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Inhibition of γ-secretases alters both proliferation and differentiation of mesenchymal stem cells

S. Vujovic*, S. R. Henderson*, A. M. Flanagan† and M. O. Clements*,‡

**Wolfson Institute for Biomedical Research, University College London, London, UK, †Institute of Orthopaedics and Musculoskeletal Sciences, University College London, Stanmore, Middlesex, UK, and ‡Department of Molecular and Applied Bioscience, School of Biological Science, University of Westminster, London, UK*

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Abstract. *Introduction*: Human mesenchymal stem cell (hMSC) proliferation and development is regulated by many signalling pathways. γ-Secretases play an important role in Notch signalling as well as other processes that are involved in developmental decisions, but their role in hMSC proliferation and cell fate decisions has not been explored. *Objective*: To investigate the role of γ-secretases in hMSC proliferation and differentiation. *Materials and methods*: Using the γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), we investigated their role in hMSC growth and differentiation to chondrogenic, osteogenic and adipogenic fates. *Results*: We found that inhibiting γ-secretases reduced the rate of hMSC proliferation, and altered hMSC differentiation *in vitro*. Addition of DAPT had an inhibitory effect on chondrogenesis resulting in impaired cartilage matrix production and altered chondrocyte morphology. DAPT treated chrodrocytic pellets had reduced levels of Hes1 and Hey1 suggesting that these effects are mediated via Notch signalling. Addition of the DAPT inhibitor to osteogenic cultures did not alter the appearance of bone markers, however, adipogenesis occurred in these cultures in a DAPT concentration-dependent manner. DAPT did not enhance adipogenesis in the presence of a potent adipogenic cocktail, but had an adipogenic effect when combined with dexamethasone only. *Conclusion*: We conclude that γ-secretases play an important role in both hMSC proliferation and differentiation.

INTRODUCTION

Human adult mesenchymal stem cells (hMSC) are bone marrow-derived cells that have the potential to differentiate into various mesodermal lineages, including bone, cartilage, muscle and fat (Pittenger *et al.* 1999). There is great interest in hMSC due to their therapeutic potential, as well as their use as a model system for human developmental processes (Le Blanc & Pittenger 2005).

During development, the tight control of stem cell self-renewal and differentiation is required to generate complex organized tissues and organs. One mechanism employed to coordinate stem cell differentiation is the activation and inhibition of specific signalling pathways. Manipulation of hMSC *in vitro*, provides an ideal model system to investigate the

Correspondence: Mark Clements, School of Biosciences, University of Westminster, 115 New Cavendish Street, London W1W 6UW, UK. Tel.: +44 207911 5800 ext 3544; Fax: +44 207911 5087; E-mail: clemenm@wmin.ac.uk

role of signalling pathways in the commitment and progression of differentiation towards specific lineages. To date, wingless-type MMTV integration site family members (Wnts) (De Boer *et al.* 2004; Gaur *et al.* 2005), bone morphogenetic proteins (BMPs) (Sottile & Seuwen 2000), mitrogen-activated protein kinase family members (Jaiswal *et al.* 2000; Tuli *et al.* 2003), transforming growth factor family β members (TGFβs) (Mishra *et al.* 2005) and fibroblast growth factors (FGFs) (Tsutsumi *et al.* 2001; Davidson *et al.* 2005) are all implicated in the regulation of hMSC differentiation. For example, there is evidence that Wnt signalling is required for hMSC self-renewal, although it also promotes osteogenesis and chondrogenesis, but is inhibitory to adipogenic differentiation (De Boer *et al.* 2004; Gaur *et al.* 2005). Hence, a complex picture is emerging whereby activation of specific signalling pathways either promotes or inhibits lineage-specific differentiation.

Notch signalling is another pathway commonly involved in developmental processes, and is evolutionarily conserved from *Drosophila* to mammals (Artavanis-Tsakonas *et al.* 1999). Notch receptors interact with membrane bound delta/jagged ligands, hence signalling is only activated upon direct cell-to-cell contact (Baron *et al.* 2002 and references therein). Binding of ligand to the Notch receptor results in proteolytic cleavage of the receptor and the release of the intracellular domain. This proteolytic cleavage is a multi-step process, with the γ-secretase enzyme, presenilin, catalysing the final step. The cleaved Notch intracellular domain translocates into the nucleus and activates transcription of Hes and Hey gene family members. Hes and Hey proteins are basic helix-loop-helix (bHLH) transcriptional regulators that act as both homo- and hetero-dimers in repressing the transcription of target genes. This inhibition can occur either directly by the Hes and Hey dimer binding to specific repressor binding sites, or indirectly by binding to bHLH transcription factors, such as myogenic differentiation agent 1, and preventing them from forming functional hetero-dimers with other bHLH family proteins (Iso *et al.* 2003).

