression, increased glutamine synthase, and glutamate transporter-1 mRNA levels as compared to astrocytes cultured in MD-10% FBS medium.

Comparison to existing methods—These observations suggest that astrocytes cultured in ABM-FGF2-EGF media compared to the usual FBS media promote quiescent and biosynthetic phenotype similar to *in vivo* astrocytes.

Conclusion—This media provides a novel method for studying astrocytes functions in vitro under physiological and pathological conditions.

Keywords

Astrocyte; basic fibroblast growth factor; epidermal growth factors; fetal bovine serum; astrogliosis; primary culture

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1. Introduction.

Astrocytes constitute about 40% of mammalian brain cells outnumbering neurons (Baxter, 2012; Herculano-Houzel, 2009). Astrocytes have been long thought to play primarily passive support roles in the function of the central nervous system (CNS). However, decades of physiological and pathological research using various state of the art techniques and equipment have shown that astrocytes are cells with functional ion channels, receptors, and transporters that play pivotal roles critical for the development and function of the CNS. For instance astrocyte plays a crucial role in maintaining brain homeostasis, neuronal development, and survival by regulating ionic and water content of the interstitial space, uptake and recycling of neurotransmitters, and producing various neurotrophic factors, antioxidant molecules and reactive oxygen species (ROS) detoxifying molecules (Araque, Parpura, Sanzgiri, & Haydon, 1999; Belanger & Magistretti, 2009). Astrocytes are also critical for the formation, maintenance and function of synapses, blood flow regulation, blood brain barrier formation, as well as providing the energy needs for neurons (Attwell et al., 2010; Belanger, Allaman, & Magistretti, 2011; Chung, Allen, & Eroglu, 2015). Additionally, astrocytes are considered important components in the regulation and modulation of sleep, as well as learning and memory (Halassa et al., 2009; Henneberger, Papouin, Oliet, & Rusakov, 2010). It is therefore not surprising that astrocyte dysfunctions have been implicated in the onset and progression of many neurodegenerative disorders (Pekny et al., 2016).

In spite of the aforementioned advances, our understanding of the mechanisms and pathways regulating astrocytic dysfunctions and their roles in the initiation and progression of neurodegenerative diseases is still rudimentary. This is as a result of the complex interwoven nature of the different cells in the CNS. Understanding brain cells at the cellular and molecular levels is the cornerstone of modern neuroscience. The complexities of brain cells structure and function require unusual methods of *in vitro* culture to determine the function of brain cells and their interaction under physiological and pathological conditions devoid of the complex brain environment. Over the years, primary astrocytes, mostly prepared and maintained in fetal bovine serum (FBS)-containing medium according to the protocol developed by McCarthy and de Vellis in 1980, named as MD method (McCarthy & de Vellis, 1980) with minor modifications, have served as a useful tool in understanding many of the complex astrocytic functions in both health and disease. However, concerns have been raised as to whether these primary astrocyte cultures are reliable in accurately mirroring events *in vivo*, and whether such obtained findings can serve as a basis for effective therapeutic targeting of astrocytes in pathological conditions.

FBS is a complex medium supplement that contains unspecified growth factors, proteins and vitamins needed to promote cell survival and proliferation (Aswad, Jalabert, & Rome, 2016). Since FBS is collected from cattle, genetic diversity of a herd, seasonal and continental variation, and animal diet as well as manufacturing procedures make the complete composition difficult to determine and inter-vendor as well as inter-batch variability become challenging to control. Recently it has been found that FBS contains significant levels of lipopolysaccharides and extracellular vesicles (EV) which have been reported to alter cell biology and phenotype (Aswad et al., 2016; Kirikae et al., 1997; Shelke, Lasser, Gho, &

Lotvall, 2014). Astrocytes *in vivo* do not contact serum as many components of serum do not cross the blood brain barrier (BBB) except in certain pathological conditions. It was reported that astrocytes cultured in serum free media had a gene profile more representative of *in vivo* astrocytes as compared with those cultured in FBS containing media (Cahoy et al., 2008; Doyle et al., 2008; Foo et al., 2011). Additionally media composition has been reported to affect astrocyte culture purity and the expression of astrocytes specific proteins (Codeluppi et al., 2011; Foo et al., 2011). Due to the physical stress (e.g. shaking; done to remove other cell types) associated with the MD method of astrocyte preparation and the presence of FBS, it has been suggested that astrocytes obtained using this culture system are in the reactive state (Du et al., 2010). There is a need to purify and culture astrocytes under conditions that mimic the *in vivo* environment thus increasing translational value of the result derived from these cultures.

Efforts have been invested in the development of serum-free primary astrocyte culture systems, including a recent method combining negative immunopanning and a serum-free heparin-binding EGF-containing medium which has been demonstrated to resemble astrocytes *in vivo* (Foo, 2013; Foo et al., 2011; Zhang et al., 2016). However, the application of this method has been dramatically limited due to the labor-intensive, costly 5/6-step negative immunopanning of microglia, oligodendrocyte, endothelium, and neurons. (Foo et al., 2011). Additionally this method was not completely devoid of serum, as astrocytes were exposed to fetal calf serum (FCS) in the process of detaching purified astrocytes from the dishes (Foo, 2013; Foo et al., 2011; Zhang et al., 2016).

A reliable serum free astrocyte culture system that greatly promote the survival of astrocytes to the same degree as FBS containing medium as well as produce astrocytes with a phenotype closely resembling *in vivo* quiescent astrocytes will increase the translational value and reliability of the results obtained from primary cultures. Aside from transferrin, selenium, hormones, and other molecular components known to perform certain functions attributed to FBS in media (Fischer, Leutz, & Schachner, 1982; Obayashi, Tabunoki, Kim, & Satoh, 2009), various growth factors have been found to play critical role for astrocyte survival and proliferation *in vitro*. Heparin-binding epidermal growth factor (HBEGF) has been shown to promote astrocyte survival and proliferation and to affect cell differentiation and morphology in serum free culture conditions (Mayer, Rossler, Endo, Charnay, & Thiel, 2009; Puschmann et al., 2014). FGF2, first purified in bovine brain (Gospodarowicz, Lui, & Cheng, 1982), has been found to maintain astrocyte in nonreactive state. Similarly, FGF signaling delays astrogliosis and accelerates astrocytes deactivation after injury (Hizay et al., 2016; Kang et al., 2014; Menon & Landerholm, 1994). In the current study, we developed a serum free FGF2-EGF containing medium for primary astrocyte culture. We employed metabolic assays, morphological and gene expression analysis to compare the phenotype of primary astrocytes under FBS containing medium and serum free FGF2-EGF containing medium culture conditions. Our study demonstrated that serum free FGF2-EGF containing medium supports astrocyte growth while mimicking an *in vivo* quiescent astrocyte phenotype.

