### Title

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# The Key Components of Schwann Cell-like Differentiation Medium and their Effects on Gene Expression Pattern of Adipose-Derived Stem Cells

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**Background:** Schwann cell-like cells differentiated from adipose-derived stem cells may have an important role in peripheral nerve regeneration. Herein, we document the individual effects of growth factors in Schwann cell-like differentiation medium.

**Methods:** There were 6 groups in the study. In the control group, we supplemented the rat adipose–derived stem cells with normal cell culture medium. In group 1, we fed the cells with Schwann cell-like differentiation medium (normal cell culture medium supplemented with platelet-derived growth factor, basic fibroblast growth factor, forskolin, and glial growth factor). In the other groups, we removed the components of the medium one at a time from the differentiation medium so that group 2 lacked glial growth factor, group 3 lacked forskolin, group 4 lacked basic fibroblast growth factor, and group 5 lacked platelet-derived growth factor. We examined the expression of the Schwann cell–specific genes with quantitative reverse transcription polymerase chain reaction and immunofluorescence staining in each group.

**Results:** Groups 3 and 4, lacking forskolin and basic fibroblast growth factor, respectively, had the highest expression levels of integrin-β4, and p75. Group 1 showed a 3.2-fold increase in the expression of S100, but the expressions of integrin-β4 and p75 were significantly lower compared to groups 3 and 4. Group 2 [glial growth factor (−)] did not express significant levels of Schwann cell–specific genes. The gene expression profile in group 4 most closely resembled Schwann cells. Immunofluorescence staining results were parallel with the quantitative real-time polymerase chain reaction results.

**Conclusions:** Glial growth factor is a key component of Schwann cell-like differentiation medium.

**Key Words:** adipose-derived stem cells, Schwann cells, peripheral nerve injury

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Peripheral nervous system has limited regenerative capacity. Although this regeneration capability can heal simple cuts of peripheral nerves satisfactorily, it is not sufficient for healing in case of larger nerve defects.[1,2] The distance and speed of regeneration in nerve defects can be improved by adding stem cells (SCs) to the defect zone.[1,3,4] However, SC therapy is limited by donor tissue availability, donor site morbidity, and long culture times.[5–9] Therefore, recent research is actively focusing on finding alternative sources for SCs.[10] Adipose-derived mesenchymal SCs (ASCs) can be differentiated into SC-like cells via coculture with SCs or more commonly with a mixture of growth factors (GFs).[11–13] Although these methods are both effective, they are usually too complicated and expensive for rapid clinical translation. A better understanding of the roles of GFs used to induce the ASCs into SC-like cells is crucial to facilitate the clinical translation of SC therapy.

In this study, we examined the individual effects of GFs in ASCs-SC differentiation medium by observing the changes in gene expression patterns of ASCs in response to the removal of the components (one at a time) from the differentiation medium.

## MATERIALS AND METHODS

All animal procedures were performed following the guidelines of Institutional Animal Care and Use Committee (IACUC approval #16662) and the National Institutes of Health and any national law on the care and use of laboratory animals.

### Harvesting of ASCs

Adipose-derived stem cells were harvested from the inguinal fat pads of female Lewis rats by enzymatic digestion as described elsewhere.[14] The cells in culture flasks were maintained in a 37°C incubator with 5% $CO_2$. Cells from passages II-IV were used for all experiments.

### Characterization of ASCs

Adipose-derived stem cells were characterized by flow cytometry and multilineage differentiation. For flow cytometry, ASCs in suspension were incubated with phycoerythrin-coupled antibodies for rat CD31 (BD Pharmingen, San Jose, CA), CD44 (eBioscience Inc, San Diego, CA), CD45 (BioLegend, San Diego, CA), and CD90 (Acris Antibodies, San Diego, CA) in the dark, at room temperature for 30 minutes. The cells were washed with wash buffer (0.5% fetal bovine serum in phosphate-buffered saline) and were fixed in neutral 4% paraformaldehyde solution for 30 minutes.

To induce adipogenic and osteogenic differentiation, ASCs were cultured in adipogenic differentiation medium (StemPro Adipogenesis Kit, Gibco) and osteogenic differentiation medium (StemPro Osteogenesis Kit, Gibco) for 14 and 21 days, respectively. For chondrogenic differentiation, ASCs micromass pellets were fed with chondrogenic differentiation medium that consisted of Dulbecco modified eagle medium with 10-ng/mL transforming GF β-3 [TGFβ-3], and 200-μmol/L ascorbic acid for 2 weeks. Adipogenic, osteogenic, and chondrogenic differentiation was confirmed with oil red O, alizarin red staining, and alcian blue staining, respectively.