The role of Notch signalling in the developmental processes of a variety of organisms has been studied by the use of γ-secretase inhibitors including L-685 458 and DAPT (Micchelli *et al.* 2003; Tian *et al.* 2003). Exposure of *Drosophila* to DAPT blocks the release of Notch intracellular domain and produces the same phenotype as that resulting from mutations in the components of the Notch signalling pathway (Micchelli *et al.* 2003). DAPT is also an accepted inhibitor of Notch signalling in *in vitro* studies (Cheng *et al.* 2003; van den Brandt *et al.* 2004; Li *et al.* 2006). However, it is known that signalling molecules other than Notch protein act as targets for γ-secretases and therefore caution needs to be taken when interpreting data from experiments using such inhibitors (Kopan & Ilagan 2004).

Various reports have implicated γ-secretases and Notch signalling as being involved in chondrogenic, osteogenic and adipogenic development (Dowthwaite *et al.* 2004; de Jong *et al.* 2004; Ross *et al.* 2004). A variety of cell types and species was used in these studies, however, the role of γ-secretases in hMSC biology has not previously been determined. To address this, we used the inhibitor DAPT to investigate the role of γ-secretases in proliferating and differentiating hMSC.

MATERIALS AND METHODS

hMSC isolation and culture

Mesenchymal stem cells were isolated from human bone marrow aspirates obtained after informed consent according to local ethical guidelines. Donors were healthy individuals between 24 and 40 years old. After aspiration, the mononuclear fraction of bone marrow was separated

by Ficoll gradient centrifugation (Ficoll-Paque PLUS, GE, Little Chalfront, UK). hMSC were obtained by washing the bone marrow mononuclear cells twice with phosphate-buffered saline (PBS) and finally re-suspending in Mesencult medium (Stem Cell Technologies, Vancouver, BC, Canada) containing 1 ng/ml bFGF (RnD Systems, Minneappolis, MN, USA) seeding 1×10^5 cells/cm^2 on tissue culture plates. The hMSC adhered to the culture dish and non-adherent cells were washed off in a medium change 2 days after isolation. hMSC were routinely passaged every 3 days using 0.25% Trypsin ethylenediaminetetraacetic acid (Invitrogen, Paisley, UK) and cells were re-seeded on tissue culture plastic at a density of $2–5 \times 10^3$/cm^2. Experiments were performed on hMSC isolated from three donors. Similar trends were observed between donors and therefore results from a single bone marrow are presented.

Growth curves

Growth curve assays were performed every 3 days in 6-well plates (surface area: ~9.6 cm^2). Cells were grown in Mesencult medium containing 1 ng/ml bFGF. Initially 60 000 cells were seeded and were allowed to grow for 3 days, when they were detached from the well using 300 μl of 0.25% Trypsin ethylenediaminetetraacetic acid, re-suspended with 1 ml of medium and were counted using a haemocytometer. After the counts, 60 000 cells were seeded. Cell counts were performed in triplicate per culture condition.

DAPT treatment

DAPT (Merck Biosciences, Darmstadt, Germany) was dissolved in DMSO (Sigma, Pool, UK) to make 20 mM and 10 mM stocks that were then added to the hMSC cultures at 1 : 1000 dilution, producing a final concentration of 10 μM DAPT and 20 μM DAPT. In all cases where DAPT treatment was used in conjunction with differentiation medium, DAPT or DMSO was applied 16 h prior to induction of differentiation and alongside the differentiation inducers throughout the differentiation. hMSC were allowed to reach confluence before DAPT or DAPT plus 100 nM dexamethasone treatment.