2. Materials and experimental methods

2.1. Materials and reagents

Cell culture dishes, plates, cell strainers were purchased from Greiner bio-one (USA) and Genesee scientific (USA). Micro cover glasses were purchased from VWR. Trypsin-EDTA, trypan blue, poly-L-Lysine solution, bovine serum albumin, transferrin, putrescine dihydrochloride, progesterone, sodium selenite, and N-Acetyl Cysteine were purchased from Sigma-Aldrich (USA). TrypLE, Dulbecco's Modified Eagle's medium (DMEM), Neurobasal medium, Penicillin-streptomycin, sodium pyruvate, Glutmax was purchased from Gibco/Life Technologies (USA). FBS was purchased from Atlanta Biologicals. Normal goat serum was purchased from Jackson Immunoresearch. Gold antifade mountant with DAPI and BD cytofix/cytoperm™ fixation and permeabilization solution were purchased from Invitrogen and BD respectively. Human FGF-basic and Human EGF were purchased from PeproTech (USA).

Primary antibodies against GFAP (monoclonal cell signaling), Vimentin (monoclonal Cell signaling), IBA-1, ALDH1L1 (polyclonal Abcam), AMPK α and AMPK β (monoclonal Cell signaling), GS and ACC (Monoclonal Cell signaling), mTOR (monoclonal Cell signaling), Actin and GAPDH (monoclonal Santa Cruz) and Alexa-fluor conjugated secondary antibodies from Invitrogen as well as non-conjugated secondary antibodies from Jackson laboratory were used for immunostaining or Western blot. PE anti-mouse/human CD11b Antibody, purchased from Biolegend and GFAP Monoclonal Antibody (GA5), Alexa Fluor 488, eBioscience™ were used to label astrocytes and microglial for flow cytometry. Information regarding primers used for quantitative Polymerase Chain Reaction (qPCR) to quantify mRNA expression of some astrocyte specific genes is listed in Table 1.

2.2. Culture media

Astrocytes cultures were prepared in differing media conditions, defined as follows. The serum containing media known as MD medium is composed of Dulbecco's Modified Eagle's medium (DMEM with 5.5 mM glucose, 4 mM L-glutamine, 1mM sodium pyruvate) containing 10% FBS and streptomycin (100 units/ml) - penicillin (100 μ g/ml). Serum free media defined as astrocyte based media (ABM) is composed of Neurobasal medium and DMEM (1:1 v/v) supplemented with 5.5 mM glucose, Penicillin (100 units/ml)-streptomycin (100 μ g/ml), 1 mM sodium pyruvate, Glutmax, bovine serum albumin (100 μ g/ml), transferrin (100 μ g/ml), putrescine dihydrochloride (16 μ g/ml), progesterone (60 ng/ml), sodium selenite (40 ng/ml), and N-Acetyl Cysteine (5 mg/ml). ABM was supplemented with 2, 5 or 10 ng/ml of Human FGF-basic, Human EGF, or a combination of both.

2.3. Primary astrocyte culture

All procedures for primary astrocyte cultures were approved by the Institutional Animal Care and Use Committee of the university of North Texas Health Science Center (UNTHSC). Primary astrocytes were prepared according to previous published methods with minor modifications (McCarthy & de Vellis, 1980; Roy Choudhury et al., 2015). Briefly, one day old pups were anesthetized by hypothermia followed by decapitation with sharp surgical scissors. Cerebral cortices were dissected and meninges were removed under

aseptic conditions. Cortical tissue was digested in TrypLE, at 37°C for 15 minutes. Cell suspension was prepared by repeated pipetting of digested cortical tissue through different bore sized Pasteur pipettes. The cell suspension was strained through 40 µM size cell strainers and cells were counted with a hemocytometer using trypan blue staining. Cells were then seeded into plates coated with poly-L-lysine in MD (10% FBS) or ABM supplemented with different growth factors referred to as ABM-FGF2, ABMEGF, ABM-FGF2-EGF for 15 minutes. Media was replaced to get rid of debris in a process referred to as variable cell attachment rate. The cells were then cultured in a humidified incubator at 37°C with 5% CO₂. Half of the media was changed every 3 days and when the cells became 90% confluent, the plates were constantly shaken for 24 hours in a CO₂ incubator at 37°C to eliminate microglia. The cells were split into new plates, incubated for 2–3 days before use, and generally used within 2 weeks at passage 1.

2.4. Cell viability assay and apoptosis analysis

Cell viability was determined by Calcein AM and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay at day 2 after primary astrocytes were prepared in different media conditions. For the Calcein AM assay, 40,000 cells were seeded in poly-L-lysine coated 12-well plates in different media conditions on the day of primary astrocyte preparation and incubated at 37°C with 5% CO₂. On day 2 of culturing, media was removed and replaced with a 1 µM solution of Calcein AM in PBS. Cells were incubated for 5 minutes at 37°C and fluorescence was measured using a Tecan Infinite F200 plate reader (excitation 485 emission 530). For the MTT assay, plates were removed from the incubator and 20 µl MTT (5 mg/ml in PBS) was added per well. The plates were agitated gently to mix the MTT into the media and then returned to the incubator for 2 hours. After 2 hours the media was removed and 100 µl of DMSO was added to each well. The plate was mixed by gentle agitation and the absorbance was measured (560 nm with a reference of 670 nm) with a Tecan Infinite F200 plate reader. For flow cytometry analysis, primary astrocytes were cultured in various media and grow to confluence. The cells were then collected and seeded in 6 well plates. On the 7th day the cells were stained with annexin-V and propidium iodide and flow cytometry analysis was performed (BD LSR II, San Jose, CA, USA).

2.5. Immunostaining of astrocytes

Cells were seeded at 3×10^4 cells/well in 24-well plates on poly-L-lysine coated coverslips in serum free ABM-FGF2-EGF or MD medium containing 10% FBS. When confluent, cells were fixed with cytofix overnight at 4°C. Cells were incubated with goat serum blocking solution and 0.2 % triton -X 100 for 1 hour. After 3 times washing with PBS, cells were incubated overnight with primary antibodies at 4°C. Cells were then washed and incubated with FITC-labelled secondary antibodies for 1 hour at room temperature and stained with DAPI. Images were obtained with a confocal microscope (Olympus fluoview FV1200). The purity of astrocytes was calculated by the percentage of cells both stained with ALDH1L1 and DAPI to cells stained with DAPI only or Iba-1, using 5 random views per slides. The cell's morphology and dimensional structure were analyzed using Imaris (bitplane) and FIJI-ImageJ as previously described (Tavares et al., 2017).

