Oxidative Stress–Associated Senescence in Dermal Papilla Cells of Men with Androgenetic Alopecia

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Dermal papilla cells (DPCs) taken from male androgenetic alopecia (AGA) patients undergo premature senescence *in vitro* in association with the expression of $p16^{INK4a}$, suggesting that DPCs from balding scalp are more sensitive to environmental stress than nonbalding cells. As one of the major triggers of senescence *in vitro* stems from the cell "culture shock" owing to oxidative stress, we have further investigated the effects of oxidative stress on balding and occipital scalp DPCs. Patient-matched DPCs from balding and occipital scalp were cultured at atmospheric (21%) or physiologically normal (2%) O_2. At 21% O_2, DPCs showed flattened morphology and a significant reduction in mobility, population doubling, increased levels of reactive oxygen species and senescence-associated β-Gal activity, and increased expression of $p16^{INK4a}$ and pRB. Balding DPCs secreted higher levels of the negative hair growth regulators transforming growth factor beta 1 and 2 in response to H_2O_2 but not cell culture–associated oxidative stress. Balding DPCs had higher levels of catalase and total glutathione but appear to be less able to handle oxidative stress compared with occipital DPCs. These *in vitro* findings suggest that there may be a role for oxidative stress in the pathogenesis of AGA both in relation to cell senescence and migration but also secretion of known hair follicle inhibitory factors.

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INTRODUCTION

Androgenetic alopecia (AGA) is the most common form of male hair loss affecting 31% of genetically predisposed men aged 40–55 years (Hamilton, 1951; Nyholt *et al.*, 2003). AGA is a polygenic disorder with multiple risk loci (Hoffmann *et al.*, 2002; Heilmann *et al.*, 2013) driven predominantly by mutations of the X-linked androgen receptor (AR) gene (Ellis *et al.*, 2001). Oxidative stress may also have a role, as hair loss is linked to a number of factors that increase cellular oxidative stress, including metabolic syndrome, alcohol consumption, smoking, and UV radiation (Severi *et al.*, 2003; Trueb, 2003a,b; Su and Chen, 2010). However, it is not known whether oxidative stress is a pathogenic contributor to AGA.

The dermal papilla (DP) contains a population of pluripotent stem cells (Hunt *et al.*, 2008; Driskell *et al.*, 2011) and has a fundamental role in regulating hair follicle development and hair growth (Oliver, 1966; Jahoda *et al.*, 1984). ARs are expressed by DP cells (DPCs) and the actions of androgens on hair growth are believed to be mediated via androgen regulation of hair growth regulatory factors (Randall *et al.*, 1991; Choudhry *et al.*, 1992; Hibberts *et al.*, 1998). In balding DPCs (BDPCs), androgens stimulate the secretion of hair growth inhibitory factors such as transforming growth factor beta 1 and 2 (TGF-β1/β2) and DKK-1 (Foitzik *et al.*, 2000; Inui *et al.*, 2002; Hibino and Nishiyama, 2004; Kwack *et al.*, 2012). In contrast, the occipital DPCs (ODPCs) from occipital, nonbalding scalp regions are classically insensitive to androgens (Inui *et al.*, 2002; Randall, 2007).

Previously, we demonstrated that BDPCs underwent premature senescence *in vitro* compared with ODPCs (Bahta *et al.*, 2008) with a concomitant elevation of $p16^{INK4a}$ and retinoblastoma protein (pRB) expression, both of which are known to mediate cell cycle arrest in response to environmental stress (Chen, 2000). A major cause of cell stress *in vitro* stems from oxidative stress caused by reactive oxygen species (ROS; Alaluf *et al.*, 2000; Grayson *et al.*, 2006). Therefore, because the concentration of oxygen in the dermis has been measured at between 1 and 5% O_2 (Wang, 2005) and cell culture is usually carried out at atmospheric levels of oxygen (21% O_2) known to cause senescence *in vitro* (Alaluf *et al.*, 2000), we have suggested that the premature senescence of BDPCs *in vitro* may be in response to stress or ''culture shock'' of tissue culture and may be indicative that BDPCs are

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Study carried out in Whitechapel, London, UK.

Abbreviations: 4-MU-Gal, methylumbelliferyl galactopyranosidase; AGA, androgenetic alopecia; AR, androgen receptor; BDPC, balding dermal papilla cell; DHT, dihydrotestosterone; DP, dermal papilla; DPC, dermal papilla cell; GSH, glutathione sulfhydryl (reduced state); GSSG, glutathione disulphide (oxidized state); ODPC, occipital dermal papilla cell; PD, population doubling; pRB, retinoblastoma protein; ROS, reactive oxygen species; SA-β-Gal, senescence-associated beta-galactosidase; TGF-β1/2, transforming growth factor beta 1/2

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more sensitive than ODPCs to environmental—including oxidative—stress. We sought to investigate the effects of physiologically relevant oxygen and oxidative stress on the growth and cell signaling potential of both BDPCs and ODPCs. We demonstrate *in vitro* a previously unreported role for oxidative stress in the pathogenesis of AGA both in relation to cell senescence and secretion of known hair follicle inhibitory factors. Furthermore, these experiments also highlight the major impact that oxygen can have on the physiology and biochemistry of cultured DPCs.