Chondrogenic differentiation

Chondrogenic differentiation was adapted from Sekiya *et al.* (2002). Chondrocyte differentiation medium comprised high glucose DMEM (Invitrogen) supplemented with 1% penicillin/streptomycin (Invitrogen), 10^{-7} M dexamethasone (Sigma), 50 μg/ml ascorbate-2-phosphate (Sigma), 40 μg/ml l-proline (Sigma), 100 μg/ml sodium pyruvate (Invitrogen), 50 mg/ml ITS + Premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, 5.35 mg/ml linoleic acid) (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA), 500 ng/ml BMP6, 10 ng/ml TGFβ3. hMSC (250 000) were suspended in 500 μl of chondrocyte differentiation media, placed in a 15-ml falcon and centrifuged at 600 ***g*** for 5 min allowing the cells to form a loose pellet (day 0). These pellets were incubated at 37 °C, 5% CO_2 for 4 days, at which time the differentiation medium was changed. Media changes were then performed every 3 days. Pellets were harvested on day 21 unless otherwise stated. Six pellets were grown per condition, and the experiment was performed in duplicate. In the case of DAPT treatment, the hMSC were exposed to either 1 μl/ml DMSO, 1 μl/ml DMSO with 10 μM DAPT or 1 μl/ml DMSO with 20 μM DAPT 16 h prior to growing in pellet cultures and throughout the differentiation process.

Pellet measurements were taken using a graticule eye-piece. For histology, pellets were embedded in paraffin wax and were sectioned at 10 μM. Slides were dewaxed in xylene for 10 min, re-hydrated in 100% ethanol 10 min, 90% ethanol 5 min, 70% ethanol 5 min and washed in distilled water. Toluidine blue was used to depict the proteoglycan content of the

pellets. The stain (0.1% w/v toluidine blue dissolved in 0.1 M sodium phosphate buffer [188 ml of 0.1 M NaH_2PO_4 and 12 ml 0.1 M Na_2HPO_4]) was applied for 45 s and was washed with distilled water. Tissues were then dehydrated by consecutive ethanol washes (70%, 90% and 100%), and were dipped in xylene for 5 min. Samples were air-dried and mounted with DPX.

Adipogenic differentiation

hMSC were grown to confluence, at which point the adipogenic medium comprising Mesencult and supplement with 1 μM dexamethasone (Sigma), 500 μM IBMX (Sigma), 2 μM indomethacin (Sigma), 10 μg/ml insulin (Roche) was added. The medium was changed every 3 days. In the case of DAPT treatment, hMSC were exposed to either 1 μl/ml DMSO, 10 μM DAPT or 20 μM DAPT 16 h prior to exposing to differentiation media and thereafter.

Adipocytes were identified using oil red O lipid stain (Sigma). This entailed removing the medium from the plates, rinsing the cells with PBS, and fixing them by applying 8 ml of 4% formaldehyde, 75 mM sodium phosphate buffer, 3% methanol for 30 min. Fixative was then removed and the plate was washed three times with PBS, before applying 8 ml of oil red O dye (600 μg/ml oil red O, dissolved in isopropanol; diluted 60 : 40 in water and filtered) and incubating in the dark for 1–4 h. Plates were rinsed with 50% ethanol and covered with PBS.

Osteogenic differentiation

hMSC were grown to confluence, at which point the osteogenic medium, comprising Mesencult supplemented with 100 nM dexamethasone, 10 μM β-glycerol phosphate (Sigma) and 50 μg/ml ascorbate-2-phosphate (Sigma) was added. The medium was changed every 3 days. In the case of DAPT treatment, hMSC were exposed to either 1 μl/ml DMSO, 10 μM DAPT or 20 μM DAPT 16 h prior to exposing to differentiation media and throughout differentiation.

A kit was used to detect the presence of alkaline phosphatase (Sigma). This involved exposing the cells to a 2% citrate/60% acetate solution for 30 s, and then rinsing twice with dH_2O. The stain (one dye capsule dissolved in 49 ml dH_2O with 2 ml Naphthol AS-MX) was applied for 30 s, after which the cells were rinsed with dH_2O for 2 min. Mayer's haematoxylin (Sigma) solution was applied for 10 min, when it was removed by two washes with dH_2O.

