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Notch independent signalling mediates Schwann cell-like differentiation of Adipose Derived Stem Cells

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ABSTRACT

Adipose derived stem cells (ASC) differentiate into a Schwann cell (SC)-like phenotype but the signalling pathways mediating this are unknown. We hypothesised that notch might be involved, given its important role in regulating SC development. Rat ASC were differentiated using bFGF, PDGF, GGF-2 and forskolin. RT-PCR analysis showed that mRNA for notch-1 and notch-2 receptors and the notch responsive gene, hes-1, were expressed throughout the differentiation process whereas jagged-1 a notch ligand, and the hey-1 gene were markedly down-regulated. In contrast delta-1 was up-regulated with differentiation and was strongly expressed by rat primary SC. Treatment of ASC with N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT), a gamma-secretase inhibitor which blocks notch signalling, had no effect on up-regulation of SC proteins S100 or GFAP during differentiation. Furthermore, when co-cultured with NG108-15 neurons, differentiated ASC cultures treated in the absence or presence of DAPT enhanced neurite outgrowth to similar levels. Differentiated ASC expressed PMP-22 but P0 was only present when co-cultured with dorsal root ganglia neurons. DAPT did not affect the expression of these myelin proteins. Thus, ASC express components of the notch signalling pathway but our studies suggest notch is unlikely to play a role in the neurotrophic activity and myelination capability of ASC differentiated into SC-like cells.

Adipose tissue is a clinically desirable source of stem cells because of its relative expendability and the ease with which it can be obtained through minimally invasive liposuction procedures. Within adipose tissue there is a heterogenous stromal stem cell population, generically named adipose derived stem cells (ASC), which have the ability to differentiate into a variety of cell types [7] including neural and glial cells [11,13,19,27]. We have shown it is possible to direct ASC differentiation towards a Schwann cell (SC)-like phenotype [12]. Other groups have also recently shown the potential of differentiated ASC to myelinate neurons *in vitro* [25]. However, to date the signalling pathways underlying this SC-like differentiation are unknown.

Schwann cells (SCs) are derived from the neural crest and develop through a number of intermediate cell phenotypes (reviewed in Ref. [22]). SC precursors are generated from neural crest stem cells and first appear in rat nerves around embryonic day 14/15. They then develop into immature SCs, a phenotype characterised by the up-regulation of markers such as GFAP and S100 (reviewed in Ref. [9]), before finally forming either myelinating or non-myelinating cells. A number of reports suggest notch signalling functions at different stages of SC development, acting both on the neural crest stem cell [10] and mediating the generation of immature SCs from the precursor stage [21]. Notch genes encoding members of a family of receptors which mediate cell–cell signalling events are expressed by neural crest cells [4]. Notch is a potent glial cell inducing cue in the neural crest, causing an irreversible switch from neuro- to gliogenesis [17]. Following binding to a ligand notch receptors are cleaved, generating an intracellular fragment (NICD) which translocates to the nucleus to regulate transcription (reviewed in Ref. [5]).

In this study we have analysed the expression of notch signalling components in ASC and investigated whether notch thus plays a role in their differentiation towards a SC phenotype. ASC were isolated from the visceral fat of adult rats as previously described [12] in compliance with the UK Animals (Scientific Procedures) Act 1986. Cells were maintained at 37 °C with 5% CO_2 in growth medium consisting of Modified Eagle Medium (α-MEM; Invitrogen, UK) with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin solution. SCs were harvested from the sciatic nerves of adult rats and maintained as previously described [1].

ASC at passage 2–4 were differentiated to a SC phenotype as previously described [12]. Cells were incubated for 2 weeks with 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech Ltd, UK),

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Table 1
Primer sequences for RT-PCR.

Molecule	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Annealing temp. (°C)
notch-1	CTTGTGAAAATGACGCCCG	CCTTATTGCCTGCATCCTCCT	66.4
notch-2	TTTGCTGTCGGAAGACGACC	GCCCATGTTGTCCTGGGCGT	67.8
jagged-1	ATGGCCTCCAACGATACTCCT	ACATGTACCCCCATAGTGGCA	64.9
delta-1	CACGAGAAAACCAGAAGC	ATGCCCGGAAAGTCTATGTG	52
hes-1	GTCCCCGGTGGCTGCTAC	AACACGCTCGGGTCTGTGCT	69.3
hey-1	AAAGACGGAGAGGCATCATCG	GCAGTGTGCAGCATTTTCAGG	54.2
β-Actin	ACTATCGGCAATGAGCGGTTC	AGAGCCACCAATCCACACAGA	67.7
HPRT	CAGGCCAGACTTTGTTGGAT	TCCACTTTCGCTGATGACAC	54.9

5 ng/ml platelet-derived growth factor (PDGF; PeproTech Ltd, UK), 252 ng/ml glial growth factor-2 (GGF-2), and 14 μM forskolin. In some experiments ASC were differentiated in the presence of a notch signalling inhibitor, 25 μM N-[N-(3,5-difluorophenacetyl-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), and medium was completely replenished every 3 days.

Total RNA was isolated from control ASC cultures and at key stages of differentiation (day 4 following retinoic acid treatment, day 5 following 24 h stimulation with glial growth factors and after 2 weeks of differentiation). A One-Step RT-PCR kit (QIAGEN, UK) was used with 1 ng RNA per reaction mix with primers [18] manufactured by Sigma, UK (Table 1). An MJ Research PTC-200 (gradient) cycler was used with previously described parameters [16] and annealing temperatures were optimised per primer set as described in Table 1.