RESULTS

Low oxygen culture increases BDPC and ODPC viability and migration

Patient-matched DPCs cultured from balding and occipital/nonbalding scalp biopsies demonstrate clear morphological differences when culture is performed in atmospheric (normoxia) versus physiological (hypoxic) O_2. Both ODPCs (Figure 1a) and BDPCs (Figure 1b) exhibited a morphology associated with stress at 21% O_2. BDPCs displayed a particularly severe phenotype, growing in a dispersed manner with flattened morphology classically associated with senescence (Alaluf *et al.*, 2000). ODPCs at 21% O_2 also had a mildly flattened morphology, although they grew in organized clusters and did not exhibit full senescent morphology (Figure 1a).

ODPCs grown at 2% O_2 maintained their healthy, spindle-like morphology and formed organized clusters with raised pseudopapillae (Figure 1c), a well-established characteristic of cultured DPCs (Almond-Roesler *et al.*, 1997). BDPCs at 2% O_2 maintained a spindle-like morphology typical of healthy fibroblasts (Figure 1d), although BDPC cultures did not form pseudopapillae.

Oxygen conditions also mediated significant changes in the migratory function of DPCs (Figure 1e). Time-lapse analysis of cells cultured in 21% O_2 revealed that ODPCs had a mean velocity of 0.12 μm per minute, which was over 8-fold faster than BDPCs (0.014 μm per minute) under the same conditions ($P<0.001$). At 2% O_2, the mean velocity of both DPCs significantly increased to 0.58 μm per minute for ODPCs and 0.34 μmper minute for BDPCs. Notably, there was still a significant difference between BDPCs and ODPCs at 2% O_2 ($P<0.01$), although low oxygen conditions appeared to reduce the relative difference in mean velocities between DPC types.

Oxidative stress decreases population doubling and increases senescence in DPCs

Measurement of population doubling (PD) and quantification of senescence-associated beta-galactosidase (SA-β-Gal) activity showed that ODPCs and BDPCs were more proliferative ($P<0.001$ for both ODPCs and BDPCs) and had reduced levels of senescence when cultured at 2% O_2 versus 21% O_2 (Figure 2). However, BDPCs underwent fewer PD than ODPCs under both oxygen conditions (12 vs. 16 PD at 2% O_2 ($P<0.001$) and 5 vs. 9 PD at 21% O_2 $P<0.001$; Figure 2a and b); furthermore, senescence was significantly higher in BDPCs at 21% O_2 than ODPCs ($P<0.001$; Figure 2c). There was no significant difference in senescence between ODPCs and BDPCs at 2% O_2.

To investigate whether the effects of oxygen on PD and senescence were reversible, we also switched cells at passage 4 from 21 to 2% O_2 and from 2 to 21% O_2 (Figure 2a and b). The slower growth rate of ODPCs and BDPCs at 21% O_2 was rescued when cells were switched to 2% O_2 ($P<0.05$ and $P<0.001$ for ODPCs and BDPCs, respectively), which correlated with the levels of cell senescence similar to those seen in cells grown continuously in 2% O_2 (Figure 2c). In contrast, PD decreased in cells switched from 2 to 21% O_2 ($P<0.01$ and $P<0.001$ for ODPCs and BDPCs, respectively) correlating

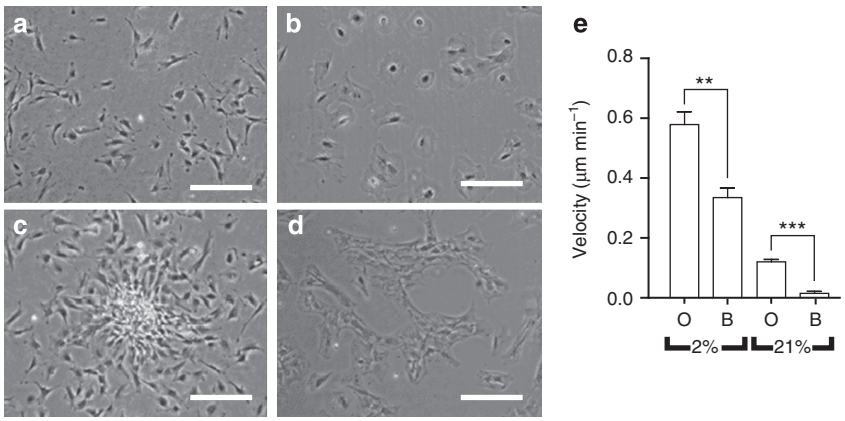

Figure 1. Dermal papilla cell (DPC) viability is directly affected by O_2 conditions. Morphology and arrangement of DPCs under varying O_2 conditions for the following: (**a**) ODPCs at 21% O_2, (**b**) BDPCs at 21% O_2, (**c**) ODPCs at 2% O_2, and (**d**) BDPCs at 2% O_2. Images are representative of cells from three individual patients. (**e**) Cell velocity was measured by culturing DPCs to 30–50% confluence and capturing images from 10 randomly selected areas at 10-minute intervals for 24 hours using an inverted light microscope contained within a cell incubator at atmospheric (95% air and 5% CO_2) or low oxygen (97% N_2, 5% CO_2 and 2% O_2) conditions. Metamorph software was used to track the velocity of 10 cells from each of the videos produced. Data are mean ± SEM. Statistical analysis was carried out using one-way analysis of variance with Bonferroni's *post hoc* test. $^{**}P<0.01$, $^{***}P<0.001$. BDPCs, blading DPCs; BDPCs, DPC, dermal papilla cell; ODPCs, occipital DPCs. Bar = 200 μm.