For Flow cytometry to determine culture purity, astrocytes cultured in the different media conditions were trypsinized and collected. Cells were incubated in 2% PFA for 15 minutes and permeabilized using 0.1% triton X 100 for 10 minutes. Supernatant was discarded after the cells were centrifuged. The cells were incubated by resuspension in Magnetic-Activated Cell Sorting (MACS) buffer with fluorochrome conjugated anti-CD11b and anti-GFAP antibodies for 1 hour. Cells were centrifuged again and supernatant removed. MACS buffer was used to re-suspend cells for analysis via flow cytometry (BD LSR II, San Jose, CA, USA).

2.6. Growth curve and cell cycle assay

Astrocytes were seeded at a density of 40,000 cells/well in 12-well plates or 25,000 cells per well in 24-well plates with different culture medium. At the indicated days after seeding, cells were harvested using trypsin-EDTA and counted using a hemocytometer. Four wells were assigned to each group and cell counting was conducted by a researcher who was blinded to the group assignment using an inverted phase contrast Zeiss Invertoskop microscope.

For cell cycle analysis astrocytes were seeded at a density of 50,000 cells/well in 12-well plates in various culture media. On day 7 after culture, cells were harvested using trypsin-EDTA and washed with buffer (PBS) twice to remove trypsin. Cells were fixed in ice-cold 70% ethanol for 24 hours at 4°C. Then, cells were incubated with propidium iodide (PI) (40 µg/ml) and RNase (10 µg/ml) for 30 minutes at 37°C. The stained cells were analyzed using a Beckman Coulter FC500 Flow Cytometry Analyzer for quantification of cell cycle distribution (G1, S or G2/M).

2.7. Metabolic assays.

2.7.1. ATP assay—Total cellular ATP levels were determined using an ATP kit (Invitrogen) as previously described in (Roy Choudhury et al., 2015). Astrocytes were seeded in 12-well plates at a density of 10×10^4 cells/well under different culture conditions and grew for 14 days. On the day of the experiment cells were treated with oligomycin for 2 hours. The cells were then detached with trypsin (Sigma-Aldrich, St Louis, MO), washed with PBS twice and lysed with ATP assay buffer (500 mM Tricine buffer, pH 7.8, 100 mM 100 mM MgSO₄, 2 mM EDTA, and 2 mM sodium azide) containing 1% Triton X-100. The ATP reaction buffer (30 µg/ml D-luciferin, 20 µM DTT, and 25 µg/ml luciferase) was added to each 10-µl cell lysate in white 96-well plates (in triplicate) along with an ATP standard. Luminescence was measured using a Tecan Infinite F200 plate reader. Protein concentration was measured simultaneously using the Pierce 660 nm Protein Assay (660 nm absorbance). ATP values were determined from a standard curve and normalized to the protein content of each sample.

2.7.2. Glycogen and lactate assays—Total glycogen level and amount of lactate produced by astrocytes cultured under various conditions was determined using a glycogen and lactate assay kit following the manufacturer's instructions (Sigma-Aldrich, St Louis, MO). Glycogen levels and lactate concentrations were normalized to the protein

concentration of the samples determined by colorimetric analysis using the Pierce 660 nm Protein assay reagent (Thermo Scientific) and a Tecan Infinite M200 plate reader.

2.7.3. Hexokinase activity—Flex station (Molecular Devices) was used to measure hexokinase (HEK) activity as previously described (Roy Choudhury et al., 2015). Astrocytes were seeded in 12-well plates at a density of 10×10^4 cells/well under different culture conditions and grew for 14 days. On the day of the experiment, cells were lysed in RIPA buffer (50 mM of Tris. HCL, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X). In a 96 well plate, 10 μ l of cell lysate was added to 120 μ l of Tris $MgCl_2$ buffer (0.05 M Tris*HCl buffer, pH 8.0 with 13.3 mM $MgCl_2$), 50 μ l of glucose (0.67 M) and 10 μ l each of ATP (16.5 mM), NAD (6.8 mM) and glucose-6-phosphate dehydrogenase (300 IU/ml). The change in absorbance was determined at 340 nm. Enzyme activities were expressed as units per milligram of protein after the protein concentration of each sample was measured.

2.7.4. Extracellular flux analysis—Oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and cell metabolic potential was monitored using Agilent Seahorse XFe96 analyzer. Astrocytes were seeded at a density of 15,000 cells/well in 96-well Seahorse XF cell culture microplates under different culture conditions for 7 days. One day prior to the experiments a sensor cartridge was hydrated in Seahorse calibrant in a non- CO_2 incubator at $37^\circ C$ overnight. One hour before the experiment, assay medium was prepared by supplementing Seahorse XF base medium with 1 mM pyruvate, 2 mM glutamine and 5.5 mM glucose. Assay medium was warmed and adjusted to pH 7.4 with 0.1 N NaOH. For the Mito stress test, oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and rotenone/antimycin were diluted in XF base medium and loaded into the accompanying cartridge port to achieve a final concentration of 1 μ M, 1 μ M and 0.5 μ M, respectively. For cell energy phenotype determination, oligomycin and FCCP were diluted in XF medium together to achieve a final concentration of 1 μ M for both oligomycin and FCCP, which was loaded into one port. Various metabolic parameters were monitored with each cycle set as mix for 3 minutes, delay for 2 minutes and then measure for 3 minutes. Values were normalized to the cell number of each well determined by Calcein AM assay. Using wave software, metabolic potential was calculated by dividing stressed OCR and ECAR by baseline OCR and ECAR respectively.

2.7.5. Glucose uptake—Using glucose analog 2-NBDG, glucose uptake in astrocytes was determined as previously described (Lin et al., 2012). Astrocytes were cultured in 96-well culture plates (10,000 cells/well) under different culture conditions. Cells were washed twice and incubated in glucose-free Krebs Ringer HEPES (KRH) buffer (129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES; pH 7.4) for 30 minutes on the day of the experiment. The astrocytes were then incubated in glucose free KRH buffer containing 100 μ M of 2-NBDG for 5 minutes. Glucose uptake was determined using a Tecan Infinite M200 plate reader (Excitation/Emission for 2-NBDG ~465/540 nm).

