SOX9 as a Predictor for Neurogenesis Potentiality of Amniotic Fluid Stem Cells

PEI-CHI WEI,a,b,* ANGEL CHAO,b,* HSU-HUEI PENG,b AN-SHINE CHAO,b YAO-LUNG CHANG,b SHUENN-DYH CHANG,b HSIN-SHIH WANG,b YU-JEN CHANG,f MING-SONG TSAI,d MARTIN SIEBER,e HUA-CHIEN CHEN,f SHU-JEN CHEN,f YUN-SHIEN LEE,g,h SHIAW-MIN HWANG,5 TZU-HAO WANGa,b,g,i

Key Words. Amniotic fluid • Stem cells • Neural differentiation • SOX9 • Cell-based therapy

ABSTRACT

Preclinical studies of amniotic fluid-derived cell therapy have been successful in the research of neurodegenerative diseases, peripheral nerve injury, spinal cord injury, and brain ischemia. Transplantation of human amniotic fluid stem cells (AFSCs) into rat brain ventricles has shown improvement in symptoms of Parkinson’s disease and also highlighted the minimal immune rejection risk of AFSCs, even between species. Although AFSCs appeared to be a promising resource for cell-based regenerative therapy, AFSCs contain a heterogeneous pool of distinct cell types, rendering each preparation of AFSCs unique. Identification of predictive markers for neuron-prone AFSCs is necessary before such stem cell-based therapeutics can become a reality. In an attempt to identify markers of AFSCs to predict their ability for neurogenesis, we performed a two-phase study. In the discovery phase of 23 AFSCs, we tested ZNF521/Zfp521, OCT6, SOX1, SOX2, SOX3, and SOX9 as predictive markers of AFSCs for neural differentiation. In the validation phase, the efficacy of these predictive markers was tested in independent sets of 18 AFSCs and 14 dental pulp stem cells (DPSCs). We found that high expression of SOX9 in AFSCs is associated with good neurogenetic ability, and these positive correlations were confirmed in independent sets of AFSCs and DPSCs. Furthermore, knockdown of SOX9 in AFSCs inhibited their neuronal differentiation. In conclusion, the discovery of SOX9 as a predictive marker for neuron-prone AFSCs could expedite the selection of useful clones for regenerative medicine, in particular, in neurological diseases and injuries. Stem Cells Translational Medicine 2014;3:1138–1147

INTRODUCTION

Amniotic fluid stem cells (AFSCs) are usually isolated from human amniotic fluid between 16 and 18 weeks of gestation [1–5]. These cells possess the capability of self-renewal and rapid expansion in culture [6] and can proliferate for more than 250 generations in culture without requiring feeder layers [4]. AFSCs have the potential for multilineage differentiation into adipocytes, osteocytes, epithelial cells, and neuronal cells [3, 4, 7, 8]. Cultured AFSCs exhibit mesenchymal markers CD73 (SH4), CD90, and CD105 (endoglin, SH2), adhesion molecules CD29 and CD44 [3, 4], and pluripotent markers Nanog, OCT4, and SSEA4 (stage-specific embryonic antigen 4) [4, 9, 10] but do not express the hematopoietic markers, such as CD34, CD45, and CD133. Directed differentiation of AFSCs has gained attention owing to their potential to develop into different lineages. However, each AFSC collection is composed of a variety of cells originated from all three germ layers; thus, individual AFSC collections each have different abilities to differentiate into various cell lineages. The discovery of predictive markers for neuron-prone AFSCs could be useful for the selection of AFSC collections to be used in stem cell-based therapeutics for neurological diseases.