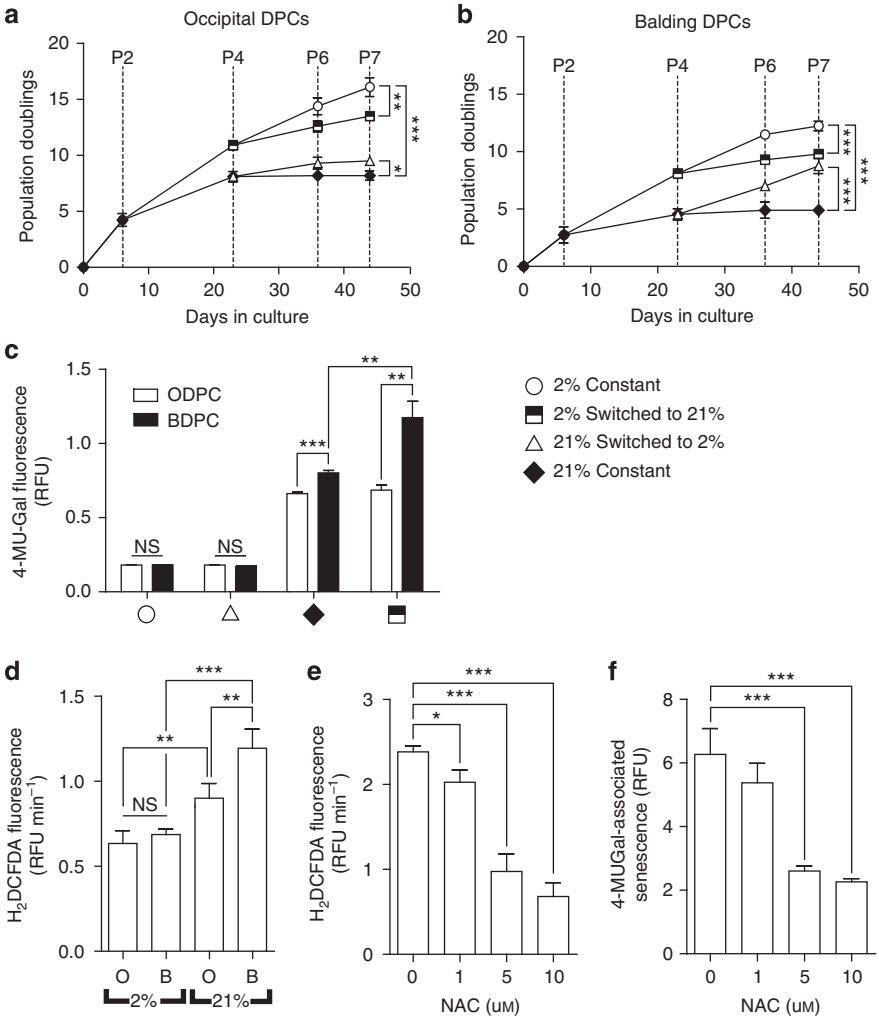

Figure 2. O_2 conditions have a direct effect on dermal papilla cell (DPC) proliferation and senescence. Population doubling rates measured at each passage for (**a**) ODPCs and (**b**) BDPCs from three individual patients in triplicate. DPCs were initially cultured at 21% O_2 before being equally split between 2 and 21% O_2 incubators at passage 2. At passage 4, DPCs were split again, and either maintained at the same condition or switched to the opposite O_2 condition. (**c**) DPCs were taken from the end point (passage 6) of samples used in **a** and **b**. Lysates were quantified for senescence-associated β-Gal activity using 4-MU-Gal substrate. (**d**) ROS levels quantified via H_2DCFDA fluorescence in DPCs from four individual patients in triplicate at 2 and 21% O_2, and in the presence and absence of *N*-acetyl cysteine (**e**).(**f**) Senescence-associated β-Gal activity was quantified in DPCs in the presence and absence of *N*-acetyl cysteine. Data are mean ± SEM. Statistical analysis was carried out using one-way analysis of variance with Bonferroni's *post hoc* test. *$P<0.05$, **$P<0.01$, ***$P<0.001$. DPC, dermal papilla cell; H_2DCFDA, hydroxyl-2-dichlorofluorescein diacetate; ODPCs, occipital DPCs; ROS, reactive oxygen species; 4-MU-Gal, methylumbelliferyl galactopyranosidase.

with a significant increase in cell senescence (Figure 2c). BDPCs were particularly sensitive to senescence when transferred from 2 to 21% O_2 ($P<0.01$). Thus, environmental O_2 levels, which are routinely used in cell culture and known to cause oxidative stress in cultured cells, have a significant impact on DPC morphology, motility, proliferation, and senescence, and physiological (2%) O_2 levels appear optimal to maintain healthy cells. Moreover, BDPCs are much more sensitive to the stress of environmental O_2 than ODPCs.

Hydroxyl-2-dichlorofluorescein diacetate was used to quantify ROS. Figure 2d shows that ROS levels were significantly higher in BDPCs at 21% O_2 compared with ODPCs under identical conditions ($P<0.01$). At 2% O_2, ROS levels were significantly lower in both BDPCs ($P<0.001$) and ODPCs ($P<0.05$) when compared with BDPCs and ODPCs at 21% O_2, but there was no significant difference in ROS levels between BDPCs and ODPCs at 2% O_2.

We demonstrate that the higher levels of ROS in the BDPCs can be suppressed in a dose-dependent manner by treating BDPCs for 24 hours with the ROS-scavenger *N*-acetyl cysteine (NAC; Figure 2e). NAC was effective at significantly reducing the levels of ROS at 1 μM ($P<0.05$) and 5 and 10 μM ($P<0.001$; Figure 2e); moreover, analysis of SA-β-Gal showed that both 5 and 10 μM NAC also resulted in a significant ($P<0.001$) reduction in senescence in BDPCs (Figure 2f).