RNA isolation and reverse transcription-polymerase chain reaction

RNA was isolated from cells using the RNAeasy kit (Qiagen, Sussex, UK). Reverse transcription-polymerase chain reactions (RT-PCR) were performed using the Superscript II enzyme kit (Invitrogen) according to the manufacturer's instructions and with 100 ng starting RNA. The subsequent PCRs were performed using DyNAzyme kits (Finnzymes, Espoo, Finland) in 25 μL reaction volumes using 2.5 μl of 10X reaction buffer containing 50 mM $MgCl_2$, 1 μl of 10 mM dNTP (Promega), 2.5 μl of 10 μM of each primer (Thermo, Ulm, Germany), 0.25 μl of 5 U/ml Taq polymerase, 1.25 μl DMSO and 1 μl cDNA. The cycling times were, 94 °C for 2 min; and then 28 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 45 s; 72 °C for 10 min. Reactions were then run on a 1.5% agarose gel and the following primers were used:

Hes1 (forward):	CTACCCCAGCCAGTGTCAAC
Hes1 (reverse):	CATTGATCTGGGTCATGCAG
Hey1 (forward):	AACTGTTGGTGGCCTGAATC
Hey1 (reverse):	CCAGTTCAGTGGAGGTCGTT
Col2a1 (forward):	TTCAGCTATGGAGATGACAATC
Col2a1 (reverse):	AGAGTCCTAGAGTGACTGAG
GAPDH (forward):	GATCATCAGCAATGCCTCCT
GAPDH (reverse):	TGTGGTCATGAGTCCTTCCA

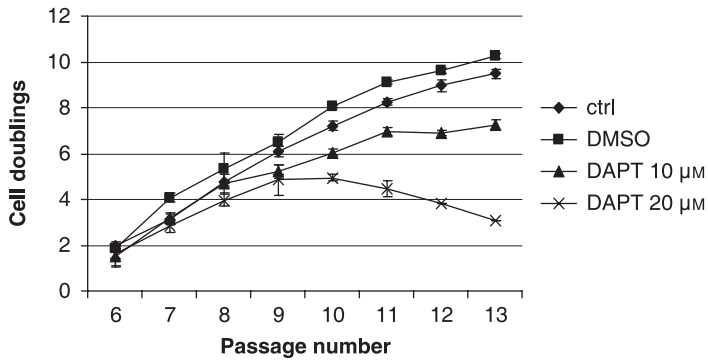

Figure 1. Inhibiting γ-secretases impairs hMSC self-renewal. hMSC were exposed to DAPT, the rates of proliferation determined (population cell doublings) and compared to DMSO treated and untreated cells (ctrl). Student's *t*-test DMSO versus DAPT-treated growth rates ***$P < 0.001$.

RESULTS

DAPT effect on hMSC proliferation

To examine the role of γ-secretases on hMSC proliferation, we exposed proliferating hMSC to the γ-secretase inhibitor DAPT at 10 and 20 μM concentrations. The addition of 10 μM DAPT slowed hMSC growth and finally inhibited cell division after six passages (Fig. 1). Increasing the concentration of DAPT to 20 μM inhibited cell division within four passages and resulted in growth arrest. Therefore, although DAPT did not immediately inhibit hMSC growth, long-term exposure led to growth arrest, implicating a role for γ-secretases in MSC proliferation *in vitro*.

DAPT effect on chondrogenesis

We examined the impact of γ-secretases on the ability of hMSC to differentiate towards the chondrocytic lineage using a pellet culture model system. The addition of DAPT to hMSC pellet cultures significantly reduced the final pellet size by ~50%, as demonstrated by graticule eye-piece measurements (Fig. 2a). The effect was not enhanced by doubling the concentration of DAPT. Toluidine blue-stained sections of the pellets, presented in Fig. 2(b), show that the control and DMSO-treated samples had accumulated large amounts of cartilage matrix and the cells within the matrix were round and set within lacunae – a morphological appearance typical of chondrocytes. The DAPT-treated pellets embodied a pronounced reduction in matrix accumulation compared to control cultures and the cells had a less uniform appearance. In addition, the DAPT-treated cultures had an outer rim of spindle cells surrounding the cartilaginous core. Exposing chondrogenic cultures to the higher concentration of DAPT (20 μM) seemed to enhance this effect.

RT-PCR analysis of RNA isolated from cartilage pellets (at day 21) detected the expression of Notch effectors Hes1 and Hey1 in control and DMSO treated pellets (Fig. 2c). Their expression was diminished by the addition of DAPT. Although the DAPT inhibitor caused a considerable decrease in mRNA levels, it did not completely obliterate the expression of Hes1/Hey1. In order to show that decrease in Hes1 and Hey1 levels was a direct consequence of DAPT treatment, rather than an indirect consequence of the impaired chondrogenic differentiation, RNA was also isolated from control, DMSO and DAPT-treated pellets, 6 h after the exposure to differentiation reagents and the same results were observed as above (data not shown).