Directed neural differentiation from stem cells has been assayed with neural markers, including NeuN, a nuclear protein expressed by nerve cells [11, 12]; nestin, the intermediate filament protein expressed in neural stem cells [13]; Tuj1 (also called neuronal class III β-tubulin), a neuronal cytoskeletal protein; and tyrosine hydroxylase, the enzyme catalyzing the conversion of the amino acid L-tyrosine to dihydroxyphenylalanine. In contrast, several genes, such as Zfp521, OCT6, SOX1, SOX2, SOX3, and SOX9, have been associated with neural stem cells. Xenopus Zfp521 is capable of directing neural differentiation, promoting embryonic stem cell (ESC) differentiation toward neural progenitors [14]. OCT6 is expressed in both the embryonic epiblast and the early neuroectoderm, and forced expression of OCT6 was shown to also increase the expression of Zfp521 [14]. Zfp521 has been shown to directly activate the early neural genes, SOX1 and SOX3 [14], critical determinants of neurogenesis [15–17]. In contrast, differentiation of stem cells into neurons depends on the ability to suppress SOX1–3 expression by proneural basic helix-loop-helix proteins [15].
SOX9 was expressed in neural crest precursors in *Xenopus* [17] and the forced expression of SOX9 promoted neural crest-like properties in chick neural tube progenitors [18].

In this study, we tested ZNF521/Zfp521, OCT6, SOX1, SOX2, SOX3, and SOX9 as predictive markers of AFSCs for neural differentiation. The efficacy of predictive markers for good neural differentiation was validated in an independent set of AFSCs and a set of dental pulp stem cells (DPSCs).

**MATERIALS AND METHODS**

**Study Design**

This study consisted of two phases: the discovery phase and the validation phase (Fig. 1). In the discovery phase, we quantified the expression levels of 6 predictive markers in 23 clones of AFSCs before induced neural differentiation. At the same time, we used an image analysis system to quantify four neural markers after these AFSCs had undergone neural differentiation and performed clustering analysis to identify well versus poorly differentiated groups. The candidate predictive markers were selected by analyzing their expression levels between the two groups. In the validation phase, an additional 18 clones of AFSCs and 14 DPSCs were tested to confirm the efficacy of these candidate predictive markers (Fig. 1).

**Isolation and Culture of AFSCs**

AFSCs were derived from amniotic fluid at 16–18 weeks of gestation. Amniotic fluid cells were cultured in alpha-minimum essential medium (α-MEM) supplemented with 20% fetal bovine serum (FBS; HyClone, Logan, UT, http://www.hyclone.com) and 4 ng/ml basic fibroblast growth factor (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com), and incubated at 37°C with 5% CO₂ [18]. After the nonadherent cells were removed, the culture medium was changed every 3–4 days. The Chang Gung Memorial Hospital institutional review board (approval no. 97-1341A3) approved this study, and each enrolled subject provided written informed consent.

**Characterization of AFSCs**

Human AFSC cells were characterized with flow cytometry using fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated antibodies (BD Biosciences, San Diego, CA; or Southern Biotech, Birmingham, AL), as previously reported [3]. Before analysis, the cells were detached with trypsin/EDTA in phosphate-buffered saline (PBS), washed with PBS, and incubated with each designated antibody for 15 minutes. For each sample, 1 × 10⁵ events were acquired and analyzed using Cell Quest software (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com).

**RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction Analysis**

Total RNA of AFSCs and neuron-like cells were isolated with Trizol reagent (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), as previously reported [19]. The mRNA of ZNF521, OCT6, and SOX family members and pluripotent genes were quantified with real-time quantitative polymerase chain reaction (RT-qPCR) using SYBR green reagents (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). The primer sequences were as follows: ZNF521: CCTGCCTATGTCATGTTGC (forward) and GGATGGTTTGAGATGTTTC (reverse); OCT6: AGGACGAAGGCGAGATTG (forward) and CGAGAGGCTCCACAAT (reverse); SOX1: ATGGGACCTTGAGGGTTTTC (forward) and GGCGCACTAACTCAGCTTTT (reverse); SOX2: ACACCAATCCATCCATCCACT (forward) and GCAAACTTCCTGCAAAGCTC (reverse); SOX3: AGTCAGGAGCAGCGAAAATG (forward) and TTCTCCATTCTCCTTGG (reverse); SOX9: CACAGCTCACTCGACCTTG (forward) and GCGCGTTGGATAGGTCATGTT (reverse). The thermocyclic conditions were 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute for a total of 40 cycles and were performed using the ABI 7900 HT RT-PCR system (Applied Biosystems). The mean threshold cycles (Ct) were calculated from duplicate reactions and normalized by glyceraldehyde-3-phosphate dehydrogenase. The ΔΔCt values were used to represent the relative gene expression, as previously described [20].

