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# *"Investigating hair follicle aging - DNA intercalation induces senescence markers, shortens anagen phase and reduces hair shaft production in healthy human hair follicles ex vivo"*

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## 1. Introduction

Hair aging extends beyond mere hair greying; it also involves a reduction in both the number and diameter of visible hairs. This decline is attributed to prolonged catagen (regression) and telogen (resting) phases of the hair cycle as well as miniaturization of hair follicles (HFs), which are, in part, caused by the loss of eHFSC activity and/or a reduced regenerative capacity of mesenchymal stem cells **[1, 2]**. Additionally, alterations in hair shaft structure, namely keratin and keratin-associated protein (KRTAP) expression, contribute to hair thinning and reduced hair shaft quality **[3]**. It is widely recognized that the aging process is driven by a combination of genetically determined, intrinsic influences as well as extrinsic influences such as UV radiation, nutrition or pollution (exposome) **[4]**.

These extrinsic factors induce various stressors, including DNA damage, epigenetic alterations, tissue damage, telomere erosion, oxidative stress, or other cellular insults which can subsequently result in cellular senescence **[4]**. Cellular senescence, a hallmark of aging, is a state where cells enter permanent growth arrest without undergoing cell death. Senescent cells undergo morphological and metabolic changes, autophagy, and DNA damage responses (DDR), and often adopt a distinctive phenotype known as the senescence-associated secretory phenotype (SASP), characterized by the secretion of pro-inflammatory cytokines, chemokines, growth factors, and proteases. The accumulation of senescent cells can lead to chronic inflammation and disruption of normal tissue structure and function **[5, 6]**

However, the mechanisms underlying cellular senescence in human HFs and its role and functional impact during HF aging remain insufficiently studied. A deeper understanding of these processes could have significant implications for developing therapies to combat senescence-related processes and age-related hair loss.

To address this gap, we treated healthy human HFs *ex vivo* with 5-Bromo-2-deoxyuridine (BrdU), a thymidine analogue that incorporates into replicating DNA during the S-phase of the cell cycle **[7]**. BrdU primarily affects highly proliferating cells, such as those in the HF epithelium, particularly in the hair matrix. Due to its incorporation into DNA, BrdU has been shown to possess toxic properties, alter cell generation and survival patterns **[8]**, and influence cell growth and differentiation by inducing senescence markers *in vitro* (e.g. increased

SA-ß-Gal activity, and γH2A.X, p53, p21, and checkpoint kinase 1 and 2 expression) **[4, 9, 10]**. Additionally, we could demonstrate before that BrdU induces a senescence phenotype and hallmarks of aging in healthy human skin organ culture.

Therefore, we here utilized BrdU as a senescence inducer and potential generator of HF aging *ex vivo*, aiming to better understand HF aging and potentially provide a platform for screening nutraceuticals and cosmeceuticals for rejuvenating hair treatments.

## 2. Materials and Methods

### *Hair follicle organ culture and treatment*

All human samples were obtained after informed, written consent and ethics committee approval (University of Münster, no 2015-602-f-S, and Comité de Bioética de la Universidad Fernando Pessoa Canarias (03 (2020-06-22). This study was conducted according to Declaration of Helsinki principles.

Amputated anagen VI HFs were microdissected from scalp skin or follicular unit extractions (FUE) **[11–14]** of 2-3 healthy donors and treated for 5 days ex vivo with three different concentrations of BrdU (10µM – low, 500µM – medium, and 1mM - high) or vehicle control at 37°C with 5% CO2 in William's complete media as previously described **[11–14]**. The culture medium was exchanged every second day and the culture was terminated by embedding the HFs in OCT matrix. 6 µm cryosections were cut with a cryostat (Leica), consecutive sections of each HF were collected, and slides were stored at −80°C.

### *(Immuno-)histochemistry and immunofluorescence microscopy*

For the histochemical visualization of melanin, Masson–Fontana staining was performed as previously described **[15]**. To stain Ki-67+/TUNEL+ cells, the ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Merck Millipore) was used following the manufacturer’s protocol, in combination with a Ki-67 staining **[12–15]**.

KRTAP3.3 expression was analysed by immunohistochemistry cryosections were incubated with rabbit anti-human KRTAP3.3 (1:100, PA5-60190, ThermoFischer) over night at 4°C. Secondary antibody incubation with biotinylated goat anti – rabbit Ig G was performed at RT for 45min and the signal was visualized with Avidin/Biotin – HRP.