2.8. Western blot and real time qPCR analysis

Astrocytes were seeded at a density of 400,000 cells/well in 6-well plates in different media conditions and grew to confluency for 7 days. For induction of astrocyte activation, 10 ng/ml TGF- β 1 was added to cells for 3 days or 100 ng/ml LPS for 15 hours. The cells were collected and lysed in RIPA buffer (50 mM of Tris, HCL, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X) with protease and phosphatase inhibitors (1:100). Protein assay reagent Pierce 660 nm (Thermo Scientific) was used to determine the protein content of the samples. The samples were resolved on SDS gel and transferred to a nitrocellulose membrane. The membranes were incubated overnight in primary antibodies against GFAP, Aldh1L1, vimentin, AMPK α and AMPK β , glycogen synthase; GS and ACC, mTOR, Actin and GAPDH followed by secondary antibody. Using Biospectrum 500 UVP imaging system, chemiluminescence signal was detected and normalized to actin.

For RNA extraction and real time qPCR, total RNAs were extracted using PicoPure® RNA Isolation Kit following the manufacturer's manual (Invitrogen). Complement DNA synthesis was performed using SuperScript® III First-Strand Synthesis System according to the manufacturer's instructions (Invitrogen). Quantitative PCR was performed using Fast SYBR® Green Master Mix (Invitrogen) on a 7300 Real-Time PCR System (Invitrogen). Data were analyzed with 7300 system software and 2^{-C_t} method was used to calculate gene expression.

2.9. Glutamate clearance/uptake assay

Primary astrocytes were plated in 24-well tissue culture plates at a density of 10×10^4 cells/well and allowed to grow confluent for one week in different media condition. On the day of the experiment, glutamate (400 μ M) was dissolved in phenol-free astrocyte medium and added into each well. Clearance was assayed at 4 and 8 hr. The assay was performed and analyzed according to manufacturer's guidelines (Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit, Life Technologies).

2.10. Scratch/migration assay

Astrocytes were seeded at a density of 100,000 cells/well in 12-well plate in different media conditions. Using a sterile 200 μ l pipette tip, straight scratches were made on the cell layer, simulating a wound. The cells were then washed with PBS to remove cell debris and replaced with fresh media. At different time points after scratch, cells were stained with Calcein AM (10 μ M), and fluorescent images taken randomly with a Zeiss fluorescent Microscope.

2.11. Astrocyte calcium signaling

Astrocytes were seeded at a density of 20,000 cells/well in poly-L-lysine coated black-walled 96-well plate and grew confluent for a few days. Cells were washed and incubated with 10 μ M Fluo-4 AM (Invitrogen F14201) for 45 minutes. Fluo-4 AM fluorescence was measured before and, after stimulating the cells with 0.1 mM glutamate, 50 μ M Adenosine and 100 μ M ATP. Calcium concentration were measured with Flex station 3 (Molecular Devices, Sunnyvale, CA) for 600 seconds. The baseline calcium signal was measured for 30 seconds followed by injection of stimulant.

2.12. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 7. Results are expressed as mean $\pm$ standard error mean (SEM). When comparing two groups a t-test was used to identify any significant differences. Significant difference among groups with one independent variable was determined by one-way ANOVA with a Turkey's multiple comparisons test for planned comparisons between groups when significance was detected. For comparison of groups with two independent variables, two-way ANOVA was used and post-hoc Bonferroni analysis was conducted for planned comparisons between groups when significance was detected. $P < 0.05$ was considered statistically significant.

3. Results

3.1. FGF2 and EGF collectively support survival and growth of primary astrocytes in serum free media.

The effect of the serum free media and the various concentrations of growth factors to promote survival and growth of astrocytes prepared and seeded using the variable cell attachment rate method was assessed (Fig. 1A). At 48 hours after seeding the cells, in the absence of growth factors, the ABM only promoted 12% cell survival. We also found that 2, 5, and 10 ng/ml FGF2 increased the survival rate to $19.14\% \pm 2.03$, $29.714\% \pm 2.52$, and 38.57 ± 2.467 , respectively, in serum free ABM as compared to $72.00\% \pm 3.901$ survival rate of astrocytes cultured in MD (10% FBS) medium. Similarly, 2, 5, and 10 ng/ml EGF increased the survival rate to $25.34\% \pm 2.95$, $40.6\% \pm 3.22$, and 47 ± 3.40 , respectively, in serum free ABM (Fig. 1B). Furthermore, a combined 2, 5 and 10 ng/ml FGF2 and EGF was more effective in promoting astrocyte survival as compared to each growth factor by increasing the survival rate to $38.12\% \pm 3.5$, $57.12\% \pm 3.52$, and $56.57\% \pm 3.45$, respectively, in the serum free ABM. No statistical significant difference in cell survival was observed between 5 and 10 ng/ml FGF2-EGF and MD (10% FBS) culture conditions (Fig. 1B). When the effect of 5 ng/ml FGF2, 5 ng/ml EGF, or 5 ng/ml ABM-FGF2-EGF together was assessed for growth rate of astrocytes cultured in different medium for 2 weeks, we observed that cells grown in ABM-FGF2-EGF proliferated faster than those grown in ABM-EGF, or ABM-FGF2 (Fig. 1C). When the growth rate of astrocytes in ABM culture conditions was compared to MD (10% FBS) for 7 days, we found that the growth rate in serum containing MD medium was higher than those in ABMFGF2 and ABM-EGF conditions but not ABM-FGF2-EGF. ($*p < 0.05$, $***p < 0.001$ day 7 comparison) (Fig. 1C). Astrocytes were seeded at various densities in MD (10% FBS) and ABMFGF2-EGF to assess if the seeding density has any effect on our growth assay. We observed no significant difference in the growth rate of astrocyte seeded in the two media conditions after 4 days in culture (Fig 1D).

We speculated that the decreased viability in different conditions could be as a result of reduced cell proliferation or increase cell death. Flow cytometry analysis of cells stained with Annexin V and PI after 7 days in the various culture conditions demonstrated that 14.2%, 18.7%, and 8.466% of ABM-FGF2, ABM-EGF, ABM-FGF2-EGF cultured astrocytes, respectively, were in their early apoptotic stages whiles 3.50%, 1.12%, and 1.53% of ABM-FGF2, ABMEGF, ABM-FGF2-EGF cultured astrocytes, respectively, were

in their late apoptotic stages (Fig. 1E). Additionally, 25%, 14%, and 12% of astrocytes seeded in ABM-FGF2, ABM-EGF, and ABM-FGF2-EGF, respectively, were in their necrotic stages of cell death. Comparatively, 14.4%, 0.65%, and 5.03% astrocytes cultured in MD (10% FBS) condition were in their early, late and necrotic apoptotic stages, respectively (Fig 1E).

