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Potential application of PBM use in hair follicle organoid culture for the treatment of androgenic alopecia

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ABSTRACT

Androgenic alopecia is a hereditary condition of pattern hair loss in genetically susceptible individuals. The condition has a significant impact on an individual's quality of life, with decreased self-esteem, body image issues and depression being the main effects. Various conventional treatment options, such as minoxidil, finasteride and herbal supplements, aim to slow down hair loss and promote hair growth. However, due to the chronic nature of the condition the financial cost of treatment for androgenic alopecia is very high and conventional treatment options are not universally effective and come with a host of side effects. Therefore, to address the limitations of current treatment options a novel regenerative treatment option is required. One promising approach is organoids, organoids are 3D cell aggregates with similar structures and functions to a target organ. Hair follicle organoids can be developed *in vitro*. However, the main challenges are to maintain the cell populations within the organoid in a proliferative and inductive state, as well as to promote the maturation of organoids. Photobiomodulation is a form of light therapy that stimulates endogenous chromophores. PBM has been shown to improve cell viability, proliferation, migration, differentiation and gene expression in dermal papilla cells and hair follicle stem cells. Therefore, photobiomodulation is a potential adjunct to hair follicle organoid culture to improve the proliferation and inductive capacity of cells.

1. Introduction

Androgenic alopecia (AGA), commonly known as baldness is an androgenic-dependant form of hair loss that occurs in genetically susceptible individuals and follows a predictable pattern of hair loss in both genders [1]. It has been estimated that 50 % of males are affected by AGA by the age of 50 [2]. AGA has a profound effect on the quality of life as it is often accompanied by body issues, decreased social interactions, depression, and anxiety [3]. AGA is a complex condition with genetic susceptibility and sensitivity to DHT (dihydrotestosterone) being the main causes for follicle miniaturization and hair loss [4]. However, chronic low-grade inflammation and oxidative stress have also been implicated and worsened by environmental factors [5,6]. Currently the first line of treatment consists of minoxidil and finasteride to slow down hair loss and promote hair growth. However, these treatments are non-curative and have a host of side effects [2]. Herbal alternatives have been identified for patients that experience side effects but have a limited effectivity [7]. Microneedling, platelet rich plasma (PRP) and low-level light therapy (LLLT) have been identified as effective adjunct treatments, but due to the chronic nature of the condition can be very expensive [8,9].

Tissue engineering is a form of regenerative medicine, with the aim to restore the structure and function of HFs (hair follicles) affected by AGA, by repairing, replacing or regenerating these structures [10]. One such regenerative approach is the use of HF organoids. Organoids are cell aggregates of an organ with similar structures and functions to the target organ in an *in vitro* 3D (three-dimensional) culture [11]. The application of HF organoids in regenerative medicine is to harvest autologous, DHT resistant cells from an AGA patient, culture and expand the cells *in* vitro and transplant the organoids back into the balding areas of the patients for HF neogenesis. However, in practice organoids pose certain challenges, such as reduced proliferation rates, reduced hair forming inductivity and failure of *in vitro* maturation [12]. Photobiomodulation (PBM) is a form of light therapy, that stimulates endogenous chromophores to modulate cellular activities [13]. Clinically PBM has been used as LLLT in clinics or in home use devices to effectively promote scalp hair growth [14]. However, studies evaluating its application in HF organoid development has not been fully investigated.

This review focuses on the use of HF organoids in hair regenerative medicine and the application of PBM to augment HF organoid

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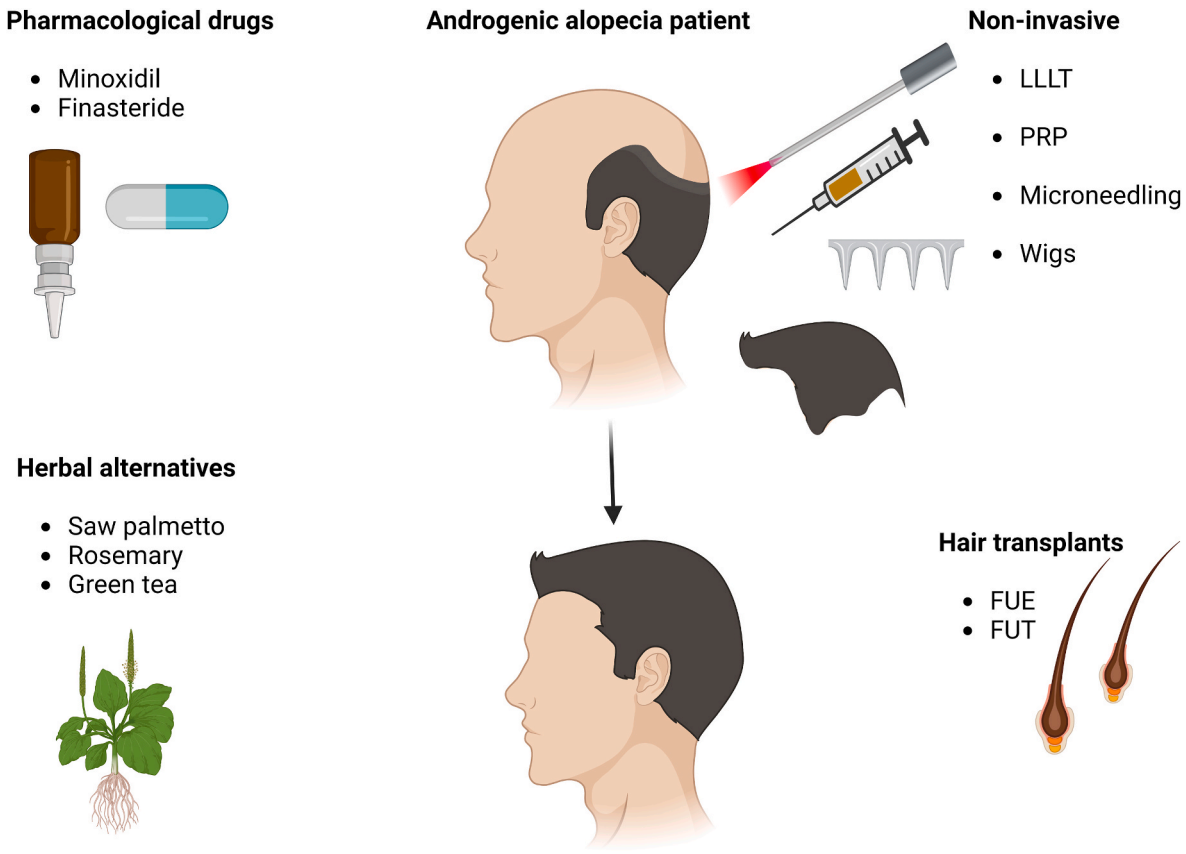

Fig. 1. Conventional treatment options. AGA patients experience androgenic-dependant hair loss. Currently available treatment options include pharmacological drugs (minoxidil and finasteride), herbal alternatives (saw palmetto, rosemary and green tea), surgery (hair transplants) and non-invasive treatments (LLLT, PRP, microneedling and wigs) to slow down hair loss, promote hair growth or conceal hair loss. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

development, by maintaining the proliferative and induction capacity of cells while in 3D culture. The review will briefly discuss the background of AGA, highlight the current forms of treatment and their drawbacks, summarize the concept of the hair growth cycle, identify current strategies in hair tissue engineering, discuss the use of HF organoids in hair regenerative medicine and the application of PBM to augment HF organoids. Followed by a current perspective and conclusion.