Catalase and glutathione are influenced by oxygen and are higher in balding than nonbalding DPCs

We also investigated the expression of catalase, an indicator of oxidative stress (Clerch *et al* 1991). Western blot analysis

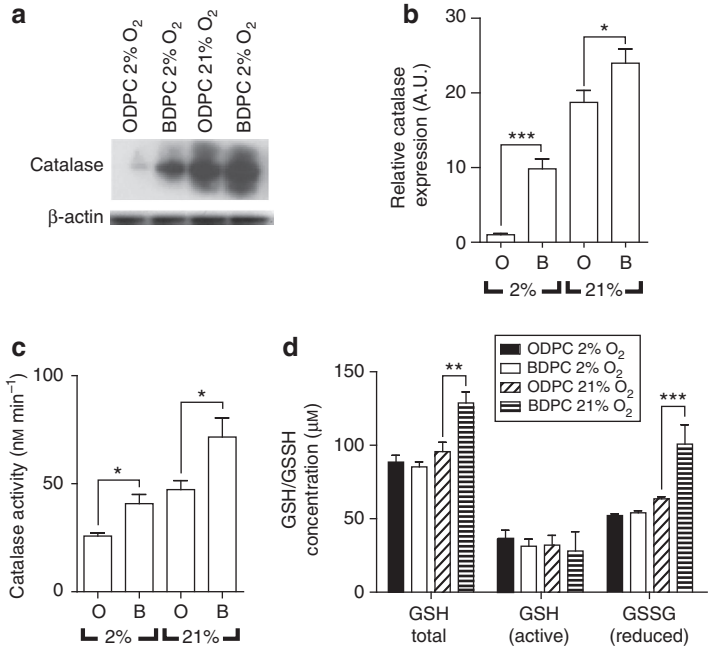

Figure 3. O_2 conditions affect antioxidant response protein expression and activity. (**a**) Western blot analysis of catalase and β-actin loading control in ODPCs and BDPCs from three individual patients cultured at 2 and 21% O_2. DPCs were lysed using RIPA buffer, and 5 μg of each lysate was run on a Sigma Nu-page electrophoresis gel. (**b**) Densitometric analysis was carried out on ($n=3$) blots using Image-J (NIH, Open source). Blots were stripped and reprobed with antibodies for β-actin to determine equal protein loading and to normalize densitometry values. (**c**) Activity of catalase quantified using Amplex Red assay in DPCs from three individual patients in triplicate at 2 and 21% O_2. (**d**) Total glutathione concentration quantified using Ellman's reagent, displaying reduced and oxidized fractions in DPCs cultured at 2 and 21% O_2. Means ± SEM. Statistical analysis carried out using the Bonferroni's *post hoc* test. *$P<0.05$, **$P<0.01$, ***$P<0.001$. BDPCs, blading DPCs; ODPCs, occipital DPCs; RFU, relative fluorescence units; RIPA, radioimmunoprecipitation assay.

(Figure 3a) and densitometry (Figure 3b) showed that catalase expression was significantly lower in ODPCs at 2% O_2 compared with BDPCs at the same passage under the same O_2 conditions ($P<0.001$). At 21%, O_2 catalase was upregulated in both ODPCs and BDPCs but remained higher in BDPCs compared with ODPCs ($P<0.05$). Catalase activity was also significantly higher in BDPCs ($P<0.05$) compared with ODPCs (Figure 3c) and was also higher at 21% O_2 compared with 2% O_2 ($P<0.05$). These data demonstrate that oxygen and cell passage have a significant impact on the stress response of DPCs but show that BDPCs appear to be more sensitive to stress than ODPCs.

Glutathione has a critical role in the elimination of ROS (Maher, 2005). Total and oxidized glutathione was quantified in DPCs at 2 and 21% O_2. Total glutathione (GSH and GSSG) concentration was significantly higher in BDPCs compared with ODPCs at 21% O_2 ($P<0.05$; Figure 3d). Total glutathione concentration was also significantly higher in BDPCs cultured at 21% O_2 compared with 2% O_2 ($P<0.01$). There was no significant difference in total glutathione in BDPCs versus ODPCs at 2% O_2 and between ODPCs at 21 and 2% O_2. When we calculated the fraction of reduced glutathione (GSSG), we observed that BDPCs had a significantly higher concentration of GSSG at 21% O_2 compared with ODPCs ($P<0.01$). No significant difference was observed between BDPCs and ODPCs at 2% O_2. ODPCs at 21% O_2 showed no statistical difference in GSH concentrations compared with those cultured at 2%. BDPCs cultured at 21% O_2 had a significantly higher fraction of their total glutathione in its reduced form compared with those grown at 2% O_2 ($P<0.001$). These data indicate that although the amount of active GSH was similar between ODPCs and BDPCs under all conditions, the overall fraction of active glutathione was smaller in BDPCs than in ODPCs and that active fraction of glutathione is lower in BDPCs than ODPCs under oxidative stress.

Oxygen regulates $p16^{INK4}$ and pRB

Western blot analysis (Figure 4a) and densitometry showed that $p16^{INK4a}$ (Figure 4a and b) and the pRB(Figure 4a and c) were expressed at much higher levels in DPCs maintained at 21% O_2 compared with 2% and that levels of $p16^{INK4a}$ and pRB were expressed at lower levels in ODPCs compared with BDPCs, thus confirming our previous data in which we showed that premature senescence of BDPCs was associated with $p16^{INK4a}$ and pRB (Bahta *et al.*, 2008).