Cell cycle distribution of astrocytes grown in serum free ABM-FGF2, ABM-EGF, ABMFGF2-EGF, and MD (10% FBS) demonstrated a significant increase in the percentage of cells in the G2/M phase of cells grown in ABM-FGF2, ABM-EGF with proportional decrease of cell number in G0/G1 phase at day 7 after culture (Fig 1F). Among the 4 culture conditions, the proportion of cells in the S phase was higher in cells cultured in ABM-FGF2 (Fig 1F). A notable observation was that after day 6 in culture, the growth rates of astrocytes in serum free medium supplemented with growth factors began to decline. We speculated that this could be as a result of decreased growth factors concentration to provide trophic support to the cells. Hence it is necessary to replace half of the media every 3 days (Fig. 1C).

3.2. Astrocyte cultures in ABM-FGF2-EGF mimic quiescent astrocyte morphology with high purity.

Using FIJI-ImageJ with simple neurite tracer (SNT) plugin, an open source software that aids in reconstructing the structure of astrocytes as previously described (Tavares et al., 2017), morphological analysis indicated that primary astrocytes cultured in serum free ABM-FGF2-EGF medium have more branching processes as compared to astrocytes cultured in MD medium (Fig. 2A). Immunostaining of astrocytes cultured for 2 weeks showed decreased GFAP expression and smaller cell size of primary astrocytes cultured in ABM-FGF2-EGF conditions as compared to MD (10% FBS) cultured astrocytes (Fig. 2B). When the purity of both cultures were compared after 2 weeks by flow cytometry of CD11b and GFAP staining, we observed that 97% and 92% of cells were GFAP positive in the primary astrocytes cultured in the serum free ABM-FGF2-EGF medium and MD (10% FBS) medium, respectively whereas 2% and 7% of the cells cultured in serum free ABM-FGF2-EGF and MD (10% FBS) medium respectively stained positively for CD11b (Fig. 2C). Immunostaining of astrocytes using ALDH1L1, neuronal marker Tuj1, and Iba1 revealed a similar pattern of purity, with most cells staining positive for the astroglial marker ALDH1L1 (Supplementary Fig. 1).

3.3. TGF- β 1 activates primary astrocytes cultured in serum free ABM FGF2/EGF medium.

Astrogliosis defined as changes in astrocytes in response to all forms of CNS injury and disease is characterized by cellular hypertrophy and upregulation of intermediated filament proteins such as GFAP (Kang et al., 2014; Sofroniew, 2009). We examined reactive astrogliosis markers in primary astrocytes cultured in different conditions using Western blot, PCR, and immunostaining. There was a significant decrease in GFAP expression in primary astrocytes cultured in ABM supplemented with either FGF2, EGF or a combination of FGF2 and EGF, as compared to astrocytes cultured in MD media with 10% FBS (Fig. 3A). Similarly, ABM-FGF2 and ABM-FGF2-EGF cultured astrocytes, but not ABM-EGF cultured astrocytes, had decreased vimentin expression as compared to astrocytes cultured in MD media with 10% FBS (Fig. 3A). When growth factors were withdrawn for 2 days before

the experiment, astrocytes cultured in ABM without growth factors have GFAP and vimentin expression levels similar to that of MD cultured astrocytes, indicating the key role of the EGF-FGF2 in maintaining primary astrocytes in their quiescent state (Fig. 3A). Consistently, Real time PCR of GFAP and vimentin in astrocytes cultured under different conditions showed a significant decrease in GFAP and vimentin expression only in astrocytes maintained in ABM-FGF2 and ABM-FGF2-EGF, but not ABM-EGF as compared to MD (10% FBS) (Fig. 3B).

TGF- β 1 has been known to induce astrocyte activation with an increase of GFAP expression (Reilly, Maher, & Kumari, 1998). Our quantitative PCR analysis indicated that TGF- β 1 treatment (10 ng/ml for 3 days) significantly increased the expression of GFAP in primary astrocytes cultured in ABM-FGF2-EGF and MD (10% FBS) medium as compared to the non-treatment groups (Fig. 3C). Consistently, GFAP and vimentin protein levels were increased in both culture conditions after insult (Fig. 3D). Similarly LPS treatment significantly increased the expression of IL-6, IL-1 β , TNF α and LCN-2; genes that are involved in inflammatory or immune responses. Astrogliosis is involved in wound healing after injury. Wound healing assay demonstrated that scratch induced primary astrocytes activation in primary astrocytes cultured in serum free ABM-FGF2-EGF medium, although the wound healing was slower than those cultured in MD (10% FBS) condition (Fig. 4A).

Increased calcium concentrations are associated with reactive astrogliosis (Fiacco & McCarthy, 2006; Scemes & Giaume, 2006). Using Fluo-4 AM we investigated the calcium waves in primary astrocytes cultured in different conditions. We observed that primary astrocytes responded to the entire stimulus in both media. Higher basal level of calcium was observed in serum containing MD medium cultured astrocytes. Nonetheless, stimulation by adenosine, ATP, and glutamate increased calcium concentration in primary astrocytes cultured in serum containing MD medium and ABM-FGF2-EGF medium (Figure. 4B).

3.4. Astrocytes cultured in serum free ABM-FGF2-EGF medium have a biosynthetic phenotype.

The effects of culture conditions on astrocyte metabolism were examined using Seahorse XFe 96 analyzer. We determined OCR and ECAR before and after injecting oligomycin, FCCP, and rotenone/antimycin A. We observed a significant increase in basal (~19%) and maximal respiration (~35%) as well as ATP production (~13%) but no significant difference in proton leak and non-mitochondrial oxygen consumption (non-MOC) in ABM-FGF2-EGF and MD (10% FBS) cultured astrocytes (Fig. 5A). Similarly, a higher baseline and stressed ECAR was observed in ABM-FGF2-EGF cultured astrocytes (Fig. 5B). Assessment of cell energy phenotype indicated a more glycolytic phenotype of the ABM-FGF2-EGF cultured astrocytes as compared with FBS-containing MD medium cultured astrocytes (Fig. 5C). We measured lactate production in astrocytes cultured under different conditions. Consistent to the glycolytic phenotype, there was a 20% increase in lactate production in ABM-FGF2-EGF cultured primary astrocytes as compared to MD medium cultured astrocytes (Fig. 5D). Our result also showed no significant difference in hexokinase activity in astrocytes cultured in both conditions (Fig. 5E). However, using PCR, we observed that the mRNA levels of

HEK-1 and MCT-1 were significantly increased in serum free ABM-FGF2-EGF cultured primary astrocytes as compared with MD medium cultured astrocytes (Fig. 5F).