2. Background of androgenic alopecia

AGA, commonly known as male pattern baldness, is one of the most common forms of hair loss. The condition mainly affects men, but it can also impact women [1]. It is characterised by a gradual and predictable pattern of hair loss. In men, it typically presents with a receding hairline and thinning in the temples, vertex and mid frontal scalp regions. In contrast women often experience a diffuse thinning across the top of the scalp without significant loss in the hairline [15]. Common trichoscopy features include: varying degrees of hair shaft thickness, perifollicular hyperpigmentation, increased number of vellus hair and a reduction in the number of hair shafts per follicle [16]. AGA is the most common type of alopecia diagnosed in hair clinics [17]. AGA shows a clear gender bias, with the highest incidence rates among males compared to their female counter parts [18]. Furthermore, the disease manifests earlier in males compared to females [15]. The condition can occur at any age, but typically starts developing after puberty and continues to progress. The incidence of AGA increases with age. By 30–40 years of age 30–50 % of males are affected and increases to 80 % after 70 years of age [2]. AGA is more common in Caucasian populations compared to Africans or Asians [19]. The epidemiology (age, gender and race) of AGA in the South African context needs to be further investigated.

AGA is a complex condition that is influenced by genetics, hormones and environmental triggers. AGA has a strong genetic link with both male and female patients reporting a family history of more than 70 % [15]. Similarly monozygotic twin studies show a heritability of 0.81 [20]. The inheritance is polygenic in nature with the *Androgen receptor* (*AR*) gene and *Ectodysplasin A2* (*EDA2R*) gene showing a strong susceptibility to AGA. Furthermore, high risk loci: 2q35, 3q25, 5q33, 20p11 and 12p12 have been reviewed [21]. The pattern of hair loss corresponds to the distribution of androgen receptors in the scalp. Balding areas tend to have higher levels of androgen receptors, as well as increased expression of 5 alpha-reductase and production of DHT [4]. The enzyme 5 alpha reductase converts the androgen, testosterone into DHT. DHT has a higher affinity for the androgen receptor and remains bound to the receptor for longer, compared to testosterone. There are two isoforms (type I and II) of this enzyme, however type II plays a dominant role in AGA [19]. Activation of the androgen receptor shortens the anagen (growth) phase in the normal hair growth cycle, with the telogen (resting) phase remaining constant or increasing in duration. In AGA, excessive activation leads to miniaturization of hair follicles. Over time the affected follicles produce thinner, shorter and less pigmented hairs, eventually leading to hair loss [2].

Inflammation and oxidative stress also play a role in hair loss, caused by AGA. Studies have found that nuclear factor kapa-β (NF-kβ) mediates the release of proinflammatory cytokines, such as TNF-α (Tumour necrosis factor-α) and IL-1β (Interleukin 1-β), creating a proinflammatory environment in the scalp. A study examining serum biomarkers for AGA found Nitric oxide (NO), a reactive oxygen species (ROS), was increased and positively correlated with NF-kβ [5]. TGF-β1 (Transforming growth factor-β1) is increased in AGA and stimulated by ROS, triggering fibrosis and suppressing hair growth [22]. Under physiological conditions DHT

increases NO, thus it is proposed that DHT increases NF-kβ [5]. Furthermore, 76 % of AGA patients have mild perifollicular inflammation, with fibrosis occurring with increased levels of inflammation [23]. Inflammation followed by fibrosis results in follicular atrophy. Lastly environmental factors (e.g. smoking and excessive drinking) produce free radicals, alter epigenetics and circulation in the scalp exacerbating the pathophysiology of AGA [6]. AGA has a significant impact on an individual's quality of life. Psychologically AGA is associated with decreased self-esteem, body image issues and depression [3]. Due to the chronic nature of the condition the financial cost of treatment for AGA is very high [9].

3. Convensional treatment options

There is currently no cure for AGA, but several treatment options have been developed to slow down the progression of AGA, conceal its effects or promote hair growth, as illustrated in Fig. 1.

3.1. Pharmacological treatment

3.1.1. Minoxidil

Minoxidil is available as an over-the-counter topical solution or foam and available in various strengths, but only the 2 % and 5 % are FDA (food and drug administration) approved for AGA [24]. The exact mode of action is not fully understood, but Minoxidil opens potassium channels, is a powerful vasodilator and increases the expression of VEGF (vascular endothelial growth factor), promoting angiogenesis. Hypothetically it allows more oxygen and nutrients to reach the hair follicles, promoting hair growth. Effectively shortening the telogen phase of the hair growth cycle and prolonging the anagen phase [2]. Minoxidil treatment is effective in slowing down hair loss, the cost of treatment is relatively low and can be used in conjunction with other treatment options for a synergistic effect. However, Minoxidil cannot stop the continuing progression of AGA and has its own side effects and draw backs. Common side effects include local irritation of the scalp, itching, redness or dryness due to the alcohol content in the formulation of the solution [9]. Initially, Minoxidil causes hair loss, by synchronizing hair growth and thereafter growth promotion takes effect. It takes weeks to see the effects and the medication needs to be used chronically to maintain the benefits. Adherence is poor due to the sticky residue left on the hair and the drying effect of the solution [25].

3.1.2. Finasteride

Finasteride is the first-line treatment for AGA. It is a 5 alpha-reductase type II inhibitor and reduces scalp DHT by 64 % and serum DHT by 68 %. It is prescribed as a daily 1 mg tablet and is highly effective at reducing hair loss and partially reversing baldness [26]. The cost of treatment is low. Side effects include sexual dysfunction, decreased libido and erectile dysfunction. Poor adherence occurs as a result of the side effects. Finasteride is not a cure and must be taken indefinitely to maintain the beneficial effects [27]. Topical finasteride is a promising alternative to oral finasteride and aims to reduce systemic effects while showing similar effectivity to the oral version [28].