TGF-β and IGF-I secretion by balding and nonbalding DPCs is influenced by oxidative stress

A critical function of the DP is its role in secreting growth factors that regulate the development and growth of the hair follicle. As TGF-β is a potent inhibitory hair growth factor and has previously been reported to be secreted by BDPCs *in vitro* in response to dihydrotestosterone (DHT; Inui *et al.*, 2002), we investigated the effect of oxidative stress and DHT on the

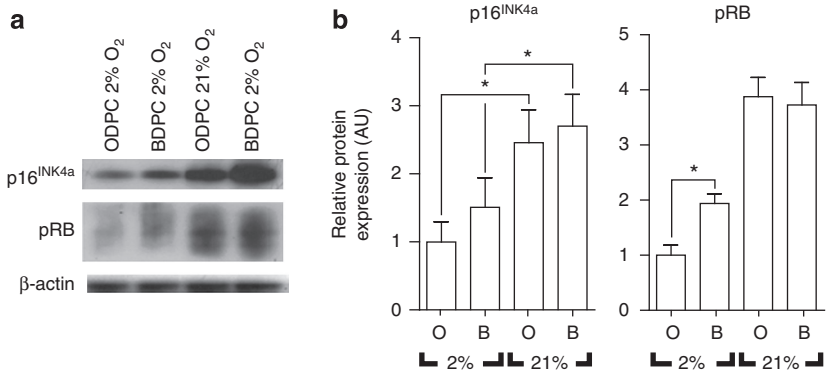

Figure 4. O_2 affects senescence-associated protein expression. (**a**) Western blot and (**b**) densitometric analyses for $P16^{INK4a}$ and pRB carried out on DPCs from three individual patients cultured at 2 and 21% O_2. Data are mean ± SEM. Statistical analysis carried out using one-way analysis of variance with Bonferroni's *post hoc* test. *$P<0.05$. DPCs, dermal papilla cells; pRB, retinoblastoma protein.

secretion of TGF-β isoforms. Oxidative stress induced by hydrogen peroxide (100 μM H_2O_2) promoted the secretion of TGF-β1, and this was significantly higher ($P<0.001$) in BDPCs (Figure 5a). Oxygen conditions alone did not independently alter TGF-β secretion (Figure 5b and c). However, DHT (100 nM) significantly increased TGF-β1 and TGF-β2 secretion compared with the vehicle control ($P<0.01$) when BDPCs and ODPCs were cultured at 21% O_2 (Figure 5b and c). Conversely, BDPCs cultured at 2% O_2 showed no change in TGF-β2 secretion (Figure 5c) and a significant decrease in TGF-β1 secretion at both 1 nM ($P<0.01$) and 100 nM ($P<0.001$) DHT (Figure 5b). ODPCs cultured at 2% O_2 also underwent a significant decrease in TGF-β1 secretion at 1 nM ($P<0.01$) and 100 nM ($P<0.05$) DHT (Figure 5b); however, no significant change in TGF-β2 secretion was observed (Figure 5c). This suggests that DPCs maintained in an environment of low oxidative stress (e.g., 2% O_2) are protected from DHT-induced TGF-β secretion. IGF, one of the main growth factors involved in the maintenance of the follicle in the anagen stage of the growth cycle, was secreted at significantly lower levels by BDPCs compared with ODPCs and also suppressed in ODPCs when cultured at 21% O_2 compared with 2% O_2 ($P<0.05$), but not in BDPCs (Figure 5d).

DISCUSSION

Here we show that environmental oxygen significantly alters DPC morphology, migration, proliferation, senescence, and TGF-β signaling. BDPCs were significantly more sensitive to oxidative stress than ODPCs, with reduced cell proliferation and migration together with increased ROS and senescence at 21% O_2. Crucially, BDPCs that were protected from oxidative stress when cell culture was performed at 2% O_2 did not secrete the negative hair growth factor TGF-β in response to DHT. This suggests that oxidative stress, as well as androgen signaling, may have an important role in the BDPC phenotype and AGA.

Remodeling of the DP is essential for hair follicle cycling and is largely dependent on the migration of DPCs between the DP and the connective tissue sheath (Tobin *et al.*, 2003). Balding follicles exhibit shrinkage and rounding of the DP (Miranda *et al.*, 2010), suggesting restricted cell migration. Here we show that BDPCs have slower migratory velocity than ODPCs under both oxygen conditions. However, oxygen significantly altered cell motility—faster migration velocity was observed at 2% O_2 in both DPC phenotypes. Therefore, these data suggest that reduced DPC migration caused by oxidative stress may inhibit hair follicle remodeling and could promote the balding phenotype.

We previously reported that BDPCs undergo premature senescence *in vitro*, caused by elevated expression of $p16^{INK4a}$ and pRB, but not p53 or p21 (Bahta *et al.*, 2008). We now show that the expression of $p16^{INK4a}$ and pRB is associated with high levels of oxygen. Environmental stress has been reported to trigger senescence via $p16^{INK4a}$ in dermal fibroblasts (Chen, 2000; Jacobs and de Lange, 2004) and is considered a key factor of skin aging (Alaluf *et al.*, 2000). Although cell passage did have an effect on the expression of senescence markers in DPCs, the differences between BDPCs and ODPCs cultured at 2 and 21% O_2 were similar at low and high passage number. This suggests that the senescence that we observed was owing to oxidative stress as opposed to replicative senescence commonly observed in dermal fibroblasts as the result of passage-induced telomere shortening (Itahana *et al.*, 2001).