ATP is generated by both glycolysis and mitochondrial phosphorylation in astrocytes. We observed a high ATP level in ABM-FGF2-EGF cultured primary astrocytes as compared to MD cultured astrocytes (Fig. 6A). We speculated that the increase the higher ATP levels observed in ABM-FGF2-EGF cultured astrocytes was likely attributed to glycolysis. To investigate this, oligomycin (ATP synthase inhibitor) was added 2 hours prior to ATP assay to inhibit oxidative phosphorylation. As expected, even with the inhibition of oxidative phosphorylation we observed 24% and 54% ATP content in MD and serum free ABM-FGF2-EGF cultured primary astrocytes, respectively, indicating that glycolysis contributed to the increase ATP production in serum free ABM-FGF2-EGF cultured astrocytes. Furthermore, glycogen content, high molecular weight glucose polymer which serve as energy reserve in astrocytes, was higher in serum free ABM-FGF2-EGF cultured primary astrocytes than in MD cultured astrocytes (Fig. 6B)

AMP-Activated protein kinase pathway (AMPK) is an important regulator of cellular energy homeostasis with predominate expression in neuron in the brain. We observed a decrease in expression and phosphorylation of AMPK α in ABM-FGF2-EGF cultured primary astrocytes as compared to MD cultured astrocytes while increase mTOR phosphorylation was observed in ABM-FGF2-EGF cultured astrocytes (Fig. 7A). Consistently, a significant decrease of ACC and GS phosphorylation, downstream of AMPK activation, was observed in ABM-FGF2-EGF cultured primary astrocytes as compared to MD cultured astrocytes (Fig. 7B). These data indicated that primary astrocyte cultured in serum free ABM-FGF2-EGF medium had a biosynthetic phenotype as compared with serum-containing MD medium cultured astrocytes.

3.5. The mRNA expression levels of astrocytes cultured in serum free ABM-FGF2-EGF condition depicts resting astrocyte phenotype.

The mRNA expression of genes commonly associated with astrocyte functions was assessed by quantitative PCR. No significant difference in S100 β (Fig. 8A) and GLAST (Fig. 8B) mRNA levels were observed in primary astrocytes cultured in ABM-FGF2-EGF and MD culture media. On the other hand, higher mRNA levels of GLT-1 (Fig. 8C), GS (Fig. 8D), GLUT-1 (Fig. 8E) were observed in primary astrocytes cultured in ABM-FGF2-EGF for 14 days as compared to MD cultured astrocytes. A decreased mRNA level of CXN-43 in primary astrocytes cultured in ABM-FGF2-EGF medium was observed as compared to serum containing MD cultured astrocytes (Fig. 8F). Comparing the mRNA expression of other important astrocytic factors, we observed no significant difference in the levels of AQP4 (Fig. 8G) and Kir4.1 (Fig. 8H) between primary astrocytes cultured in serum free ABM-FGF2-EGF medium and astrocytes cultured in serum containing MD media.

Astrocytes are involved in the uptake and clearance of neurotransmitters at the synaptic cleft. Using an assay kit to measure the amount of glutamate present in the media after the addition of 400 μ M glutamate for 4 and 8 hours. We observed a significantly higher rate of glutamate clearance by ABM-FGF2-EGF cultured (70.17 ± 3.962) astrocytes at 8, but not 4, hours as compared to MD cultured astrocytes (10% FBS) (53 ± 4.926) (Fig. 8I). Higher

glucose uptake was also observed in serum free ABM-FGF2-EGF cultured primary astrocytes as compared to MD (10% FBS) astrocytes (Fig. 8J).

4. Discussion

In vitro systems allowing maintenance and experimentation on primary astrocyte cultures have been in use for decades, which are essential for understanding astrocyte functions in health and to mimic various pathological conditions. The use of a serum containing media makes astrocytes obtained via this method exhibit a flat and fibroblast like morphology (Foo et al., 2011; Puschmann et al., 2014; Zhang et al., 2016). Additionally the use of FBS in the medium makes it difficult to study the effects of certain compounds and drugs on astrocytes *in vitro*, as serum components may antagonize or potentiate the actions of certain agents under investigation (Barnes & Sato, 1980). Astrocytes obtained by this method also have gene profiles significantly different from astrocytes *in vivo* (Cahoy et al., 2008; Doyle et al., 2008). Correcting the limitations of this astrocyte culture method and producing astrocytes which closely resemble *in vivo* astrocytes will ensure results obtained using primary culture can be attributed to events occurring *in vivo*.

Culture media are to ensure cell growth and proliferation. We report that our serum free media promotes proliferation of astrocytes similar to astrocytes cultured in FBS containing medium. Consistent with recently established serum free primary culture methods (Foo et al., 2011; Puschmann et al., 2014; Zhang et al., 2016), we observed that the morphology of astrocytes cultured in the serum free ABM culture condition was similar to *in vivo* resting astrocytes with a greater degree of branching.

Studies have indicated that different sera leads to different degrees of microglia contaminations as serum components may affect proliferation of microglia and hence astrocytes growth (Codeluppi et al., 2011). Our current study used a serum free-ABM-FGF2-EGF culture condition with a variable cell attachment rate method to produce a culture of high purity. Similar to earlier studies conducted by (Foo et al., 2011) using immunopanning to directly isolate and culture astrocytes in a serum free medium, astrocytes cultured in our serum free ABM-FGF2-EGF medium have similar degree of purity.

Glucose metabolism in the brain occurs in a compartmentalized way. Astrocytes are the primary contributors to glycolysis, the cytosolic and anaerobic arm of glucose metabolism, while neurons are more responsible for oxidative metabolism (Belanger et al., 2011). Energy metabolism of astrocytes is coupled to neurons as the end product of glucose metabolism in astrocytes, lactate, is released into the extracellular space through (Monocarboxylate transporters) MCT1 and taken up by neurons through MCT2 to be used for oxidative metabolism to generate ATP (Falkowska et al., 2015). Our study indicates that astrocytes cultured in serum free media have increased mitochondrial respiration, enhanced glycolysis, increased lactate levels, and ATP content compared to astrocytes in serum containing media. We speculated that the increase of ATP and lactate production as well as the enhanced glycolysis could be due to increased expression of HK1, MCT1, GLUT-1, and increased glucose uptake observed in serum free ABM-FGF2-EGF astrocytes.

AMPK is an important regulator of multiple metabolic pathways. AMPK activation stimulates catabolism and concomitantly inhibits anabolism (Hardie, 2011). Given the increase ATP and glycogen content of primary astrocytes cultured in the serum free ABM-FGF2-EGF condition, it might not be a surprise that our serum free culture condition will impact AMPK signaling. In the brain, AMPK are predominately localized and activated in neurons. We observed that primary astrocytes cultured in serum free ABM-FGF2-EGF condition have a decreased AMPK activation as compared to astrocytes cultured in MD media evidenced by lower AMPK phosphorylation. Consistent with the lower AMPK activation in primary astrocytes cultured in serum free medium, we observed a concomitant increase anabolism evidenced by decrease phosphorylation and activation of acetyl CoA carboxylase (ACC) and glycogen synthase. Consistently, serum free media could produce astrocytes with a biosynthetic or anabolic phenotype similar to resting *in vivo* astrocytes.