**Induction of Neural Differentiation and Characterization of Neural Markers**

AFSCs were cultured in neural differentiation medium (Cellular Engineering Technologies Inc., Coralville, IA, http://www.
celleng-tech.com) supplemented with 10% FBS (HyClone) at 37°C with 5% CO₂ for 2 days. Neural markers were identified using immunofluorescent microscopy and quantified using the IN Cell Analyzer 1000 (GE Healthcare Life Sciences, Pittsburgh, PA, http://www.gelifesciences.com). Noninduced and induced cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100, and then incubated with tyrosine hydroxylase (polyclonal rabbit; Santa Cruz Biotechnology Inc., Dallas, TX, http://www.scbt.com), TuJ1 (neuronal class III β-tubulin, monoclonal mouse; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), NeuN (monoclonal mouse; Merck Millipore, Billerica, MA, http://www.millipore.com), nestin (monoclonal mouse; Merck Millipore). After washing, the cells were incubated with designated secondary antibody-conjugated Alexa 488 fluorescent dye or Alexa 546 fluorescent dye (Molecular Probes, Invitrogen, Carlsbad, CA, http://www.invitrogen.com). The nuclei were stained with 4’,6-diamidino-2-phenylindole (Molecular Probes, Invitrogen) in PBS. Images were captured and quantified using the IN Cell Analyzer 1000 [21] (GE Healthcare Life Sciences). To have better microscopic resolution on some images, we used the Leica TCS SP2 laser scanning confocal system (Leica, Mannheim, Germany, http://www.leica.com) for immunofluorescent microscopy, as previously reported [22].

Small Interfering RNA Transfection
Human AFSCs were transfected with small interfering SOX9 (Santa Cruz Biotechnology) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer’s instructions. SOX9 was confirmed by RT-qPCR and Western blotting after 72 hours of transfection.

Western Blot Analysis
The procedure of Western blot analysis has been previously reported [22, 23]. In brief, 50 μg of each protein sample was used for 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with SOX9 antibody (Abnova, Taipei, Taiwan, http://www.abnova.com), followed by the secondary antibody-conjugated horseradish peroxidase (Santa Cruz Biotechnology), and visualized with enhanced chemiluminescence reagents (Merck Millipore). The image analysis was quantified using the UN-SCAN-IT software (Silk Scientific Inc., Orem, UT, http://www.silkscientific.com), and relative intensity was normalized using the corresponding actin intensity.

Culture of Dental Pulp Stem Cells
DPSCs were obtained from Taiwan Bionet Corp. (Taipei City, Taiwan, http://www.babybanks.com) and cultured as previously reported [24]. In brief, cells were cultured in α-MEM supplemented with 10% FBS (HyClone) with 5% CO₂ in a 37°C incubator. The culture medium was changed every 3 days for optimal cell proliferation. The induction of neural differentiation was identical to that of the AFSC, and the induced neural cells were analyzed at 24 hours of induction using immunofluorescent microscopy and IN Cell Analyzer 1000, as designated.

Statistical Analyses
Statistical methods included the Mann-Whitney U test, Pearson’s correlation analysis, and receiver operating characteristic (ROC) curve analysis using SPSS statistical software, version 18.0 (SPSS Inc., Chicago, IL, http://www-01.ibm.com/software/analytics/spss/). Hierarchical clustering analysis was done with the free academic software Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#ctv). Differences were considered statistically significant when p values were <.05.

RESULTS
Identification of MSCs and Pluripotent Markers in AFSCs
Twenty-three AFSCs were isolated from human amniotic fluid. All the AFSCs expressed high levels of CD29 (integrin β1), CD44 (HA receptor), CD73 (SH4), CD90, and CD105 (endoglin, SH2) staining but only minimal levels of hematopoietic stem cell markers (CD34, CD45, and CD133) (Fig. 2A). CD133 has been regarded as one of the neural stem cell markers [25, 26]. Our findings of very low levels of CD133 in AFSCs are identical to those reported by De Coppi et al. [4]. Such minimal CD133 expression levels in AFSCs, however, did not exclude their capability for neural differentiation [3, 4]. AFSCs also exhibited some pluripotent stem cell markers, such as Nanog, OCT4 (Fig. 2A), and SOX2 (Fig. 2B). These results indicated that 23 AFSCs displayed some characteristics of both embryonic and mesenchymal stem cells.