To analyze protein expression by immunofluorescence, cryosections were incubated with rabbit anti-human phospho-Histone H2A.x *Ser139 (1:800, 2577S, Cell), mouse anti-human p21 (1:100, 55643, BD Biosciences), rabbit anti-human CXCL10 (1:200, ab9807, abcam), anti-basic hair keratin K85 guinea pig (1:200, GP-hHb5, Progene), anti-basic hair keratin K86 guinea pig (1:100, GP-hHb6S, Progene) overnight at 4°C and visualized by secondary antibody incubation at RT for 45 min using Alexa Fluor secondary antibodies (Thermo Fisher, UK) **[13, 14, 16, 17]**.

Counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) (1 µg/ml) was performed to visualize nuclei. Images were taken using a Keyence fluorescence microscope (BZ9100; Osaka, Japan), maintaining a constant set exposure time throughout imaging for further analysis **[13, 14, 18]**.

Staining immunoreactivity or the number of positive cells were counted in the corresponding reference areas depicted in the different figures. Analyses were carried out with ImageJ software (National Institutes of Health, Bethesda, MD, USA) **[13, 14, 16, 19, 20]**.

*Melanin clumping analysis*

Melanin pigments were stained as black dots using Masson Fontana; the presence of abnormal large melanin clumping (i.e. melanin positive conglomerates that were larger than keratinocyte nuclei) was counted in the HM up to 10 lines of cells above the end of the dermal papilla **[21, 22]**.

*Hair cycle staging and scoring*

Microscopic hair cycle staging was performed at the end of the culture on the basis of morphology, Masson-Fontana histochemistry, and Ki-67/TUNEL immunostaining, as previously described **[23]**. Hair cycle scoring was calculated using a standardized, arbitrary score (anagen = 100; catagen = 200; early catagen = 300, mid-catagen = 400) **[24]**. Thus, a lower score means that the HFs are more established in anagen, and a higher score means that the HFs are more progressed to catagen.

*Hair follicle elongation/hair shaft production*

To determine HF length, each HF was measured from the end of the connective tissue sheath to the end of the distal outer root sheath at different time points (Day 0, Day 1, Day 3, Day 5 and Day 6), using a digital light microscope at 50X magnification (VHX900; Keyence Corporation, Osaka, Japan) and affiliated software as described before **[13]**.

*Lactate dehydrogenase assay*

Cytotoxicity was studied by lactate dehydrogenase (LDH)-based assay **[21]** using the Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. LDH measurements were performed using a POLARstar Omega microplate reader (BMG Labtech).

*Statistical analysis*

Statistical analysis were performed using Graphpad Prism 9 (GraphPad Software Inc). Data were tested for Gaussian distribution with D'Agostino Pearson omnibus normality test. Data was not normally distributed and non-parametric tests were used: Kruskal-Wallis test, including Dunn`s multiple comparison testing fixed on vehicle. Data are expressed as mean ± SEM, or fold-change of mean ± SEM. p-values<0.05 were regarded as significant.

**3. Results**

Since BrdU can potentially be toxic **[8]**, we first analzed LDH release into the medium and melanin clumping and confirmed that none of the tested BrdU concentrations exerted cytotoxicity in healthy human HFs *ex vivo*.

Next, we assessed the protein expression of the DNA damage sensor γH2AX in the HF epithelium and found a significant increase upon treatment with the low and medium BrdU concentrations. Treatment with the high BrdU concentration tended to increase the expression of the SASP indicator CXCL10 in the outer root sheath, while the low and medium concentrations significantly enhanced CXCL10 expression in the pre-cortical hair matrix. Furthermore, all three BrdU concentrations significantly and dose-dependently enhanced the expression of the cell cycle inhibitor p21 in the hair matrix, where keratinocyte proliferation and differentiation occur, an essential step for hair shaft production **[25]**. These data confirm

that treatment with BrdU can indeed induce a senescent phenotype in healthy human HFs *ex vivo.*