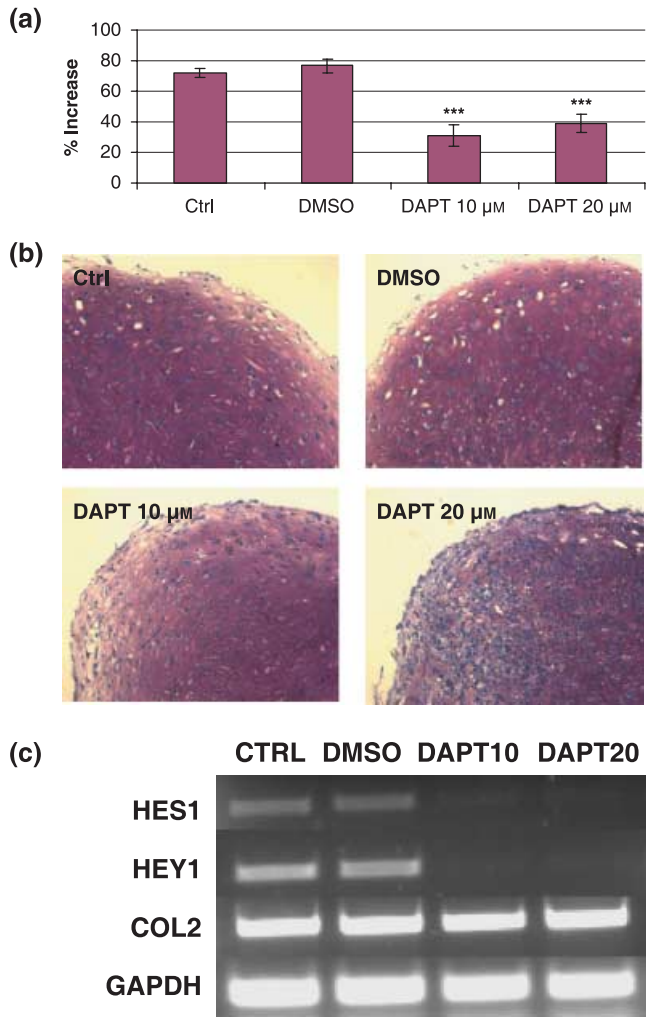

Figure 2. DAPT treatment significantly reduces the size of chondrogenic pellets, decreases matrix accumulation and reduces the levels of HES1 and HEY1. (a) Chondrogenic pellet diameters were measured on day 7 and day 21 and the percentage increase between these two measurements plotted. Student's *t*-test DMSO versus DAPT-treated pellets $***P < 0.001$. (b) A transmitted light photomicrograph of toluidine blue-stained sections of pellets (c) Gel electrophoresis showing gene expression as assessed by RT-PCRs performed on RNA isolated from chondrogenic pellets.

DAPT effect on osteogenesis and adipogenesis

DAPT did not alter osteogenic differentiation, as assessed by alkaline phosphatase and alizarin red staining under transmission light microscopy (Fig. 3a and data not shown). However, it was noted that addition of DAPT to osteogenic cultures induced formation of a large number of cells containing lipid droplets, which had typical adipocyte morphology under higher magnification (Fig. 3a and 3a inset). While it is common to note an occasional adipocyte in bone-forming cultures, adipogenesis was significantly increased in the DAPT-treated cultures, demonstrated by a 7- and 12-fold rise in adipocyte numbers in the presence of 10 μM and 20 μM DAPT, respectively (Fig. 3b).

Treatment of hMSC with either 10 μM or 20 μM DAPT in adipogenic cultures had no visible effect on adipocyte development, as assessed by oil red O staining (Fig. 4a). Therefore, to