AFSCs-Derived Neuron-Like Cells Expressed Specific Neural Markers
At 24 hours of induced neural differentiation, the cells changed morphology and grew axons. We further characterized the differentiated cells for the expression of NeuN, nestin, TuJ1, and tyrosine hydroxylase with fluorescent microscopy. Immunofluorescent staining was performed on both the undifferentiated AFSCs and the differentiated cells at 24 and 48 hours of neural induction. Positive staining for NeuN, nestin, TuJ1, and tyrosine hydroxylase was detected in the neuron-like cells (Fig. 3A, 3B). However, nestin was also expressed in undifferentiated cells (Fig. 3B). These results demonstrated that neuron-like cells derived from AFSCs expressed specific neural proteins.

Classification of AFSCs Into Good Versus Poor Neurogenesis Groups
The percentages of NeuN, nestin, TuJ1, and tyrosine hydroxylase positive cells in each AFSC-derived neuronal cells were quantified with IN Cell 1000 Analyzer (supplemental online Table 1). Using a clustering analysis (Fig. 3C) based on four neuronal markers (supplemental online Table 1), we grouped 23 AFSCs into the good neurogenesis group (n = 12) and the poor neurogenesis group (n = 11). Subsequent ROC curve analyses were used to identify the cutoff values of the neural differentiation markers that resulted in the highest sum of sensitivity and specificity (Fig. 3D; Table 1). The day 1 cutoff values for good neural differentiation were set at 71% for TuJ1, 43% for NeuN, 48% for tyrosine hydroxylase, and 33% for nestin to achieve the best discrimination between the good versus poor neurogenesis groups (Table 1). The day 2 cutoff values were not used in the subsequent experiments because they were found to be too time-consuming, although the areas under the curve (AUCs) were good for tyrosine hydroxylase and nestin (Table 1).
We also examined the expression of the four neural markers in several AFSCs for longer durations after induced differentiation. To confirm that expression of the four neural markers truly represent neural differentiation, we also analyzed the mRNA expression of G-protein-regulated inward-rectifier potassium channel 2 (GIRK2) [4] and microtubule-associated protein 2 (MAP2) [27] in these specimens. In general, the positive percentages in the four neural markers on days 3 and 4 were similar to those on day 2 (supplemental online Table 2). The expression of GIRK2 and MAP2 on days 3 and 4 was generally higher than that on day 2 (supplemental online Figure 1; supplemental online Table 8).

**AFSCs With Good Neurogenic Potential Expressed High mRNA Levels of SOX2 and SOX9**

RT-qPCR analysis revealed that the AFSCs with greater gene expression of SOX2 and SOX9 had better capability for neurogenesis of the 23 original collections ($p < .05$; Fig. 4A–4F). The use of the [39-TCT] values of 31.0 and 21.2 for SOX9 and SOX2, respectively, achieved the highest sum of sensitivity and specificity (supplemental online Tables 1, 3). Analyzed using the ROC curve method, the AUC of SOX9 and SOX2 was 0.87 and 0.84, respectively, significantly different ($p < .05$) from the AUC of 0.5 characteristic of pure guesses (Fig. 4G).

**SOX9 Is a Useful Predictive Gene in an Independent Group of 18 AFSCs**

In an independent set of 18 AFSCs, the use of the [39-TCT] value of greater than 31.0 for SOX9 predicted 11 AFSCs of good neurogenic potential (supplemental online Table 4). The percentages of neural marker positive cells were quantified using the IN Cell 1000 Analyzer (supplemental online Table 4). Neurally differentiated AFSCs with three or more neural markers greater than the day 1 cutoff values (Table 1) were categorized into the good neurogenesis group (supplemental online Table 4). The results revealed that the sensitivity and specificity of the use of SOX9 for discriminating the good versus poor neurogenic potential of 18 AFSCs was 100% and 70%, respectively (supplemental online Table 5). Although the use of a [39-TCT] value of greater than 31.0 for SOX9 could achieve 73% of the positive predictive value (PPV) in the independent group of 18 AFSCs (supplemental online Table 5), the use of a [39-TCT] value of greater than 21.2 for SOX2 only resulted in 53% of the PPV (supplemental online Table 6). Because of these results, we decided to further test functional assays of SOX9 in AFSCs with small interfering RNA (siRNA) knock-down technology.