Catalase was significantly higher in BDPCs than in ODPCs at passage 2, possibly in response to ROS. Catalase has previously been associated with hair follicle aging. However, in contrast to BDPCs, graying hair follicles express lower levels of catalase and higher levels of ROS compared with healthy, pigmented follicles (Wood *et al.*, 2009; Kauser *et al.*, 2011). ROS produced by the melanocytes in graying follicles may be responsible for creating an oxidative environment that could affect DPCs especially in balding scalp. The elevated levels of catalase present in the BDPCs did not translate to a reduction in total ROS nor reduced levels of senescence, suggesting that the BDPCs may be deficient in other antioxidants or in their ability to handle ROS.

In addition to catalase, we also showed that glutathione levels were higher in BDPCs compared with ODPCs at 21% O_2. Glutathione is an ROS-sensitive signal modulator that

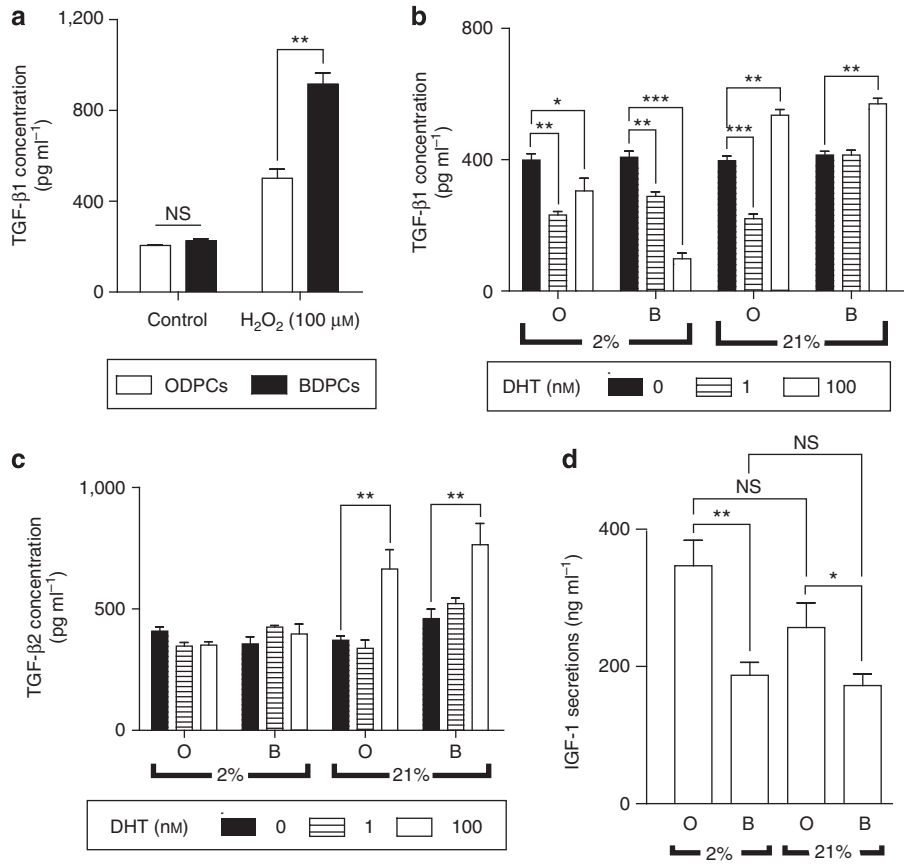

Figure 5. Oxidative stress induces changes to dermal papilla cell (DPC) growth factor secretion. (**a**) ELISA analysis was used to determine TGF-β1 secretion for DPCs from three individual patients at 2% O_2 in response to H_2O_2-induced oxidative stress. Following this, BDPCs from three patients were cultured at both 2 and 21% O_2 in the presence of DHT or ethanol vehicle control to quantify the secretion response of (**b**) TGF-β1, (**c**) TGF-β2, and (**d**) IGF. Data are mean ± SEM. Statistical analysis carried out using one-way analysis of variance with Bonferroni's *post hoc* test. *$P<0.05$; **$P<0.01$; ***$P<0.001$. DPCs, dermal papilla cells; TGF-β1/β2, TGF, transforming growth factor beta 1/2.

senses the oxidative equilibrium of the cell (Maher, 2005). Reduced (active) GSH regulates intracellular signaling by blocking the promoter-binding sites of AP-1 and SP-1 (Vayalil *et al.*, 2007). When ROS is abundant, GSH is oxidized forming a dimer (GSSG) with altered binding function of intracellular transcriptional mediators (Maher, 2005). Therefore, the proportion of active GSH is not only critical as an antioxidant but also for regulating transcription in response to oxidative stress. There was no difference in GSH and GSSG levels between BDPCs and ODPCs at 2% O_2, indicating limited oxidative stress and no associated change in intracellular signaling. However, BDPCs exposed to 21% O_2 had significantly higher levels of GSSG and proportionally less GSH. There is therefore scope for further examination of the role of GSH and whether it is able to modulate DHT signaling in DPCs.