Immunocytochemistry and Western blotting were performed as previously described [12]. Cells were incubated with mouse anti-glial fibrillary acidic protein (GFAP; 1:200; Chemicon, USA) and rabbit anti-S100 (1:500; Dako, Denmark) overnight at 4 °C and then treated with goat anti-mouse CY3 (1:200; Amersham, UK) or goat anti-rabbit FITC (1:100; Vector Labs, UK) conjugated secondary antibodies. For Western blotting, 10 μg protein per sample was resolved with sodium dodecyl sulphate–polyacrylamide gels, transferred to nitrocellulose and membranes were blotted overnight at 4 °C with either mouse anti-GFAP (1:200; LabVison, USA) or mouse anti-S100 (1:750; Chemicon, UK) antibodies.

For neuron co-culture differentiated ASC were plated at a density of 10,000 cells and allowed to settle for 24 h. DAPT notch inhibitor treated cultures were subsequently washed extensively to eliminate possible direct effects of the drug on neurite outgrowth. NG108-15 neurons (ECACC; Porton Down, UK) were then added to the ASC monolayers at a density of 1000 cells and the co-cultures were maintained for a further 24 h. Fluorescent immunocytochemistry using mouse anti-neurofilament protein antibody (1:500; Abcam, UK) as previously described [12] was used to visualise NG108-15 neurite outgrowth on the ASC. The average length and neurite number per NG108-15 cell body was determined. An average of 100 NG108-15 cell bodies were analysed for each condition in each experiment ($n = 3$) and data are presented as mean ± SEM. Kruskal–Wallis one-way ANOVA with Dunns comparison test was used to determine the statistical significance between data, $^{*}P < 0.05$.

Dorsal root ganglia (DRG) neurons were harvested from adult rat spinal cord as previously described [16]. Dissociated neurons were plated in modified Bottenstein and Sato's medium on 6-well plates pre-coated with poly-D-ornithine and laminin-1. After 2 h, 150 ng/ml nerve growth factor was added to allow neurons to develop neurites for 3 days. After 3 days of culture, neurons were co-cultured with differentiated ASC which were directly seeded over the neurons in the absence or presence of DAPT. After 24 h, 100 μg/ml brain derived neurotrophic factor (Autogen Bioclear, UK) and 50 μg/ml ascorbate were also added and left to activate the myelination process for 14 days at which point RNA was extracted from the cells and protein zero (P0) and peripheral myelin protein-22 (PMP-22) transcripts assessed.

RT-PCR analysis showed that untreated proliferating ASC expressed notch-1, notch-2, jagged-1, hes-1 and hey-1 mRNA but not delta-1 mRNA (Fig. 1A). ASC treated with a protocol to induce differentiation to the SC phenotype maintained expression of notch receptors and hes-1 but following retinoic acid treatment there was a progressive reduction in jagged-1 and hey-1 levels (Fig. 1A). In contrast delta-1 ligand was up-regulated at the end of the differentiation process. SCs showed relatively high levels of notch-1, jagged-1 and delta-1 mRNA when compared to differentiated ASC (Fig. 1A). When ASC were treated with the gamma-secretase inhibitor, DAPT, there was a noticeable reduction in the transcript levels of the notch responsive gene, hes-1 (Fig. 1B), indicating notch signalling could be functionally inhibited.

ASC were differentiated in the absence or presence of DAPT notch inhibitor for a period of 2 weeks and then assessed by immunocytochemistry and Western blot analysis for the expression of glial GFAP and S100 proteins (Fig. 2). GFAP was expressed at varying intensity throughout the entire differentiated ASC population and was co-expressed with S100 protein in 48.05 ± 7.29% of the cells (Fig. 2A). There was no significant difference in the expression levels when the cells had been treated with DAPT which was confirmed by Western blot analysis (Fig. 2B). Lysates from both ASC differentiated in the absence or presence of DAPT were probed with an S100 antibody and showed a 15 kDa band with similar intensity levels. This band was absent in the undifferentiated ASC. A 55 kDa band corresponding to GFAP was observed in both sets of differentiated lysates but was absent in undifferentiated samples (Fig. 2B). SC lysates showed an additional lower band which is likely to represent a proteolytic GFAP fragment or alternate transcript. When differentiating growth factors were removed after the 2-week treatment, expression of SC markers was maintained suggesting a stable phenotype (data not shown).

Differentiated ASC enhance neurite outgrowth in a co-culture model with the NG108-15 motor neuron-like cell line [12]. We thus tested the ability of ASC differentiated in the presence of DAPT to perform this function (Fig. 3). Control cultures of NG108-15 neurons extended 0.38 ± 0.04 neurites/cell with a mean length of 41.00 ± 7.60 μm. These parameters were significantly ($P < 0.05$) enhanced to 1.64 ± 0.03 neurite/cell with a length of 147.72 ± 9.44 μm in the presence of differentiated ASC. DAPT treated ASC showed a small but insignificant decrease to 1.29 ± 0.15 neurite/cell and length 133.48 ± 1.26 μm, suggesting notch signalling does not mediate the ability of differentiated ASC to promote neurite outgrowth. Next we used a co-culture with DRG neurons to investigate whether notch signalling plays a role in the ability of differentiated ASC to produce myelin proteins. DRG extended many neurites which made contact with the differentiated ASC (Fig. 4A). Analysis of mRNA showed that co-culture with DRG neurons up-regulated expression of P0 myelin protein which was unaffected by the inhibition of notch signalling (Fig. 4B). Differentiated ASC expressed PMP-22 in the absence of DRG neurons and this was moderately up-regulated by co-culture (Fig. 4B). DAPT