### SC-like Differentiation of ASCs

We have used a previously published protocol for the SC-like differentiation of ASCs.[7] Adipose-derived stem cells in culture flasks

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**TABLE 1.** Contents of the Differentiation Medium Used in Each Study Group

| Groups | PDGF (5 ng/mL) | bFGF (10 ng/mL) | Forskolin (14 μmol/L) | GGF (252 ng/mL) |
|---|---|---|---|---|
| Control | − | − | − | − |
| Group 1 | + | + | + | + |
| Group 2 | + | + | + | − |
| Group 3 | + | + | − | + |
| Group 4 | + | − | + | + |
| Group 5 | − | + | + | + |

were divided into 6 groups. In the control group, the cells were supplemented with cell growth medium (Dulbecco modified eagle medium +10 % fetal bovine serum +1 % antibiotic/antimycotic solution). In all the other groups, the cells were initially treated with 1-mmol/L β-mercaptoethanol for 24 hours and 35-ng/mL all-trans-retinoic acid (RA) for 72 hours. Afterward, the cells were fed with corresponding differentiation medium for 2 weeks (Table 1). Fresh medium was added every 3 days.

## Immunofluorescence Staining for SC-Specific Proteins

We carried out immunofluorescence (IF) staining to detect SC-specific proteins S100, p75, and integrin-β4 in differentiated ASCs. The cells in each group were fixed with 4% paraformaldehyde and incubated with primary antibodies for S-100 (Acris Antibodies), p75 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), and integrin-β4 (Santa Cruz Biotechnology Inc) for 1 hour at room temperature. Alexa Fluor 480 (AF480)-labeled antigoat IgG (Invitrogen, Eugene, OR) was used as secondary antibody. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and the images were captured under a fluorescence microscope.

## Real-Time qRT-PCR

We detected the fold changes in the gene expression levels of SC-specific proteins S100, p75, and integrin-β4 in each group with