In the next step, we examined whether and how these molecular changes would translate into HF function. We found significantly lower hair matrix keratinocyte proliferation following treatment with all three BrdU concentrations. This was accompanied by significantly higher hair matrix keratinocyte apoptosis upon application of the low and medium concentrations of BrdU. The reduced proliferation of hair matrix keratinocytes by BrdU was reflected in a dose-dependent inhibition of hair shaft production after 5 and 6 days ex vivo. Furthermore, BrdU reduced hair shaft quality, with the greatest effect observed at the highest concentration. This was evidenced by significantly decreased expression of Keratin 85 (K85) and a trend towards reduced Keratin 86 (K86) expression in the pre-cortical hair matrix. Additionally, the medium and high BrdU concentrations significantly lowered protein levels of keratin-associated protein 3.3 (KRTAP3.3) in the inner root sheath. Finally, microscopic hair cycle staging confirmed catagen promotion by all three BrdU concentrations. This was also reflected by a significantly lower hair cycle score, which is calculated by attributing an arbitrary score to each hair cycle phase, where a lower score indicates that more HFs are in the anagen phase **[26]**. Thus, these findings demonstrate that the senescent phenotype induced by BrdU has also functional implications on healthy human HFs *ex vivo*.

## 4. Discussion

We could demonstrate that BrdU induces a senescent phenotype with functional implications for healthy human HFs *ex vivo*. Treatment with BrdU increased expression of the DNA damage marker γH2AX, the cell cycle inhibitor p21, and the SASP indicator CXCL10, without exerting cytotoxicity. This was functionally reflected by reduced hair matrix keratinocyte proliferation and increased - apoptosis, decreased hair shaft production, diminished hair shaft quality as well as catagen promotion.

Our findings suggest that BrdU primarily affects pre-cortical and outer root sheath keratinocytes, as evidenced by the highest expression of γH2AX, p21, and CXCL10 in these compartments.Marginal numbers of p21+ and γH2AX+ cells were also observed in the dermal papilla, dermal stalk, and dermal cup. Interestingly, HF miniaturization is characterized by an increased emigration of dermal papilla fibroblast and p21 and $p16^{ink}$ expression are increased in AGA-affected HFs. However, if BrdU can induce fibroblast emigration or expression of senescence marker in the DP and if or how this plays a role during HF aging remains to be elucidated.

The reduction in hair matrix keratinocyte proliferation could be explained by the increase in p21 expression, since it functions as cell cycle inhibitor **[27]**. Given the absence of positive cells for senescence markers in the germinative hair matrix, which is the main site of keratinocyte proliferation **[25]**, we hypothesize that the p21-positive cells observed in the hair matrix were initially located in the germinative hair matrix at the beginning of the *ex vivo* culture and subsequently migrated to the hair matrix during the culture process. This “abnormal” upward movement of the hair matrix keratinocytes, during the HF organ culture, together with the inability of senescence cells to undergo apoptosis **[4]** could explain the formation of a hair shaft of reduced quality.

Indeed, aging hair is associated with a rougher hair fiber caused by structural changes in the surrounding cuticle or less shiny hair **[28]**. We here demonstrate that BrdU treatment does in decrease K85, K86 and KRTAP3.3 expression, as marker for hair shaft quality. This further

supports our BrdU-induced HF senescence model as useful tool to investigate hallmarks of aging, not only in the “living” HF but most likely also in the “dead” hair shaft.

Senescent cells resist to apoptosis, while treatment with senolytics such as Dasatinib, Quercetin, and Fisetin induce apoptosis in senescent cells thereby potentially reversing the senescent phenotype. A recent study demonstrated that injection of human dermal papilla cells, pre-treated with Dasatinib and Quercetin, into the skin of hairless mice, increased HFs regeneration by eliminating senescent cells **[29]**. These findins indicate a potential to induce hair re-growth by reducing cellular senescence. However, the influence of senescent cells on human hair loss and the effect of compounds targeting HF senescence to combat senescence-induced hair loss is currently underexplored. Therefore, it is worth investigating whether senolytics can decrease expression of senescence markers and whether they can mitigate hair loss.

Here, we show that BrdU functions as a non-cytotoxic accelerator of aging stress in healthy human HF organ culture. Among the concentrations tested, BrdU exhibited a dose-dependent effect, with a median concentration that induces senescence while (most likely) permitting phenotype rescue, potentially making it an ideal candidate for use in a screening platform. Future steps will involve validating our model with established rejuvenating agents, as well as employing it to evaluate the efficacy of selected novel cosmeceuticals or nut-raceuticals in the context of HF aging or loss.

## 5. Conclusion

Our preliminary data demonstrate that BrdU increases the expression of DNA damage markers, cell cycle inhibitors, and SASP proteins, indicating cellular senescence, which leads to decreased hair shaft production and –quality, reduced hair matrix keratinocyte proliferation and apoptosis, as well as premature catagen induction. Thus, our BrdU-induced aging, model presents a great tool for studying senescence-induced aging mechanisms and for testing senolytic compounds.

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