3.2. Herbal alternatives

Herbal alternatives are sought as a natural complementary approach to pharmacological drugs for AGA. Saw palmetto (*S. repens*), extracted from palm tree berries is rich in beta sitosterol and fatty acids. Natural inhibitors of the 5 alpha-reductase enzyme. In a study saw palmetto improved hair growth in 38 % of participants compared to 68 % improvement when using finasteride [7]. However, saw palmetto is prescribed as an alternative to patients experiencing side effects from finasteride. Green tea (*C. sinensis*) contains phytochemicals such as epigallocatechin gallate, which has an anti-inflammatory, proliferative and anti-apoptotic effect on DP (dermal papilla) cells [29]. Rosemary (*R. officinalis*) improves blood circulation and vascularity. Similar results were obtained when comparing rosemary use in AGA to Minoxidil [30]. Herbal treatments may vary in their quality, potency and safety.

3.3. Surgery

A hair transplant is a surgical procedure, involving the extraction of hair follicles from a donor region (usually the occipital and parietal regions of the scalp) and transplanting them into a recipient region of balding. The occipital and parietal hair follicles are less susceptible to the effects of DHT [31]. There are two types of transplants, namely follicular unit transplant (FUT) and follicular unit extraction (FUE). The main difference being the harvesting method of the follicles. FUT involves the removal of a strip of scalp from the donor region. The strip is then dissected into individual follicular units, consisting of one to four hair follicles. FUE involves the extraction of individual follicles transplanted into the recipient area [32]. The final result is a natural appearing look, and the full effect is seen within 12 months post-transplant. Transplants are an effective option for patients with moderate to significant hair loss. However, the recovery time is lengthy, the procedure carries risks (infection, scarring, and nerve damage), transplants can only be considered if there is sufficient follicles in the donor region and is expensive since insurance plans do not cover the procedure [9].

3.4. Non-invasive

3.4.1. Photobiomodulation (PBM) or low-level light therapy (LLLT)

LLLT is a non-invasive form of light therapy that makes use of non-ionising radiation to stimulate endogenous chromophores to modulate cellular activity. This treatment option can be performed in hair clinics or at home. Typically LLLT uses lasers with a wavelength of 650–1100 nm (red to infrared light) [14]. Light in this wavelength penetrates into the skin and displaces inhibitory nitric oxide from mitochondrial cytochrome *c* oxidase to increase ATP (Adenosine triphosphate) production, ROS (Reactive oxygen species) production and induction of transcription factors [33]. Ultimately LLLT modulates the hair growth cycle by stimulating telogen hair follicles to re-enter into the anagen phase, prolong the duration of the anagen phase and increase cellular proliferation rates. The net effect is increased hair density, shaft diameter and reduced shedding with an efficiency rating of roughly 80 % [14]. LLLT can be combined with minoxidil or finasteride to greatly enhance the hair growth properties of both treatments modalities [8]. LLLT is well tolerated by patients, however the costs are more expensive compared to pharmacological drug [34].

3.4.2. Platelet-rich plasma (PRP)

Whole blood is removed from a vein in the patient, usually 10 mL, and centrifuged at a low speed to concentrate the platelets in the plasma layer. The platelet-rich plasma is removed, the platelets are activated and then re-injected into the balding areas in the scalp [35]. The activated, concentrated platelets release a variety of growth factors that promote hair growth and follicle health by stimulating cell proliferation, angiogenesis and reducing local inflammation. PRP increases hair density and thickness [36]. PRP is a minimally invasive procedure, high patient satisfaction rates and effectivity, treatment frequency is lower and has less side effects. Side effects include pain, scalp tenderness and a burning sensation, but these subside quickly after the procedure. However, PRP protocols have not been standardised, is not curative and needs to be administered at regular intervals, more expensive than pharmacological drugs and is usually used as an adjunct to a treatment plan [9].

3.4.3. Microneedling

Microneedling is a minimally invasive procedure that uses several micro needles to damage the epidermis, using a dermaroller or

dermapen. The epidermal damage triggers the release of growth factors by platelets and white cells, responding to the damage. These growth factors and cytokines promote hair growth [37]. Microneedling can also be used in conjunction with other treatments e.g., topical minoxidil or PRP and enhances their absorption into the scalp through micro channels created by the needles. Microneedling reduces hair loss, increases hair density and shaft thickening [38]. Microneedling is a fast, easy and inexpensive procedure, with minimal side effects. It can be performed in a hair clinic or at home and the results are greatly enhanced when using it as part of a combination treatment [39].

3.4.4. *Quiff, wigs, camouflage*

Non-surgical, non-drug options have been developed to reduce or mask the visibility of thinning or balding areas for AGA patients. Hairstyles can be used to strategically hide or cover thinning areas. Hair fibres are natural or synthetic colour matched fibres that adhere to existing hair fibres and give the illusion of fuller and thicker hair. Hairpieces, quiffs or wigs come in different styles, lengths and materials. They can be attached usings clips, adhesives or worn like a hat. These options are temporary and can range from affordable to expensive [4].

Before selecting the most appropriate treatment option for a specific patient, the clinician needs to take several factors into account: side effects, cost, effectivity, severity of hair loss and practicality leading to compliance. These above-mentioned treatment options are not universally effective and come with a host of side effects or disadvantages. Thus, indicating the need for regenerative treatment options with permanent effects.

4. Hair growth cycle

Hair follicles are mini organs constantly undergoing cycles of growth and remodelling, throughout life. Hair serves several biological functions such as thermoregulation, protection against ultraviolet radiation, sensory perception and personal identity [40]. The hair growth cycle consists mainly of three phases: Anagen (growth phase), Catagen (transition phase) and Telogen (Resting phase).

The anagen phase lasts for 1–6 years in the human scalp. The DP cells secrete signalling factors to stimulate quiescent HF stem cells (hair follicular stem cells and melanocyte stem cells) in the bulge region [40]. A small number of these HF stem cells undergo asymmetrical division and differentiation into transient amplifying cells. These progeny cells migrate down and differentiate into follicular keratinocytes forming the matrix cells, inner root sheath and lower part of the HF [41]. The melanocyte stem cells divide and produce mature melanocytes, generating and distributing pigmented granules to the follicular keratinocytes. During this phase the hair shaft continues to grow, becoming longer and thicker [40]. The catagen phases lasts for 1–4 weeks and starts at the end of the anagen phase. The lower two thirds of the hair follicle shrinks and regresses. As the keratinocytes and melanocytes in the matrix of the follicle undergo apoptosis, leaving the DP and bulge intact [42]. The DP condenses and migrates upward towards the bulge. At the end of the catagen phase the terminal hair base is keratinised and the hair shaft detaches from the bulb, forming a club hair [43]. During the telogen phase the club hair remains in the orifice and the HF stem cells are quiescent. The DP is in contact with the bulge, allowing for epithelial (HF stem cells in the bulge) – mesenchymal (dermal papilla cells) interactions (EMIs), triggering the formation of the second germ layer and induction of the next anagen phase [44]. During the exogen phase the club hair is shed from the HF orifice. Roughly 50–100 hairs are lost per day [42].