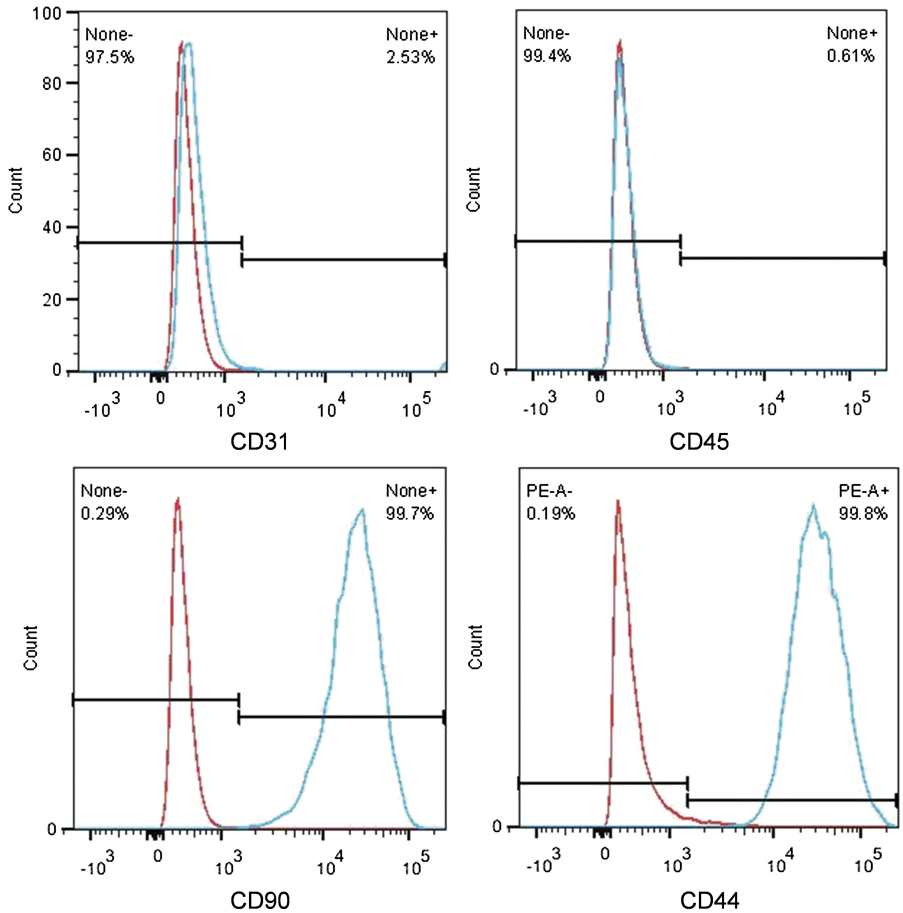


**FIGURE 1.** Flow cytometric analysis revealed that ASCs expressed MSC markers (CD90 and CD44), but they were negative for endothelial cell marker (CD31) and leukocyte marker (CD45).

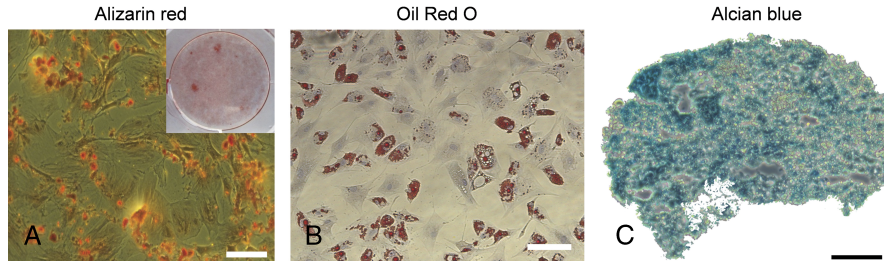


**FIGURE 2.** Adipose-derived SCs were differentiated into osteogenic (A), adipogenic (B), and chondrogenic (C) lineages to demonstrate their multipotency. Microbars, 100 μm (A); 50 μm (B, C).

real-time quantitative reverse transcription (qRT) polymerase chain reaction (PCR) (qRT-PCR). Primers were TaqMan primers all from Applied Biosystems. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control.

## Statistical Analysis

All results were compared using the one-way analysis of variance test and, if necessary, the Tukey test ($P < 0.05$ was considered significant).