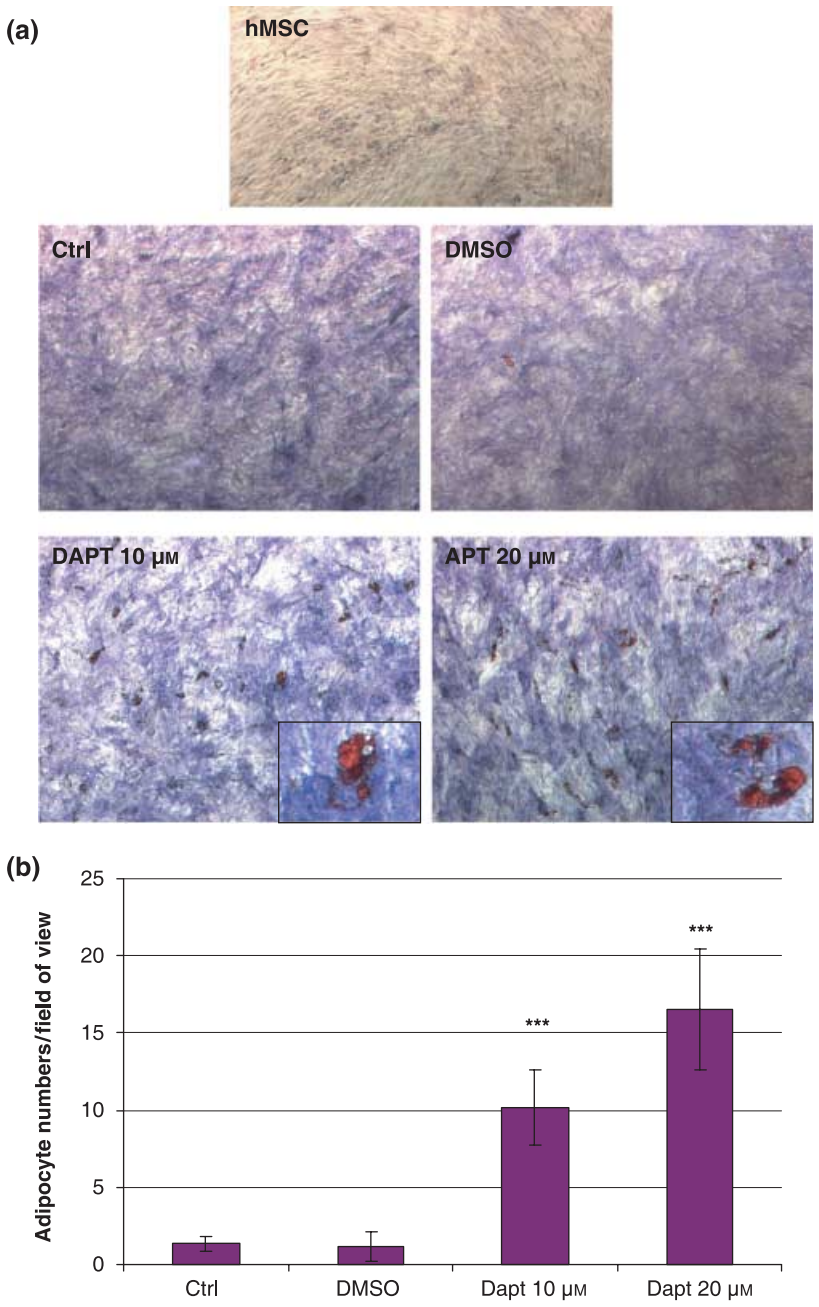

Figure 3. DAPT treatment induces adipocyte formation in osteogenesis inducing medium. (a) A transmission light photomicrograph showing alkaline phosphatase and oil red O staining of untreated hMSC and those that have undergone osteogenesis (Ctrl) or osteogenesis in the presence of the DMSO vehicle, 10 μM DAPT or 20 μM DAPT. Inset, higher magnifications of the observed adipocytes. (b) Quantification of number of adipocytes per field of view seen in (a). Student's *t*-test DMSO versus DAPT-treated cultures ***$P < 0.001$.

elucidate further the adipogenic effect of DAPT seen in the osteogenic cultures above, we exposed confluent hMSC to the DAPT inhibitor alone and in the presence of 100 nM dexamethasone. DAPT by itself was not a sufficient stimulus to induce adipogenesis but together with 100 nM dexamethasone, lipid laden cells appeared as in the osteogenic cultures, indicating that the combination of these two chemicals was sufficient to induce adipogenesis (Fig. 4b). Figure 4(c) illustrates quantification of this result.

DISCUSSION

Development of complex tissues and organs requires tight control on progenitor cell proliferation and differentiation. This control is mediated by the complex interplay of different signalling pathways. γ-Secretases target a range of signalling pathways, but play a pivotal role in regulating Notch signalling, which is an evolutionary conserved pathway that influences the balance between proliferation and differentiation of a diverse range of progenitor cells (Sarmento *et al.* 2005). By using the γ-secretase inhibitor DAPT, we show for the first time that γ-secretases are involved in both the proliferation and differentiation of hMSC to mesodermal tissues.