5. Hair tissue engineering in regenerative medicine

Regenerative medicine utilises an interdisciplinary approach, by combining the fields of biology, engineering and medicine to develop revolutionary solutions for tissue repair, replacement or regeneration [45]. The main aim of hair tissue engineering in regenerative medicine is to restore the structure and function of HFs affected by AGA, by repairing, replacing or regenerating these structures *in vitro* or *in vivo* [10]. Tissue engineering includes the use of growth factors, stem cell therapy, biomaterials and gene therapy.

As reviewed growth factors (such as vascular endothelial growth factor, transforming growth factor-β, insulin-like growth factor and platelet derived growth factor) are required at various stages of the hair growth cycle [46]. PRP increased the hair density and average anagen hair count in AGA patients, with signs of neoangiogenesis and cell proliferation [47,48]. PRP is a rich source of growth factors and cytokines, when released these molecules can activate various signalling pathways of gene transcription, promoting hair growth both *in vivo* and *in vitro* [49]. Fibroblast growth factor (FGF)-10, FGF-2 and FGF-1 induced telogen to anagen transition in C57BL/6 mice, via increased expression of the β-catenin and Sonic hedgehog pathways. Resulting increased hair density, number and size of hair follicles [50]. HF germs have been successfully cultured in media containing several growth factors e.g. FGF [51]. However, this is not economical for large scale production. PRP added to C57BL/6 mice embryonic epithelial and mesenchymal cell co-culture was able to upregulate DP cell trichogenic genes *in vitro* and increased the hair generation efficiency *in vivo* with 1.5 fold [52].

Stem cells are undifferentiated cells with an ability to self-renew and differentiate into several cell lineages to facilitate tissue repair and maintain homeostasis [53]. Stem cell therapy aims to reverse the pathogenesis of AGA, through stem cell transplants, stem cell derived conditioned media (CM) and exomes [54]. Ethical concerns have been raised regarding the use of embryonic stem cells and safety risks have been highlighted due to the potential tumorigenicity associated with induced pluripotent stem cells [55]. Thus, adult stem cells such as HFSCs and ADSCs have gained more attention. Autologous hair transplants, a form of HFSC transplant is currently the main treatment option for moderate to severe alopecia. It involves the transplant of donor hair follicles, containing HFSCs and DP cells, into balding recipient areas [31]. However, it is limited by the number of native follicles available for transplant. Alternatively, concentrated cell suspensions of HFSCs and DP cells re-injected into the balding areas of the scalp showed a 29 % increase in hair density within 23 weeks. This is a relatively inefficient process, as the harvest yields low cells counts of 5 % and 2.6 % of DP cells and HFSCs respectively [56]. The low increase in hair density will not be clinically relevant for cases of severe AGA.

Similar studies have shown that autologous cell suspensions of adipose-derived stem cells and progenitor cells increased hair density, expanded the number of follicles and enhanced healing [57,58]. The stromal vascular fraction (SVF) is a heterogenous group of stem and stromal cells derived from adipose tissue. Patients receiving intradermal injections of SVF cells showed an increase in hair density, hair shaft thickness, reduced hair loss and increased hair count [59,60]. *In vivo* the HF matures in close proximity to adipose tissue, suggesting adipose tissue may assist the stem cell niche during hair cycling via paracrine signalling [61]. CM is the culture medium in which cells, especially stem cells, have been cultured and contain the cell secretome (cell secretions), rich in growth factors, cytokines and proteins [45]. It is estimated that 80 % of the regenerative capacity of stem cells are mediated through paracrine signalling via their secretome [62]. Adipose-derived stem cell (ADSC) CM increased hair density and anagen hair growth rate [63]. Similarly, ADSC exomes increased the number of regenerated follicles and enhanced the maturity of the follicles *in vivo* [64]. Furthermore, ADSC-derived proteins protect DP cells from the effects of androgen-induced cell injury and produces growth factors (Platelet derived growth factor and vascular endothelial growth factor) that promote hair growth [65]. Thus, the positive effect on hair growth occurs as a result of growth factor-induced proliferation of dermal papilla cells, HFSCs, keratinocytes within the hair matrix and neovascularisation [66,67]. Injections of SFV cells, ADSCs or their

Table 1
Summary of various biomaterials used for HF organoid engineering in regenerative medicine.

Material	Method	Outcome	Reference
Silk-gelatin hydrogel	Human DP spheroids cocultured with HF keratinocytes and HF stem cells	Increased cellular proliferation. Increased viability. Increased expression of DP cell specific markers	[71]
Matrigel	Human DP cells co-cultured with dermal sheath cup cells and hair matrix cells	Stimulates high trichogenic inductivity in DP cells. Enhanced survival, proliferation and expression of hair inductive genes.	[72]
	Mouse epidermal and dermal cells.	Generated primitive anagen HFs, expressing antigens similar to native anagen HFs.	[73]
	Mouse epithelial and mesenchymal cells.	The supplementation of Matrigel increased hair sprouting efficiency.	[75]
Matrigel or collagen hydrogel	Human foetal/adult epithelial cells and Human foetal/adult mesenchymal cells in various combinations.	Cells cultured in the absence of Matrigel, or collagen failed to for HF structures. Adult HF keratinocytes and adult dermal papilla cells in 2 % Matrigel showed the greatest growth efficiency. AGA cultures of Adult HF keratinocytes and adult dermal papilla cells form less efficient HF structures.	[74]
Collagen type I hydrogel	Human neonatal dermal keratinocytes, fibroblasts, umbilical vascular endothelial cells and DP cells.	Low hair follicle induction efficiency, improved by transfection of DP cells with *Lef-1*	[76]
Collagen hydrogel beads	Human DP cells, encapsulated as hair beads, and cocultured with mouse epithelial cells.	HF specific genes were highly upregulated in hair beads compared to conventional spheroid culture. Similarly, the hair beads demonstrated a higher hair generating efficiency.	[79]
3D-bioprinted gelatin/ alginate hydrogel	Encapsulated fibroblasts, umbilical vein endothelial cells, DP cells and epidermal cells	Immature HFs developed in the 3D-bioprinted structures, *in vitro*. Organised HFs, with directionality developed *in vivo*.	[77]
Chitin-alginate fibrous hydrogel scaffold.	Human DP cells and epidermal keratinocytes.	Upregulated genes involved in EMIs and formed HF like structures *in vivo*.	[78]

secretions can be used as an ameliorative treatment option for the management of AGA but cannot cure AGA or generate new follicles and injections need to be administered regularly to maintain the positive effect on hair growth.