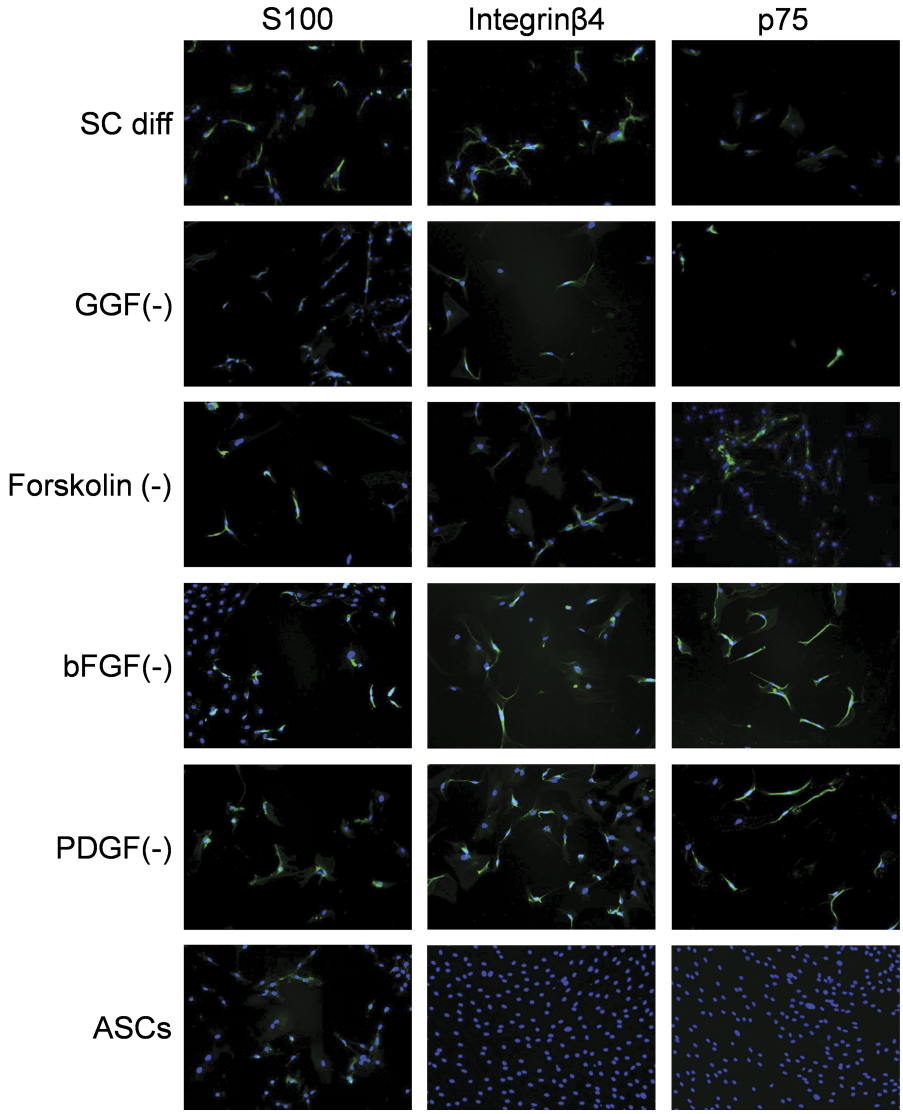


**FIGURE 3.** Results of IF staining after 2 weeks of culturing in different differentiation mediums. In parallel with qRT-PCR results, the most intense staining for integrin-β4 and p75 were in groups 3 and 4. The staining intensities for integrin-β4 and p75 in group 2 were lower. AF480 was used as secondary antibody and nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Microbar, 200 μm.

## RESULTS

### Characterization of ASCs

Adipose-derived stem cells expressed mesenchymal stem cells (MSC) markers CD90, and CD44 but lacked endothelial cell marker CD31 and leukocyte marker CD45 on their surface (Fig. 1). Additionally, ASCs differentiated into adipogenic, osteogenic, and chondrogenic lineages as a proof of their multipotency (Fig. 2).

### IF Staining for SC-Specific Proteins

S100 was positive in all the groups, but the staining in groups 1, 3, 4, and 5 was stronger than in the other groups (Fig. 3). The weakest staining for S100 was in group 2, which lacked glial growth factor (GGF). Staining for p75 and integrin-β4 was strongest in groups 4 and 5, whereas there was no staining in undifferentiated ASCs. Overall, the IF staining patterns in groups 4 and 5 were most compatible with SC phenotype.

### Real-Time qRT-PCR: Fold Changes in the Expression Levels of SC Proteins

We used the $\Delta\Delta C_t$ method to calculate the fold changes in gene expression levels relative to the control group. The cells treated with complete differentiation medium showed a 3.2-, 1.1-, and 1.7-fold increase in expression levels of S100, p75, and integrin-β4, respectively (Fig. 4). Fold increase in S100 expression in groups 2, 3, 4, and 5 were 1.2; 2.6; 2.7; 0.9, respectively. Overall, the highest expression of S100 was in groups 1, 3, and 4 ($P < 0.05$). Fold increases in p75 expression in groups 1 to 5 were 1.1, 1.8, 5.5, 7.6, and 3.7, respectively. The difference between groups 3 and 4 and the other groups were statistically significant ($P < 0.05$). Fold increases in integrin-β4 expression in groups 1 to 5 were 1.7, 2.1, 5.8, 4.8, and 3.8, with the highest values in groups 3 and 4 ($P < 0.05$). The expression levels of all SC-specific genes in group 2, which lacked GGF, were significantly lower than groups 3 and 4, demonstrating the key role of GGF in SC-like differentiation (Fig. 4). The gene expression profile in groups 3 and 4 were most compatible with a SC genetic profile.