However, our results could not completely exclude the potential value of SOX2 for predicting the neurogenic capacity of AFSCs, because nearly equal usefulness of SOX2 and SOX9 was shown in the analysis that specifically focused on the top and bottom quadrants of SOX expression. To achieve sufficient case numbers for analysis, we put together the 23 AFSCs of the discovery phase (supplemental online Table 1) with the 18 AFSCs of the validation phase (supplemental online Table 4) to analyze SOX9. We also combined the 23 AFSCs of the discovery phase (supplemental online Table 3) and the 18 AFSCs of the validation phase (supplemental online Table 6) for SOX2. In the top quadrant 10 AFSCs, the use of SOX9 correctly predicted 8 cases of good neurogenesis, and SOX2 predicted 7 cases. In the bottom quadrant 11 AFSCs for SOX9 (two AFSCs had the identical [39-TCT] value) and 10 AFSCs for SOX2, both markers predicted 100% correctly.

Figure 2. Characteristics of mesenchymal stem cell and pluripotent markers in AFSCs. (A): AFSCs expressed high levels of CD29, CD44, CD73, CD90, CD105, OCT4, and Nanog but only minimal levels of CD34, CD45, and CD133, shown using flow cytometric analyses. (B): Immunofluorescent microscopy indicated that AFSCs were positive for SOX2. Scale bars = 75 μm. Abbreviations: AFSC, amniotic fluid stem cell; DAPI, 4’6-diamidino-2-phenylindole.
Early-Passage AFSCs Exhibited Higher SOX9 Expression and Possessed Better Neurogenic Potential Than Late-Passage AFSCs

We found that AFSCs of early passages 1–5 expressed higher SOX9 mRNA than those of late passages 35–40 (Fig. 5A, 5B). Similarly, expression of neural markers (nestin, NeuN, TuJ1, tyrosine hydroxylase) was significantly higher in the early-passage AFSCs than those in late passage (Fig. 5C).

Knockdown of SOX9 in AFSCs Suppressed Neuronal Differentiation

To confirm that suppression of SOX9 in AFSCs could inhibit neural differentiation, we knocked down SOX9 in four AFSCs with good

Figure 3. Induced neural differentiation of AFSCs. (A): AFSCs were induced to differentiate into neural cells, which were analyzed for specific markers, such as NeuN (left), TuJ1 (middle), and tyrosine hydroxylase (right) on day 2. (B): Nestin was expressed in small numbers of AFSCs before induction (left), with more in neural cells after induction for 24 hours (middle) and 48 hours (right). Scale bars = 75 μm. (C): Clustering analysis of four neuronal markers discriminated 12 AFSCs with good neural induction (red points) from 11 AFSCs with a poor induction rate (green dots). (D): Receiver operating characteristic curve analysis was used to identify the cutoff percentage of each neural marker for discriminating good versus poor neurogenesis capability of AFSCs (the results are summarized in Table 1). From the results on day 1, the AUC of TuJ1, NeuN, tyrosine hydroxylase, and nestin was 0.75, 0.78, 0.88, and 0.92, respectively. All AUCs of the four assays were significantly different (p < .05) from the AUC of 0.5 that is characteristic of pure guesses. Abbreviations: AFSC, amniotic fluid stem cell; AUC, area under the curve; DAPI, 4′,6-diamidino-2-phenylindole; Nes, nestin; TH, tyrosine hydroxylase.
Table 1. Cutoff positive percentage of neural markers as criteria for neural differentiation capability

| Neural markers | Tuj1 | NeuN | Tyrosine hydroxylase | Nestin |
|----------------|------|------|----------------------|-------|
|                | Day 1 | Day 2 | Day 1 | Day 2 | Day 1 | Day 2 | Day 1 | Day 2 |
| Percentage     | >71%  | >80%  | >43%  | >41%  | >48%  | >50%  | >33%  | >26%  |
| AUC            | 0.750 | 0.742 | 0.780 | 0.720 | 0.879 | 0.955 | 0.917 | 0.856 |
| p value        | <.05  | NS    | <.05  | NS    | <.05  | <.05  | <.05  | <.05  |

Abbreviations: AUC, area under the curve; NS, not significant.