Secretion of TGF-β in response to oxidative stress is the underlying pathophysiology of pulmonary fibrosis (Cui *et al.*, 2011), heart disease (Yeh *et al.*, 2011), and photoaging of skin (Debacq-Chainiaux *et al.*, 2005). We chose to measure total TGF-β—as opposed to active TGF-β—as the most suitable measure of TGF-β, as it gives a more accurate indication of the overall bioavailability of the growth factor (Koli *et al.*, 2001). TGF-β1 and TGF-β2 are negative regulators of hair growth (Foitzik *et al.*, 2000; Hibino and Nishiyama, 2004), and H_2O_2-induced senescence of fibroblasts causes sustained overexpression of TGF-β1 and TGF-β2 via a pRB-regulated pathway (Frippiat *et al.*, 2001). We showed that TGF-β1 and TGF-β2 secretion by BDPCs was stimulated by an acute dose of H_2O_2, and therefore that oxidative stress is able to stimulate secretion of known hair growth inhibitory factors. However, the fact that we observed no increase in TGF-β secretion under 21% O_2 suggests that the conditions of oxidative stress experienced *in vitro* are insufficient to affect TGF-β secretion by cultured DPCs.

We also investigated the effects of oxygen on secretion of IGF-I, a positive regulator of hair growth *in vitro* (Philpott *et al.*, 1994). BDPCs secreted significantly less IGF-1 than ODPCs. As IGF-1 has been shown to maintain *in vitro*–cultured human hair follicles in anagen, reduced IGF-1 secretion by BDPCs may result in impaired hair growth. Although oxygen had no effect on IGF-1 secretion in BDPCs, secretion of IGF-1 by ODPCs was significantly lower under 21% oxygen compared with 2%. This suggests that BDPCs' secretion of IGF-I may be dependent on environmental stimuli.

ARs are found in both balding and nonbalding DPCs, with balding cells expressing a higher number of receptors (Hibberts *et al.*, 1998). Androgens have been shown to stimulate TGF-β1 secretion in AR-transfected BDPCs (Inui *et al.*, 2002). In our study, DHT stimulated TGF-β secretion from DPCs only at 21% O_2, suggesting that oxidative stress is an essential component of androgen response in AGA. In addition, our observation that at 2% oxygen DHT had significantly reduced TGF-β1 secretion in ODPCs and BDPCs. The reasons for this effect are unknown, although it has been shown in the prostate that DHT inhibits TGF-β signaling and secretion and that this is mediated via stromal cells (Kyprianou and Isaacs, 1989; Placencio *et al* 2008). AR-transfected BDPCs but not ODPCs respond to synthetic androgen R-1881 by producing TGF-β1 (Inui *et al.*, 2002). In contrast, we also show that at 21% O_2 ODPCs also secrete TGF-β1 and TGF-β2 in response to DHT. ODPCs are classically described as androgen-insensitive; however, their insensitivity stems from a lack of 5α-reductase, which converts testosterone to the more active DHT, rather than from a lack of AR. Indeed, Hibberts *et al.* (1998) have shown that ODPCs express AR, although at lower expression levels than the BDPCs. The use of DHT in our experiments would bypass 5α-reductase testosterone metabolism and supports the theory that androgen sensitivity in the DP is predominantly controlled by 5α-reductase (Kaufman, 1996). We observed significant effects of DHT on TGF-β secretion after only 1 hour of DHT treatment. Whether these rapid effects are being mediated via AR modulation of gene expression is not known. We ruled out the role of the nongenomic androgen response element GPCR6A (Pi *et al.*, 2010) via quantitative reverse transcriptase in real time PCR analysis (Supplementary Figure S1 online). However, ARs are able to mediate changes in cell biology via nonclassical pathways that involve direct activation of mitogen-activated protein kinases, and this may explain the rapid response that we report here (Lange *et al.* (2007) for review).

In our experiments, we required 100 nM DHT to observe any effect on TGF-β secretion. Although supraphysiological, similar levels of DHT have been used in other studies (Kwack *et al.*, 2008; Shin *et al.*, 2013). Indeed, Inui *et al.* (2002) showed that cultured DPCs lose AR expression in culture, and in their study they had to transfect their DPCs with AR to show stimulation of TGF-β secretion.

In addition to elucidating the effect of oxidative stress on DPCs, these data demonstrate the necessity of optimal cell culture conditions to assess DPC signaling. Tissue culture is routinely carried out at atmospheric levels of 21% O_2, vastly higher than the physiological levels of 1–5% O_2 found within the dermis *in vivo*. These supraphysiological levels of oxygen accelerate the fibroblast transition into a postmitotic, senescence state (Alaluf *et al.*, 2000; Chen, 2000). Our findings highlight that this is also the case for DPCs and demonstrate that these cells should be cultured under hypoxic conditions (2% O_2). It is now well established that hypoxia is a characteristic of the stem cell niche (Mohyeldin *et al.*, 2010) and the DP is known to contain stem cells (Jahoda, 2003), and, moreover, that normoxic conditions of 21% O_2 promote stem cell differentiation (Mathieu *et al.*, 2014). This may explain why cultured DPCs rapidly lose their inductive capacity (Jahoda *et al.*, 1984).

In conclusion, we present a potential link between oxidative stress and impaired DP function. We also highlight the beneficial use of low oxygen environment for DPCs, which would aid the expansion and maintenance of viable cell cultures for a greater number of passages, a finding that may be of major benefit to researchers wanting to screen large numbers of compounds, or clinicians looking to expand allogeneic grafts for re-implantation.

MATERIALS AND METHODS

Cell culture

Patient-matched punch biopsies (2 mm^2) were taken from balding (frontal) and nonbalding (occipital) scalps of men at stages IV–VI on the Hamilton–Norwood scale (Norwood, 1975) undergoing hair transplant surgery for AGA. Ethical approval for this study was obtained from the East London and City Health Authority (LREC 09/H0704/40), and biopsies were taken with written, informed patient consent. All experiments adhered to the Declaration of Helsinki principles.