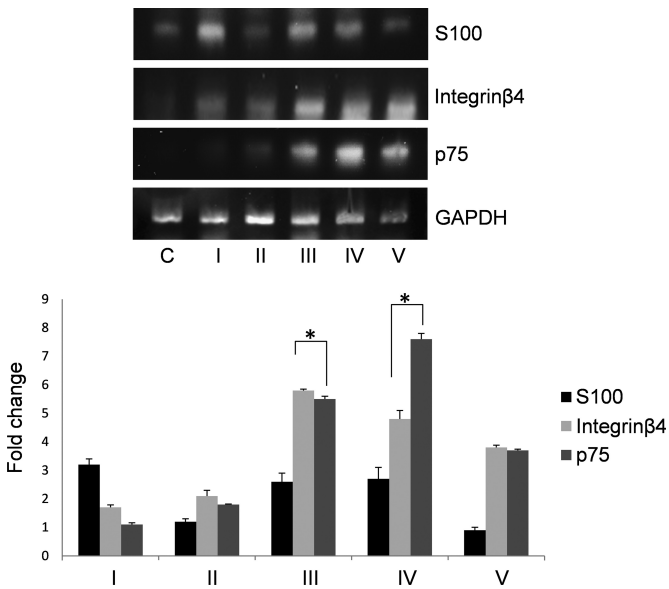


**FIGURE 4.** The fold changes in the expression levels of SC-specific genes were significantly higher in groups 3 and 4 in comparison to other groups. However, S100 was expressed in significant levels even in undifferentiated ASCS group. Differentiation medium lacking GGF (group 2) did not induce the expression of SC-specific genes, demonstrating the key role of GGF in SC-like differentiation. Agarose gel electrophoresis of PCR product revealed intense bands corresponding to p75 and integrin-β4 in groups 3 and 4.

## DISCUSSION

The most commonly used differentiation protocol for SC-like differentiation of ASCs uses a cocktail of forskolin, platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) after a pretreatment with β-mercaptoethanol and RA.[7] The rationale for adding GFs to the SC differentiation medium originates from studies that are carried out mostly on central nervous system cells.[15–20] Some of these GFs have several other functions in other bodily systems. However, the studies exploring the individual roles of these GFs in the process of SC-like differentiation of MSCs are limited.

Basic fibroblast growth factor plays a central role in proliferation, migration, and differentiation of oligodendrocyte progenitors in the central nervous system.[16,21] In a recent study, Zhu et al claimed that bFGF is a key regulator of SC-like differentiation of MSCs, but omission of GGF from the SC differentiation medium did not affect the induction of SC-like phenotype or the expression level of the S100 in induced MSCs.[22] Alone, bFGF could induce SC-like morphological changes in MSCs and contribute to increased S100 expression. However, naive MSCs express neuronal and glial proteins even before differentiation, and S100 expression is mainly modulated by other factors of the SC-like differentiation medium during the differentiation.[23] Therefore, it is possible to maintain a high level of S100 expression during SC-like differentiation of MSCs even without bFGF[22] as also shown in our study. Moreover, our data suggested that bFGF has no significant role in SC-like differentiation of ASCs as group 4, lacking bFGF in differentiation medium, still exhibited an increased expression of SC-specific genes *p75* and *integrin-β4*.

Unlike Zhu et al, we also could not detect any SC-specific protein expression in GGF-omitted group. GGF, also known as heregulin, serves as a lineage determination signal that directs neural crest cells to develop into SC.[24] An extensive line of research has revealed that GGF is crucial for promoting the SC growth and survival, migration along the extending axon, and myelination.[20,25,26] Our data confirmed the central, indispensable role of GFF in SC-like differentiation of ASCs. The reason for the discrepancy between our findings and the findings of Zhu et al can be the different types of MSCs (bone marrow-derived stem cells vs ASCs) used in these 2 studies, longer duration of differentiation (1 week vs 2 weeks) and also higher concentrations of forskolin and GGF that we used in our study.

Forskolin stimulates the myelination-associated gene expression and myelin production in SCs via elevating the intracellular cyclic adenosine monophosphate in proliferating SCs.[27–30] However, we observed a satisfactory level of SC-specific protein expression in forskolin (−) group (group 3), which could be explained by the effects of forskolin mainly focusing on myelination. Of note, we did not perform a myelination study or an animal study to evaluate the function of the SC-like cells differentiated from ASCs. Therefore, SC-like cells obtained in group 3 may not be functional, although they exhibit increased expression of SC-specific genes.

We could not detect any detrimental effect of PDGF omission on the SC-like differentiation of ASCs. We also could not find strong evidence in the literature to support the importance of PDGF in the SC-like differentiation process. Stimulation with serum or other GFs including PDGF, insulin like growth factor, and FGF increased proliferation but did not induce the expression of myelination-associated genes in SCs.[27] An interesting finding suggesting a role for PDGF in SC development is the increased expression of PDGF receptors on SC precursor cells. However, increased receptor expression was uncorrelated with the proliferation or differentiation of SC precursors in the peripheral nervous system, suggesting a different role of PDGF in SC development.[31]

In conclusion, our data suggested that GGF is a key component of SC-like differentiation medium, but the omission of bFGF and PDGF from the differentiation medium did not have a significant detrimental effect on the SC-like differentiation of ASCs.

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