Figure 4. Discriminative gene expression of ZNF521, OCT6, SOX1, SOX2, SOX3, and SOX9 between AFSCs of good neural differentiation and those of poor differentiation. (A–F): Real-time quantitative polymerase chain results of ZNF521, OCT6, SOX1, SOX2, SOX3, and SOX9 in 23 AFSCs are presented as \([39\text{-delta CT]}\) values. AFSCs with GD expressed significantly greater SOX2 and SOX9 levels than AFSCs with PD. (G): Analyzed with receiver operating characteristic curve analyses, the AUC of SOX2 (left) and SOX9 (right) was 0.84 and 0.87, respectively. Abbreviations: GD, good differentiation; PD, poor differentiation.
neurogenic potential using siRNA and then performed neural induction. When SOX9 was successfully knocked down (Fig. 5A, 5B), the expression of the neural markers was significantly ($p < .05$) inhibited during neural differentiation (Fig. 5C).

Positive Correlation Between Tyrosine Hydroxylase and SOX9 in DPSCs

To test whether SOX9 could be used to predict the neurogenic potential in other types of stem cells, we analyzed SOX9 levels and neural markers in 14 DPSCs obtained from milk teeth. The induced neural cells derived from DPSCs also expressed specific proteins, including NeuN, nestin, Tuj1, and tyrosine hydroxylase (Fig. 6A; supplemental online Table 7). The use of a $[39 \Delta Ct]$ value of SOX9 greater than 31.0 identified five DPSCs of good neurogenic potential. In contrast, the results from the neural markers categorized 13 DPSCs into the good differentiation group. Although the use of this criterion only resulted in a sensitivity of 38.5%, both the positive predictive value and specificity for discriminating good versus poor neurogenic potential of DPSCs achieved 100% (supplemental online Table 5). The results of Pearson’s correlation revealed that SOX9 expression levels were positively correlated with the protein levels of Tuj1 and tyrosine hydroxylase (Fig. 6B), although the correlation between SOX9 and NeuN or...
nestin did not reach statistical significance (data not shown). These results in DPSCs further strengthen the idea that SOX9 could be a useful predictive marker of other stem cells for successful neurogenic differentiation.

**DISCUSSION**

We have previously isolated multipotent MSCs in second trimester amniotic fluid and showed their differentiation into neural lineages [2, 3]. To our knowledge, this report is the first to show SOX9 as a predictive marker for the neurogenesis of AFSCs, which could prove clinically useful in selecting MSCs for future treatment of neurological diseases and injuries.

The stem cells used for treating nervous system disorders have been derived from embryonic stem cells, adult bone marrow, umbilical cord blood, muscle, dental pulp, and neurogenic regions of the brain [28–32]. The use of human embryonic and fetal stem cells is often hampered owing to ethical concerns, and the sources of brain tissue for stem cell transplantation are scarce [31]. However, amniotic fluid is safely and easily obtainable and contains a heterogeneous population of stem cells that might match well for tissue repair and transplantation. Compared with adult bone marrow MSCs, AFSCs were shown to have higher proliferation capacity [3, 5, 6, 33, 34] and to expand more rapidly to form neurospheres [3, 34]. Despite their high proliferative rate, AFSCs also do not display any karyotypic abnormalities, in vitro transformation, or in vivo tumorigenic effects [4, 33].

The advent of human induced pluripotent stem cells (iPSCs) raised the hope that, without the concern of immune rejection, personalized stem cells could be generated as the source of cell therapy. Although iPSCs promised pluripotent properties and expansion potentiality, several problems of iPSCs have been encountered, including somatic mutations [35], copy number variation [36], immunogenicity [37], and the risk of tumor formation [38]. Hence, technical variables, gene delivery methods, and culture conditions are essential issues that need to be addressed in the selection process for good clones of iPSCs for medical applications [39]. Given the fast pace of iPSC research, these difficulties will be soon resolved [40]. Nevertheless, different sources of well-characterized MSC, such as AFSCs, remain currently important for the development of cell therapies.