Dermal papillae were microdissected from the hair follicles using a stereoscopic microscope, as described previously (Bahta *et al.*, 2008). Identical numbers of BDPCs and ODPCs were explanted and maintained in DP medium: Williams E medium, supplemented with 2 mM L-glutamine, 10 μg ml^{-1} insulin, 100 ng ml^{-1} hydrocortisone, 100 units per ml of penicillin G, and 100 mg ml^{-1} streptomycin supplemented with 15% (v/v) fetal calf serum (reagents from (Sigma-Aldrich, Poole, UK) at 37 °C in a humidified atmosphere of 5% CO2/95% air. At passage 2, DPCs were divided and either maintained at 21% O_2 or switched to a Sci-tive Stem Cell Station (Ruskinn, Bridgend, UK). Cells were maintained under physiological oxygen conditions 5% CO_2, 93% N_2, and 2% O_2. Proliferation rates were determined using Nucleocassette cell counters (Chemometec, Königswinter, Germany). Population doubling rates were calculated as follows: $PD = \log_2 (N_H/N_S)$, where N_S is the number of cells seeded and N_H is the number of cells harvested upon passage.

Cell migration assay

DPCs were seeded at a density of 1×10^4 into 6-well plates. The plate was placed on a robotically controlled platform on a Nikon inverted light microscope inside a thermostatically controlled chamber maintained at either 21 or 2% O_2 as above. Ten randomly selected points were chosen from each well and photographed using the Metamorph software (Molecular Devices, Wokingham, UK). The software then recorded images at these chosen points every 10 minutes for 24 hours. The resultant images were then sequenced into a time-lapse video, and image analysis was carried out using the Metamorph software to assess the velocity of individual cells' movements.

SA-β-Gal assay

Cytochemical detection of SA-β-Gal activity was carried out using a modified version of the 4-methylumbelliferyl galactopyranosidase assay (4-MU-Gal; Gary and Kindell, 2005). Briefly, DPCs were lysed in buffer (40 mM citrate, 40 mM sodium phosphate, 5 mM 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate, and protease inhibitor cocktail (Roche, Lewes, UK); adjusted to pH 6.0). Lysates were centrifuged for 5 minutes at 12,000 G, and the

supernatant was mixed (1:1) with 4-MU-Gal reaction buffer (40 mM citrate, 40 mM sodium phosphate, 300 mM NaCl, 4 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 1.7 mM of 4-MU-Gal; adjusted to pH 6.0). Samples were then incubated at 37 °C for 1 h before 50 µl aliquots were taken and mixed with sodium bicarbonate (400 mM) stop solution. Fluorescence was measured at 360/465 nm every 5 minutes for 1 hour using a Synergy HT microplate reader with KC4 software (BioTek, Bedfordshire, UK).

Protein extraction and immunohistochemistry

DPCs were homogenized in radioimmunoprecipitation assay buffer containing protease inhibitors (Roche, Lewes, UK), centrifuged, and the supernatants were stored at −80 °C until analysis. Samples were separated using a Nu-PAGE 10% (v/v) Bis-Tris gel (Invitrogen, Paisley, UK) according to the manufacturer's instructions and then transferred to a HyBond nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) and incubated with mouse anti-$p16^{INK4a}$ (Santa Cruz Biotechnology, Wiltshire, UK), mouse anti-pRB (Millipore, Watford, UK), rabbit anti-catalase, or goat anti-β-actin (Abcam, Cambridge, UK), and thereafter with the appropriate horseradish peroxidase–conjugated secondary antibodies (DakoCytomation, Glostrup, Denmark). Membranes were imaged using ECL-Plus chemiluminesence solution and light-sensitive Hyperfilm (GE Healthcare). Densitometric analysis was carried out using the Image-J software (Public domain, NIH).

ROS assay

DPCs were seeded at a density of 1×10^4 per well in an opaque 96-well plate and left to adhere overnight. Cells were washed twice with phosphate-buffered saline and incubated with 50 µl of phenol red–free DP-specified medium containing 25 µM hydroxyl-2-dichlorofluorescein diacetate and placed in the incubator for 1 hour. Fluorescence was measured at 485/527 nm every 2 minutes for 30 minutes using Synergy HT microplate reader with the KC4 software (BioTek). Fluorescence was calculated as the slope of relative fluorescence units per minute.

Catalase assay

The catalase assay was carried out using the Molecular Probes: Amplex Red catalase assay kit (Invitrogen) according to the manufacturer's instructions. Fluorescence was measured at 530/560 nm using Synergy HT microplate reader (BioTek).

Glutathione assay

Total and reduced glutathione were measured simultaneously using an Ellman's reagent (Ellman and Lysko, 1979) multiwell kit (Cayman Scientific, Ann Arbor, MI) according to the manufacturer's instructions. Samples were colorimetrically quantified, and absorbance was measured at 405 nm at 5-minute intervals for 30 minutes using the Synergy HT microplate reader (BioTek).

ELISAs

IGF, TGF-β1, and TGF-β2 were measured using the Quantikine ELISA kits (R&D, Abingdon, UK) according to the manufacturer's instructions. DPCs were incubated in DP medium containing charcoal-stripped fetal bovine serum (15%) for 24 hours before testing. Plates were read at an absorbance wavelength of 450 nm using a Synergy HT microplate reader (BioTek).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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