Biomaterials are substances that are engineered to interact with biological systems for different applications, especially tissue engineering. Various biomaterials have been engineered to mimic the native extracellular matrix [68]. However, hydrogels are a favoured material as they provide cells with an optimal environment for cell to cell interaction, survival, proliferation, differentiation, they are biocompatible, degradable, have a low immunogenicity and can be modified in composition [69,70]. Different microenvironments have been generated using natural hydrogels composed of silk-gelatin [71], Matrigel [72–75], collagen [74,76], gelatin-alginate [77] and chitin-alginate [78], as summarised in Table 1. Three-dimensional cell culture of HF organoids, using hydrogels, significantly increase the viability and proliferation of HF organoid cells. As well as increase the expression of HF organoid genes. This is attributable to the generation of an environment that is more representative of the *in vivo* environment and allows for EMIs. HFs have been successfully cultured *in vitro* using conventional 3D culture, but these HF structures are immature [74]. This approach has been improved by encapsulating mesenchymal and epithelial cells into a collagen microgel (termed hair beads) for further development *in vitro* and transplantation *in vivo* [79]. Advances in biomaterials have allowed for the 3D-bioprinting of HF organoids using hydrogel. The printed HFs develop into immature structures but demonstrate better directionality in hair growth when transplanted *in vivo* compared to conventional 3D culture [77]. HF have been developed as an associated structure in skin organoid models, using human induced pluripotent stem cells. However, this process is relatively ineffective for large scale HF generation as required by AGA patients [80]. Furthermore, hydrogels have been designed to be transplanted along with cultured cells *in vivo* and slowly release growth factors to stimulate and support HF growth [81]. Thus, hydrogels enhance the culture of HFs, can be co-transplanted and designed to release growth factors to support follicle growth *in vivo*.

Gene therapy is based on the genetic modification of cells to enhance the expression of certain growth factors or signalling pathways to increase hair production [53]. Wnt signalling regulates HF morphogenesis and cycling *in vivo*. Retrovirus induced overexpression of Wnt1a in bone marrow mesenchymal stem cells CM accelerates the telogen to anagen transition, increases the number of hairs and expression of trichogenic proteins [82]. Transfection of DP cells with Lef-1 enhanced gene expression in DP cells and differentiation of HFSCs [76]. The gene editing tool CRISPR/Cas9 showed potential to reduce the mRNA expression of the *SRD5A2* gene, responsible for the production of the 5 alpha reductase enzyme [83]. Gene editing does show promise as a potential treatment option for AGA. However, it is still in early stages of development, delivery of gene editing tools into the scalp is still relatively in efficient, gene editing protocols require vigorous safety assessments and gene editing possess a risk for mutations. Increased number of patients, drug side effects and shortage of donor follicles resulted in urgent need for a regenerative treatment modality [40,84,85].

6. Hair regenerative medicine using hair follicle organoids

Organoids are 3D, self-organising cell aggregates with similar structures and functions to a target organ. These 3D structures can be used to model human diseases ethically, test drugs for treatment or used for tissue engineering in regenerative medicine [11]. The application of HF organoids in regenerative medicine is to harvest DHT resistant cells from an AGA patient, culture and expand the cells *in* vitro, generate a sufficient number of organoids and transplant the organoids back into the balding areas of the patients for HF neogenesis, as illustrated in Fig. 2 and summarised in Table 2.

HF organoids require an epithelial and mesenchymal component to allow for cell interactions needed for HF morphogenesis and cycling [90]. DP cells form the mesenchymal component of an active HF and serve as the regulatory body for hair cycling [40]. These cells pose several challenges to regenerative medicine. DP cells need to be harvested and isolated from human HFs through microdissection, a relatively ineffective method [91]. A large amount DP cells are required, but these cells proliferate slowly in *in vitro* cultures. Thus, the combination of ineffective harvest and slow proliferation rate makes it difficult to generate a sufficient number of cells [92]. DP cells lose their trichogenic inductivity in traditional two dimensional or monolayer cultures [85,93]. HF stem cells in the bulge rebuild the HFs after each telogen phase, by giving rise to keratinocytes and melanocytes [41]. In HF organoids