It has been previously reported that astrocytes cultured in serum containing media have higher GFAP levels (Du et al., 2010). Studies have shown that astrocytes express several FGF2 receptors and FGF2 signaling maintains astrocytes in their resting state (Kang et al., 2014; Reilly et al., 1998). Consistently, we observed a decrease of GFAP and vimentin levels in primary astrocytes cultured in serum free FGF2-EGF medium as compared with astrocytes cultured in FBS containing medium, indicating that primary astrocytes cultured in the serum free FGF2-EGF media are in their resting state. Nevertheless, reactive astrogliosis could be induced in primary astrocytes cultured in serum free FGF2-EGF containing medium by pro-inflammatory cytokine and scratch injury (Okada, Hara, Kobayakawa, Matsumoto, & Nakashima, 2018).

Calcium waves can be stimulated in astrocytes by several stimuli including ATP, adenosine, glutamate, and KCl (Foo et al., 2011; Kimelberg et al., 1997). The increased calcium levels observed in the absence of stimuli in serum containing MD media was similar to what was reported in previous study (Foo et al., 2011). Increased calcium waves has been found to be associated with reactive astrocytes or astrocytes in pathological conditions (Fiacco & McCarthy, 2006; Scemes & Giaume, 2006). Consistently, primary astrocytes cultured in serum free ABMFGF2-EGF medium has lower calcium as compared with astrocytes cultured in serum containing MD medium. However, stimulation of adenosine, ATP, and glutamate increased calcium concentration in primary astrocytes cultured in serum containing MD medium and ABM-FGF2-EGF medium.

Astrocytes play a critical role in the uptake and metabolism of glutamate thereby regulating glutamate level at the synaptic cleft via glutamate transporters expressed at presynaptic astrocytic processes (Tani et al., 2014). Glutamate taken up by astrocytes can serve as a transmitter precursor for the synthesis of the inhibitory amino acid GABA via its conversion to glutamine by glutamine synthase (Tani et al., 2014). In various neurodegenerative disorders characterized by astrogliosis, this important astrocytic function is lost leading to glutamate induced neurotoxic death (Pekny et al., 2016). Additionally activated astrocytes both *in vivo* and *in vitro*, have also been shown to have decreased GLT-1 expression making them less efficient in clearing glutamate, while quiescent astrocytes express higher GLT-1 (Hughes, Maguire, McMinn, Scholz, & Sutherland, 2004; Tawfik et al., 2006). In our study we observed that primary astrocytes cultured in serum free ABM-FGF2-EGF medium have

higher GLT-1 mRNA expression levels as compared to astrocytes cultured in FBS containing MD medium. Consistently, an increased clearance of glutamate as well as an increase in GS mRNA expression were observed in primary astrocytes cultured in the serum free medium ABM-FGF2-EGF medium as compared with FBS containing MD medium. One other astrocytic protein, whose expression is increased in reactive astrocytes and various diseases as well as in injuries, is CNX-43 (Cronin, Anderson, Cook, Green, & Becker, 2008; Koulakoff, Mei, Orellana, Saez, & Giaume, 2012; Lee, Lindqvist, Kiehn, Widenfalk, & Olson, 2005). We found a decrease level of CNX-43 mRNA levels in astrocytes cultured in serum free medium. Interestingly there have been conflicting reports on the regulation of astrocyte glutamate transporters by CNX 43 levels (Figiel, Allritz, Lehmann, & Engele, 2007; Unger, Bette, Zhang, Theis, & Engele, 2012). Our findings are consistent with recent data that showed decreased levels of connexin 43 regulates the increase in the expression of astrocyte glutamate transporters (Unger et al., 2012). Taken together, the increased GLT-1 and CNX-43 expression and glutamate clearance provided additional evidence that our serum free ABM-FGF2-EGF medium maintains astrocytes in their quiescent state. Indeed, it has been indicated that both FGF2 and EGF reduced CNX-43 expression in astrocytes (Reuss, Dermietzel, & Unsicker, 1998; Ueki et al., 2001) and that EGF induce GLT-1 expression (Zelenaia et al., 2000).

5. Conclusion

We demonstrated that the novel serum free ABM-FGF2-EGF medium supports astrocytes growth and enhanced glycolytic metabolism with higher glycogen content, lower GFAP and vimentin expression, and increased glutamate transporter mRNA levels as compared to astrocytes cultured in the MD-10% FBS medium. Our study suggests that our serum free culture method produces quiescent astrocytes with a biosynthetic phenotype and morphology similar to *in vivo* resting astrocytes. Additionally ABM- FGF2-EGF cultured primary astrocytes could be activated by various pathological conditions. Thus our developed serum-free and EGF/FGF2-containing astrocyte basal medium will provide a critical tool for defining the precise function of astrocytes under physiological and pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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6. References

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Highlights

- Astrocytes cultured in fetal bovine serum containing medium have reactive phenotype.
- FGF2 and EGF synergistically promote astrocytes growth in our serum free medium.
- A Serum free FGF2-EGF medium promotes quiescent phenotype in astrocytes cultures.
- Astrocytes in our FGF2-EGF medium have biosynthetic phenotype as *in vivo* astrocytes.
- Astrocytes in FGF2-EGF medium had higher process morphology as *in vivo* astrocytes.

Figure 1. FGF2 and EGF synergistically support the survival and growth of primary astrocytes in serum free media.

(A) Schematic diagram depicts the variable cell attachment experimental procedure of astrocyte culture (B) Viability assay done after seeding 40,000 cells in ABM (supplemented with different amount of growth factors) and MD (10% FBS) for 48 hours. The Y axis represent % of viable cells determined from the seeded number (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs MD (10% FBS)), (# $p < 0.05$, ### $p < 0.001$ vs ABM (no growth factor) $n = 4$). (C) Growth curve assay of primary astrocytes cultured in different medium for 2 weeks. 40,000 cells were seeded in 12-well plates and cells counted daily for 7 days (* $p < 0.05$, *** $p < 0.001$ vs MD (10% FBS) $n = 6$) (D) Different numbers of cells were seeded in 12-well

plate and growth was analyzed by Calcein AM at day 4 after seeding (n= 6). (E) Flow cytometry and quantitatively analysis of Annexin V and PI stained primary astrocytes at 7th day after culture in MD and ABM media (*p< 0.05, **p<0.01, ***p<0.001 n= 3). (F) Cell cycle analysis of primary astrocytes cultured in ABM (supplemented with different growth factors) and MD (10% FBS). Represented comparison among 4 groups *p< 0.05, **p<0.01, ***p<0.001 n= 3.