Here, we demonstrate that DAPT treatment of proliferating hMSC resulted in growth arrest, pointing to a role for γ-secretases in hMSC self-renewal. γ-secretases have also been implicated in the self-renewal of HSCs, where the interplay between Wnt and Notch pathways is required to maintain cell proliferation (Duncan *et al.* 2005). It is possible that a similar conserved mechanism is involved in hMSC proliferation, because Wnt signalling is also known to be active and to be required for hMSC expansion (Etheridge *et al.* 2004; Duncan *et al.* 2005).

Besides the role of γ-secretases in hMSC proliferation, we show that it is also involved in cell fate decisions. The addition of DAPT to hMSC undergoing chondrogenesis significantly impaired their differentiation. Inhibiting γ-secretases did not inhibit pellet formation but reduced pellet size due to decreased matrix production. Addition of DAPT also caused morphological changes in the chondrocytes, in particular in the outer layers of the pellet where they did not appear rounded or produce the lacunae as seen with the untreated control pellets, possibly because of the partial penetrance of the inhibitor. The expression of downstream Notch target genes Hes1 and Hey1, normally present during hMSC chondrogenesis, was also dramatically reduced, although not completely abolished, during DAPT treatment. This suggests that the effect of the γ-secretase inhibitor is mediated *via* Notch signalling. Residual expression of Hes1 and Hey1 in the chondrocytic pellets may be related to cells in the core of the pellet where the DAPT may have failed to penetrate. These results are in accordance with previous findings where DAPT treatment was reported to impair proteoglycan production in chondroblasts isolated from joints, but in contrast the results obtained with the ATDC5 cell line where Hes1 over-expression lead to a suppression of chondrogenic differentiation (Watanabe *et al.* 2003; Dowthwaite *et al.* 2004). One explanation for this might be that a transient Notch signal is required for chondrogenesis, and that either blocking or continually activating Notch signalling results in inhibition of chondrogenesis.

γ-Secretases also appear to be involved in the cell fate choice between osteogenesis and adipogenesis as the addition of DAPT to osteogenic cultures caused adipocyte formation. The adipogenic effect can also be achieved by addition of DAPT and 100 nM dexamethasone but not by the addition of DAPT alone. The extent of adipogenic induction in these cultures is dependent on the DAPT concentration as the number of adipocytes nearly doubled when the DAPT concentration was increased from 10 μM to 20 μM. When DAPT was added to hMSC differentiating