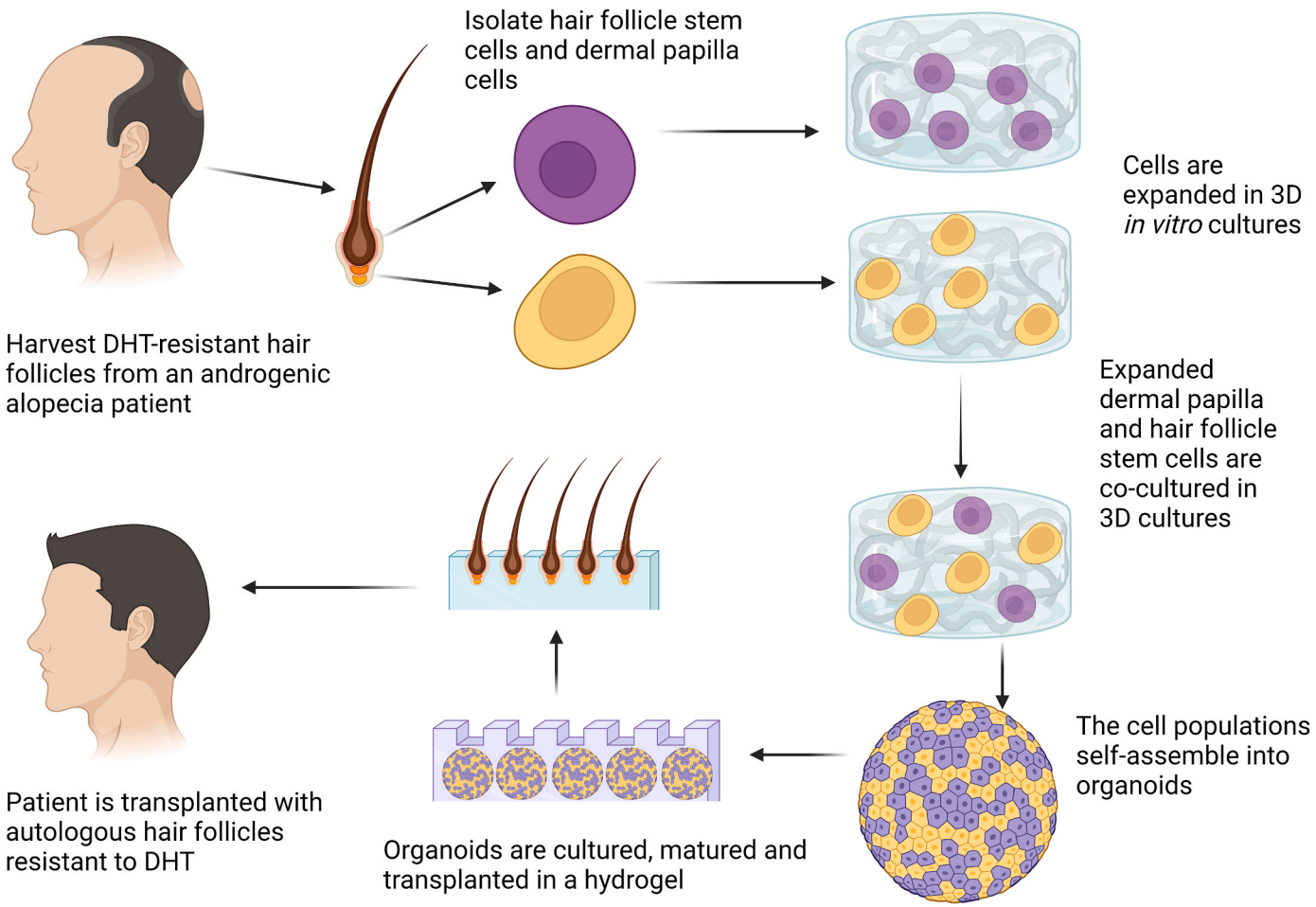

Fig. 2. Hair follicle organoids in regenerative medicine. The application of hair follicle organoids is to harvest DHT-resistant hair follicles from an AGA patient and expand the epithelial (hair follicle stem cells) and mesenchymal (dermal papilla cells) cell populations in a 3D *in vitro* culture. The populations self-assemble via EMIs and mature into hair follicles. The autologous, DHT resistant hair follicles are transplanted pack into the blading areas of the AGA patient.

Table 2
Studies on hair follicle organoids in regenerative medicine.

In vivo or *in vitro*	Cell type	Methodology	Outcome	References
In vitro	Human foetal/adult epithelial cells and Human foetal/adult mesenchymal cells	1:1 co-culture of epithelial and mesenchymal cells in a 2 % suspension of either Matrigel or collagen	Cells cultured in the absence of Matrigel, or collagen failed to for HF structures. Adult HF keratinocytes and adult dermal papilla cells in 2 % Matrigel showed the greatest growth efficiency. AGA cultures of Adult HF keratinocytes and adult dermal papilla cells form less efficient HF structures.	[74]
In vitro	Epidermal and dermal cells from newborn mice.	Co-culture (1:1) of murine epidermal and dermal cells in Matrigel matrix.	HF organoid was regenerated in Matrigel with structures similar to those of native anagen hair.	[73]
In vitro	Human neonatal foreskin keratinocytes and foetal scalp dermal cells.	Cells were co-cultured (2:3) in 3D droplets.	Cells self-organised into early hair pegs but failed to progress further.	[86]
In vivo		Cells were injected subcutaneously into nude mice in a patch assay.	Complete hair follicles, with hair shafts.	
In vivo	Human foetal dermal progenitor cells and newborn foreskin epidermal progenitor cells.	The cells were cultured, transferred onto PET membranes and grafted onto the dorsal skin of nude mice.	Dermal and epidermal cells (1:1) suspended for 24 h in solution, before being cultured demonstrated the highest hair forming potential.	[87]
In vivo	Mouse epidermal stem cells and skin-derived precursors (dermal cells).	The cells were encapsulated with Matrigel and transplanted into an excisional wound on nude mice.	The cell populations repaired the wound, with de novo hair follicle formation. PI3K/Akt is required for de novo hair follicle regeneration.	[88]
In vivo	Human dermal papilla cells, mouse epithelial cells and human umbilical cord vascular endothelial cells and (4:4:1).	Hair follicle germs were cultured in a micro-well chip and transplanted into a wound of nude mice.	The organoids developed similarly to other studies in morphology. Vascular endothelial cells localised in the dermal papilla. Pre-vascularisation of HF organoids greatly enhanced hair generation.	[89]

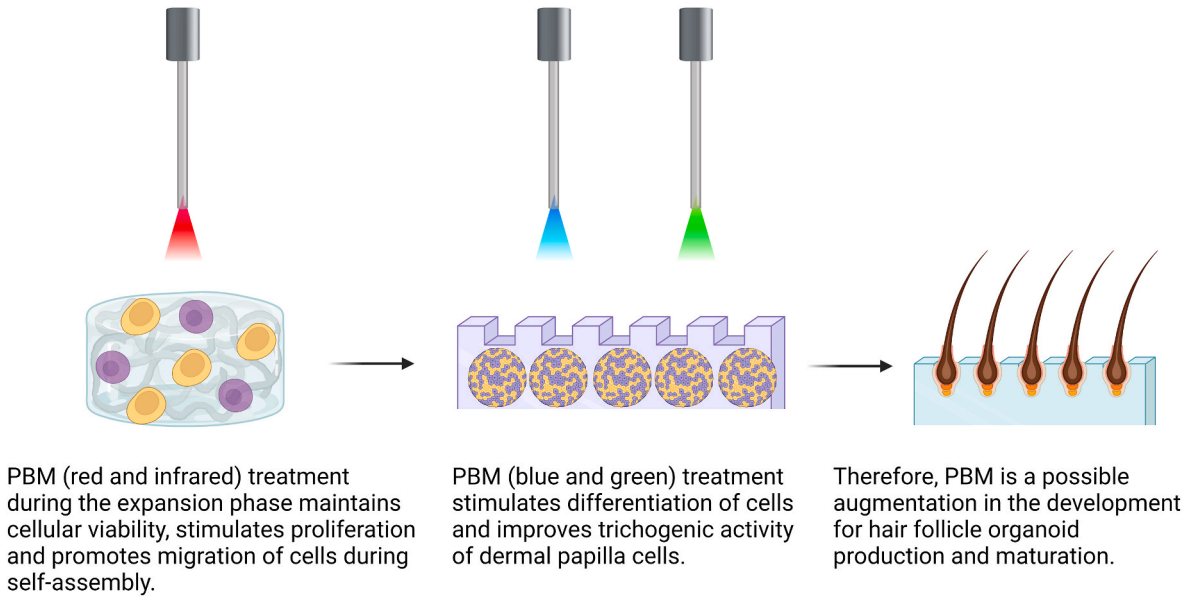

Fig. 3. The application of PBM in hair follicle organoid development. PBM can be used to augment the development of hair follicle organoids *in vitro*, by promoting cellular viability and maintenance of trichogenic activity. Furthermore, PBM stimulates migration, proliferation and differentiation.

they form the epithelial component. It remains a challenge to harvest an adequate number of these stem cells and due to their slow cycling nature, *in vitro* proliferation is ineffective [69,93].