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Figure 2. Astrocyte cultured in ABM-FGF2-EGF mimic resting astrocyte morphology with high purity.

(A). Representative florescent microscopic images of ALDH1L1 immunostaining in primary astrocytes cultured for 2 weeks and quantitative analysis of number and length of astrocyte process in MD and ABM culture condition. (B) Representative image of GFAP staining of astrocytes cultured for 2 weeks in MD (10% FBS) and ABM-FGF2-EGF; bar graph showing quantitative Imaris software analysis of astrocyte size (* $p < 0.05$ vs. MD (10% FBS) $n = 8$). (C) Flow cytometry analysis of GFAP and CD11B staining of primary astrocytes cultured in MD and ABM culture condition. (** $p < 0.01$ vs MD (10% FBS) $n = 3$)

Figure 3. Serum free ABM FGF2/EGF medium maintains primary astrocyte in their resting state which could be activated by TGF- β 1.

(A). Representative and quantitative Western blot analysis of ALDH1L1, GFAP, vimentin expression after 7 days of culture in serum free ABM Containing 5 ng/ml EGF/FGF2 or MD (10% FBS). (* $p < 0.05$ vs. MD 10% FBS $n = 6-8$). # $p < 0.05$ vs ABM (no growth factor), $n = 6$. (B) Real-time PCR analysis of ALDH1L1, GFAP and vimentin expression in astrocytes culture in MD media condition and ABM condition. (* $p < 0.05$, ** $p < 0.01$ vs. MD 10% FBS, $n = 6-8$). (C) Real-time PCR analysis of GFAP and vimentin expression in astrocytes culture in MD media condition and ABM media condition and activated with TGF β (10 ng/ml), Represented comparison among 4 groups * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ $n = 6-8$. (D) Representative and quantitative Western blot analysis of GFAP and vimentin expression after astrocytes were culture in MD media condition and ABM media condition and activated with TGF β (10 ng/ml). Represented comparison among 4 groups * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ $n = 6-8$.

Figure 4. Activation of primary astrocytes cultured in serum free ABM-FGF2-EGF medium. (A) Astrocytes maintained in serum free ABM-FGF2-EGF and MD (10% FBS) for 14 days and then seeded for scratch assay, with cell migration monitored from day 0 to day 3 after scratch (B) Astrocytes calcium response to different stimuli measured by Fluo 4AM Fluorescence (Fluo 4 F).

Figure 5. Primary astrocytes cultured in serum free ABM-FGF2-EGF has enhanced glycolysis and increased extracellular acidification and oxygen consumption rate.

Seahorse extracellular flux analysis of Oxygen consumption rate (OCR); bar graph indicate basal and maximal respiration, proton leak, non-mitochondrial oxygen consumption (non-MOC) and ATP production linked to mitochondrial respiration. (B) Extracellular acidification rate (ECAR); bar graph indicate baseline and stressed ECAR. (C) Cell metabolic potential of astrocytes cultured in MD (10%) and ABM-FGF2-EGF. (D) Lactate production of astrocytes cultured in different media condition (E) quantitative analysis of hexokinase activity in astrocytes (F) Real-time rtPCR analysis of hexokinase and MCT1 expression of astrocytes after cells were cultured in different media condition. (* $p < 0.05$ vs MD (10% FBS), $n = 6-10$).

Figure 6. Primary astrocyte cultured in serum free ABM-FGF2-EGF have increased total ATP content, glycogen storage and increased glucose uptake.

(A) Total ATP level of primary astrocytes cultured in MD (10% FBS) and ABM-FGF2-EGF

(B) Glycogen assay of primary astrocytes cultured in MD (10% FBS) and ABM-FGF2-EGF

Figure 7. ABM cultured primary astrocytes have lower AMPK activation as compared with MD cultured astrocyte.

(A) Representative western blots and quantitative analysis of total and phosphorylated AMPKα, pAMPKα, AMPKβ, pAMPKβ, and mTOR, pmTOR after 2 weeks of astrocyte culture in ABM-FGF2-EGF and MD (10% FBS) (n=6 *p< 0.05). (B) Representative Western blots and quantitative analysis of total and phosphorylated ACC, pACC, GS, pGS after 2 weeks of astrocyte culture in ABM-FGF2-EGF and MD (10% FBS) (*p< 0.05 n=6).

Figure 8. Comparison of mRNA expression profile of astrocyte specific factors of Serum free ABM-FGF2-EGF astrocytes with MD (10% FBS) astrocytes. Real time PCR analysis of astrocytes associated factors (A) S100β (B) GLAST (C) GLT-1 (D) GS (E) GLUT-1 (F) CXN-43 (G) AQP4 (H) Kir4.1 (* $p < 0.05$, ** $p < 0.01$ vs. MD 10% FBS $n = 6-8$). (I) Glutamate clearance measured at different time points in primary astrocyte cultured in MD and ABM conditions. (J) Quantitative analysis of 2-NBDG uptake shows increased 2-NBDG uptake in primary astrocytes cultured in ABM-FGF2-EGF. (* $p < 0.05$ *** $p < 0.001$ vs MD (10 FBS), $n = 6-8$).

Table 1

Markers quantified by qPCR.

	Gene name	Physiological function
ALDH1L1	Aldehyde dehydrogenase 1 family, member L1	Astrocyte specific marker
GFAP	Glial fibrillary acid protein	Astrocyte intermediate filament
VIM	Vimentin	Astrocyte intermediate filament
GS	Glutamine synthase	Converts glutamate to glutamine
HEK1	Hexokinase type 1	Phosphorylates glucose
HEK2	Hexokinase type 2	Phosphorylates glucose
HEK3	Hexokinase type 3	Phosphorylates glucose
MCT1	Monocarboxylate transporter 1	Lactate transport
Kir4.1	Inward rectifier potassium channel 10	Potassium uptake or buffering
AQP4	Aquaporin-4	Mercurial-insensitive water channel
CNX-43	Gap junction alpha-1 protein	Gap-junction component (coupling)
CNX-30	Gap junction beta-6 protein	Gap junction component (coupling)
GLUT1	Glucose transporter member 1	Glucose uptake
GLT-1	Glial high affinity glutamate transporter), member 2	Glutamate transporter
GLAST	Glial high affinity glutamate transporter), member 3	Glutamate transporter
IL-6	Interleukin-6	Cytokine
IL-1 β	Interleukin - 1 β	Cytokine
LCN-2	Lipocalin-2	Inflammatory mediator
TNF α	Tumor necrosis factor- α	Cytokine