Preclinical studies of amniotic fluid-derived cell therapy have been successful in the treatment of neurological diseases and injuries, including Parkinson’s disease, peripheral nerve injury, spinal cord injury, and brain ischemia [41–46]. Glia-derived neurotrophic factor-modified amniotic fluid mesenchymal stem cells (AFMSCs) enhanced regeneration of sciatic nerve crush injury [41]. AFMSCs helped nerve regeneration by suppressing inflammatory cytokines and macrophage deposits [42]. Injection of AFSCs into mouse brain ventricles ameliorated focal ischemia-reperfusion brain injury [43]. Transplantation of AFSCs promoted differentiation of endogenous neuronal progenitors and improved recovery of brain function after ischemic brain injury [44]. AFSCs also promoted remyelination for locomotor recovery in an early spinal cord injury [46]. Transplantation of human AFSCs into rat brain ventricles showed benefits on the symptoms of Parkinson’s disease [45], further highlighting the minimal immune rejection of AFSCs, even between species. AFSCs appear to be a promising therapeutic resource for cell-based regenerative therapy [47, 48]. AFSCs, however, contain distinct heterogeneous...
pool of cell types; thus, each preparation of AFSCs will behave uniquely [2-4, 33]. Various panels of genes have been suggested to be markers for distinct human ESC-derived neural differentiation lineages [28]. For instance, neural cell adhesion molecule, Tuj-1, Snai1, dHAND, and SOX9 are markers for ESC-derived neural precursors [49]. In addition, cell surface marker CD15/CD24/CD29 profiles were shown to be useful for the identification of human ESC neural lineage of neural stem cells, neural crest, and neurons [50]. Although the existence of neuronal progenitor cells such as nestin and brain-derived neurotrophic factor were described in amniotic fluid [7], currently, no useful marker is available to predict neurogenesis for AFSCs.

Although the results of this study cannot exclude the potential usefulness of SOX2 for predicting the neurogenesis potentiality of AFSCs, our results suggest that SOX9 could be a useful predictor. The SOX proteins are a group of transcription factors with an SRY box, a 79-amino acid motif that encodes a high-mobility group DNA binding domain [17]. SOX9 is required for nervous system development [17] and maintenance of multipotent neural stem cells [51]. Together with Snai2, SOX9 has been shown to be involved in epithelial-mesenchymal transition in avian neural crest [52]. These functions support our findings that AFSCs with higher SOX9 expression have better capability for neuronal differentiation.

CONCLUSION

AFSCs are a safe and reliable source of human stem cells that might be easier to use than iPSCs for cell-based therapeutics.

The discovery of SOX9 as a predictive marker for neuron-prone AFSCs can expedite the selection of useful clones for cell-based therapies of neurological diseases and injuries.

ACKNOWLEDGMENTS

We thank the Core Instrument Center of Chang Gung University for help on the IN Cell Analyzer 1000 Image System, Huan-Ting Lu (Bionet Corp.) and Li-Feng Hsu (Bioresource Collection and Research Center) for excellent technical assistance, and Dr. Shihyee Mimi Wang (White Memorial Hospital, Los Angeles, CA) for English editing. The study was supported in part by the following grants: Ministry of Science and Technology MOHW102-TD-PB-111NSC106 (to T.-H.W.), DOH102-TDC-111-006 (to A.C. and T.-H.W.), BMRP133 (to H.-S.W.), and Chang Gung Medical Foundation CMRP3GA0471-3 (to T.-H.W.).

AUTHOR CONTRIBUTIONS

P.-C.W.: conception and design, collection and/or assembly of data, data analysis and interpretation; A.C.: conception and design, manuscript writing; H.-H.P., A.-S.C., Y.-L.C., S.-D.C., H.-S.W., Y.-J.C., M.-S.T., M.S., H.-C.C., S.-J.C., and Y.-S.L.: collection and/or assembly of data, data analysis and interpretation; M.-H.H. and T.-H.W.: conception and design, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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