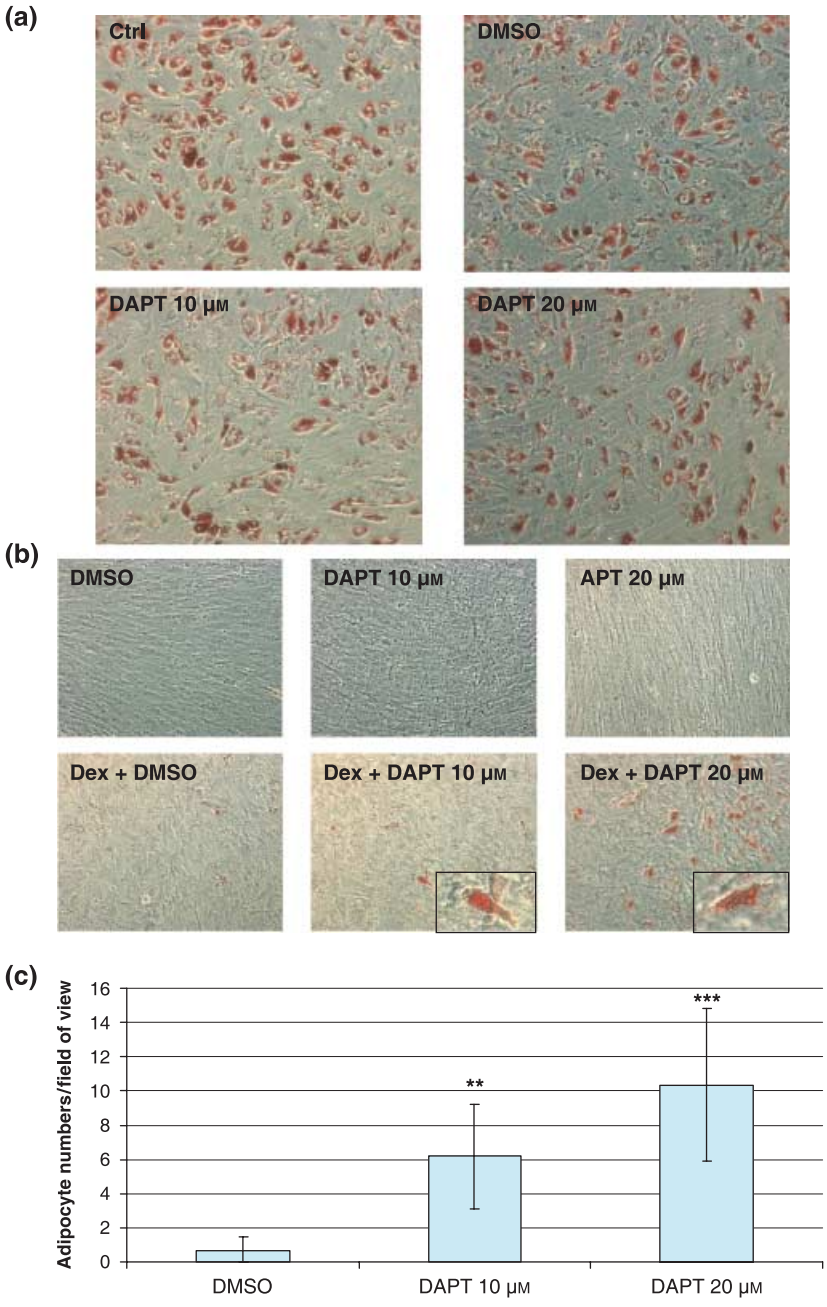

Figure 4. Adipocyte formation is not induced by DAPT treatment alone but is induced by a combination of 100 nM dexamethasone and DAPT. (a) A transmission light photomicrograph showing oil red O stained adipogenic differentiation of hMSC induced by a complex adipogenic cocktail (Ctrl) and in the presence of DMSO or DAPT. (b) A transmission light photomicrograph of oil red O stained of confluent hMSC exposed to DMSO and DAPT alone or in combination with 100 nM dexamethasone (Dex). (c) Quantification of adipocytes per field of view in (b). Student's *t*-test DMSO versus DAPT-treated cultures $^{**}P = 0.002$, $^{***}P < 0.001$.

in adipogenic media, no further enhancement of adipogenesis was seen. These cells were exposed to a very potent adipogenic cocktail, and it is therefore probable that they had already reached their full adipogenic capacity. Previous studies on the role of Notch signalling in adipogenesis have produced contradictory results. Inhibition of Notch signalling in the murine preadipocyte cell line 3T3L1 impaired adipogenesis, while murine embryonic fibroblast and murine embryonic stem cell adipogenesis was unimpaired (Nichols *et al.* 2004; Ross *et al.* 2004). In contrast, our observations argue that γ-secretases play an inhibitory role for human adipogenesis. As, only a small proportion of hMSC differentiated to adipocytes in response to DAPT and dexamethasone, other factors are required for efficient commitment of cells to fat.

DAPT is a specific γ-secretase inhibitor, but the function of such enzymes is not exclusive to the Notch pathway (Iwatsubo 2004; Kopan & Ilagan 2004). We therefore can not conclude that all the effects we observed are solely due to Notch signalling. However, in our chondrogenic cultures we observed a concomitant inhibition of extracellular matrix production and a significant decrease in Hes1 and Hey1 mRNA expression (downstream targets of Notch signalling) upon addition of DAPT, implying a direct role for Notch signalling in this process. We observed no decrease in Hes1 and Hey1 in response to DAPT treatment in our proliferation assay, nor in our adipogenic and osteogenic cultures, as the level of their expression were below detectable threshold of our RT-PCR analysis (data not shown). Because the Hes and Hey family have been shown to play a part in chondrogenic, adipogenic and osteogenic differentiation in a number of cell lines, it seems more plausible that our findings are due to the disruption of Notch signalling (Watanabe *et al.* 2003; Ross *et al.* 2004; Zamurovic *et al.* 2004 de Jong *et al.* 2004). Further experiments need to be performed to conclusively demonstrate that the DAPT effects reported in this paper are mediated solely by Notch. These experiments are complicated due to the low transfection efficiency of primary MSC preventing the use of molecular tools.

Taken together, our results infer a role for γ-secretases in the control of hMSC proliferation and cell fate determination. Signalling pathways dependent on γ-secretases are likely to interact with the other signalling pathways already implicated in hMSC function, as a means of regulating stem cell function. Our findings provide a platform on which to investigate further the factors regulating hMSC differentiation.

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