Three dimensional cultures allow the cells to self-aggregate forming an environment organised similarly to that of their *in vivo* niche and enhances their trichogenic inductivity [90]. Hydrogels have been used *in vitro* to culture HFs and tend to improve the hair inductivity capacity of DP cells [73,74]. Mouse derived HF organoids have been regenerated in a 3D Matrigel co-culture and generated structures similar to native hair in the anagen phase, although immature in nature [73]. Human neonatal keratinocytes and dermal cell culture was able to generate early hair pegs, an early stage of hair follicle morphogenesis, *in vitro*. However, the pegs did not develop and mature further in 3D droplet culture. However, when the cells were transplanted into nude mice complete HF were generated. The authors highlighted low DP inductivity as a possible cause [86]. Most HF organoid studies are based on embryonic or murine cells in combination with human adult cells due to the low inductivity of adult cells in generating HF organoids [94]. However, adult human epithelial and mesenchymal cells recently demonstrated the highest efficiency in generating hair-sprouts in a 3D culture using Matrigel. Indicating the importance of 3D cultures, appropriate EMI's from co-culture and low passage number [74]. Furthermore, de novo HFs were generated, when a mouse epidermal and dermal cell matrix was transplanted into an *in vivo* mouse wound model [88]. Indicating that organoids require additional signals from the native tissue environment and more research is required relating to the signalling pathways from other cell populations.

It is possible to generate HF organoids *in vitro*, but these structures are usually immature and only fully mature in an *in vivo* environment [95]. However, in clinical practice the aim is to culture large amounts HF organoids to a mature state and transplant it back into an AGA patient, similarly to current hair transplants [69]. A large number of hair follicle organoids (roughly 5000) can be generated by *in vitro* 3D culture using prefabricated microwells and collagen hydrogel, with a hair generation efficiency of 65 % when transplanted *in vivo*. Furthermore, microwell culture allowed for the special transplantation of the organoids and resulted in spatially aligned HFs [51]. Organoids generated with AGA patient keratinocytes and DP cells generate less efficient HF structures due to the intrinsic pathophysiology mechanisms of AGA [74]. Indicating that clinically applicable organoids for HF neogenesis, in AGA patients, will require stronger stimulation to produce optimal HF organoids for transplantation. Currently the key challenges of organoids is the expansion of suitable cell populations *in vitro*, while maintaining their proliferative and inductive potential [12]. Aggregation before *in vivo* transplantation, pre-vascularisation (addition of vascular endothelial cells) and the use of a biomaterials (Matrigel) in HF organoids enhances the hair growing activity [74,87,89]. However, more methods of stimulation are required to maintain the inductivity, EMIs and proliferation of cultured AGA patient cells, to ensure successful organoid generation and maturation for transplantation.

7. PBM mechanisms and application in HF organoids

PBM is a form of light therapy, that uses lasers to produce low intensity light capable of stimulating endogenous chromophores [13]. PBM is gaining traction in the regenerative medicine space. However, PBM has not been standardised due to uncertainty in the mechanism of action and large variation in the laser parameters [96]. Although, in clinical practice PBM, previously known as LLLT, has been used successfully in hair regeneration by promoting hair growth, increasing shaft diameter and hair density, as well as an assistive technique in compound treatment regimens to stimulate hair growth [8,14].

The effect of red (600–700 nm) and near infrared light (780–1100 nm) is mediated by the release of nitric oxide from cytochrome *c* oxidase in the electron transport chain [97]. Thereby increasing the mitochondrial membrane potential and ATP (adenosine triphosphate) production [98]. Additionally, there is an increase in ROS production and serves as a signalling molecule in low concentrations [99]. Opsins (OPN) are light sensitive receptors, linked to transient receptor potential (TRP) cation channels [100]. Opsins are found in various cell types, such as keratinocytes, melanocytes, dermal fibroblasts and hair follicle stem cells [13]. Opsins are activated by specific wavelengths of light, triggering TRP channels to open and allow the influx of cations, especially calcium, into cells [101]. With the downstream effect of activating signalling cascades and gene transcription. Both OPN-2 (Rhodopsin) and OPN-3 (Encephalopsin) are present in hair follicles and respond to blue and green light. *In vitro* PBM (453 nm; 3.2 J/cm^2) prolonged the anagen phase and promoted proliferation in HF cells [102].

The Wnt/β-catenin canonical signalling pathway is the main regulator of HF growth and regeneration, by facilitating EMIs. Activation of

Table 3
Photobiomodulation use in hair regeneration.

Wavelength	Fluency	Methodology	Outcome	Reference
655 nm	3 J/cm^2	C57 mice were used in a scratched dermis model for *in vivo* work. DP cells harvested from mice, cultured and exposed to PBM for *in vitro* work.	HFs showed increased proliferation. *In vitro* PBM treatment had no effect on proliferation, but increased migration and exosome secretion in DP cells, via the activation of AKT/GSK-3β/β-catenin pathway.	[113]
660 nm	8 J/cm^2	Primary human DP cells were cultured and exposed to PBM.	PBM promoted DHT treated DP cell growth and viability. PBM modulated key hair growth pathways, such as Wnt and Sonic Hedgehog.	[116]
655 nm	2.9 J/cm^2	DP cells from HFs were harvested from AGA patients before and after receiving scalp PBM to measure changes in protein expression.	Proteins involved in transcription, protein synthesis, cell energy, ECM, cell to cell adhesion and angiogenesis was upregulated.	[115]
635 nm	8 J/cm^2	C57BL/6 mice were used for *in vivo* work and Lgr5-CreER: β-catenin$^{flox/flox}$ mice were used to model β-catenin knock out. Mouse HFSCs and SKPs were used for *in vitro* work.	PBM stimulates quiescent HFSCs. PBM-induced ROS activity stimulates the AKT/β-catenin pathway. PBM stimulates Wnt secretion.	[110]
636 nm	2.5–10 J/cm^2	Photodynamic treatment in C57BL/6 mice were used for the *in vivo* work.	PBM stimulation caused a transient increase in ROS. PBM treatment increased the number of proliferating cells in the bulge and dermal regions. Lef1 expression occurred earlier in the PBM treated group.	[111]
660 nm	3 J/cm^2	Twenty-five human males were exposed to PBM twice a week.	PBM stimulated an increase in hair density.	[109]
655 nm and 630 nm	4.8–7.4 J/cm^2	C57BL/6 mice were irradiated.	PBM stimulated hair growth and showed more anagen transitions compared to a minoxidil control. PBM treated groups showed increased proliferation in follicular keratinocytes and increased β-catenin expression in DP cells.	[112]
453 nm	3.2 J/cm^2	Primary human ORS keratinocytes HFs *in vitro*	PBM interacts with OPN3 and increases HF survival time *ex vivo*, stimulates proliferation of the ORS keratinocytes.	[114]
660 nm	4 J/cm^2	Three human males with AGA received PBM (12 events) and microneedling (2 events).	PBM and microneedling increased hair density with 26 % after four weeks.	[108]

this pathway mediates the transition of telogen to anagen phase, promoting proliferation and differentiation of HF stem cells and DP cells [103,104]. HFSCs reside in a Wnt-restricted environment, showing increased expression of Wnt inhibitors and down regulated Wnt promoters [105]. In brief the DP cells secrete Wnt (Wingless-related integration site) ligands, which bind to the frizzled receptor and complexes with Lrp5/6 (Lipoprotein receptor-related protein 5/6) coreceptors. This complex prevents the degradation of β-catenin and causes it to accumulate in the cytoplasm of HFSCs. The stabilised β-catenin acts as a transcriptional co-factor, complexing with Lymphoid enhancer factor (LEF)/T cell factor (TCF) and triggering the transcription of several Wnt target genes required for HFSC activation, proliferation and differentiation [106,107]. The PI3K/AKT signalling pathway is another important regulator in the cross talk between epithelial and mesenchymal cells for HF growth. Similarly, the PI3K/AKT signalling pathway is required for telogen to anagen phase progression and stimulates proliferation in HF stem cells [88]. In brief a relevant ligand activates PI3K (phosphoinositol 3-kinase) and tiggers the phosphorylation and activation of AKT, AKT affects glucose metabolism and promotes protein synthesis, proliferation and differentiation of HFSCs [103].

PBM treatment (660 nm; 3–4 J/cm^2) in patients with AGA showed a positive effect on hair growth, increasing the average hair density of patients. Furthermore, PBM used in combination with microneedling had a synergistic effect [108,109]. PBM treatments (635 nm; 8 J/cm^2) (636 nm; 2.5–10 J/cm^2) was sufficient increase the expression of Lef1, a marker of telogen to anagen transition, in mice HFs (*in vivo*) and stimulate HFSC proliferation. With the effect of speeding up HF growth and creating longer HFs in the experimental group (within 7 days) compared to the control group (within 14 days) [110,111]. Thus, indicating that PBM has a positive effect on hair follicle growth and cycling in both AGA patients and animal models. By triggering the transition from telogen to anagen. PBM treatment significantly increased the expression of β-catenin, a key regulator of the hair cycle, in the HF bulge (HFSC region), DP cells and secondary hair germ layers [110,112]. Furthermore, PBM increased ROS production, activating the PI3K/AKT/GSK-3β signalling pathway and prevented β-catenin degradation [110,113]. PBM also promoted the expression and secretion of Wnt ligands in SKPs, further activating the β-catenin signal. However, in a β-catenin knockout model the positive effect of PBM and HFSC was negated. Similarly, removing ROS or inhibiting Wnt secretion negates the positive effect of PBM [110]. Thus, indicating that PBM's positive effects on hair growth (telogen to anagen transition) is mediated by increased expression of the Wnt-β-catenin pathway. Currently PBM mostly focuses on light in the red and near infrared spectrums. However, PBM can also interact with OPNs in the hair follicle. PBM (453 nm; 3.2 J/cm^2) interacts with OPN3 and increases HF survival *ex vivo* and stimulates the proliferation of ORS (outer root sheath) keratinocytes *in vivo* [114]. These molecular mechanisms result in an increase in proliferation within the hair follicle bulge and DP regions [111,113]. Furthermore, PBM (655 nm; 2.9 J/cm^2) significantly upregulates the expression of proteins involved in transcription, protein synthesis, cell metabolism, ECM production and

cell-cell interaction in AGA DP cells [115]. Similarly, PBM (660 nm; 8 J/cm^2) promoted growth, viability and increased inductivity in DP cells treated with DHT *in vitro* [116]. It is evident that PBM has a positive effect on hair growth, by upregulating critical hair growth pathways and stimulates cell proliferation, viability and differentiation, as illustrated in Fig. 3 and summarised in Table 3. However, the use of PBM in the development of HF organoids is lacking.

8. Perspective and conclusion

In conclusion AGA is a chronic condition with moderately effective treatments options available. However, their effectivity and side effects remain problematic [27]. Current treatment options need to be utilised indefinitely to retain their positive effects and are non-curative. Hair transplants are currently used for AGA patients with moderate to severe AGA and are limited by the number of follicles available for transplant [9]. Thus, a new approach using regenerative medicine is required to harvest DHT resistant hair follicles from AGA patients and expand the resident cell populations *in vitro*. Thereafter these cells can be 3D cultured and developed into HFs for autologous transplantation back into the patient, to develop into HFs *in vivo*.

However, there are several challenges to this approach. The harvest of DP cells and HFSCs is a relatively ineffective process, and these cell populations proliferate slowly [92,93]. A sufficiently large number of cells are required for successful organoid generation and transplantation. DP cells rapidly lose their hair inductivity capacity *in vitro* and DP cells from AGA patients already have an inertly ineffective inductivity capacity due to the pathophysiology of the disease [74,85]. Lastly, current HF organoid models have a low hair generation efficiency and do not mature *in vitro* [73].

Studies available on the application of PBM on HFs have shown a positive effect on hair growth in AGA patients, mouse models and *in vitro* applications on DP cells, keratinocytes and HFSCs [109,112,114]. PBM is capable of increasing the expression of both the Wnt/β-catenin and PI3K/AKT signalling pathways, two of the key regulators of telogen to anagen transition [110]. PBM maintains viability, promote migration, stimulate proliferation and enhance the inductivity capacity of normal and DHT exposed DP cells [116]. Furthermore, PBM stimulates the activation, proliferation and differentiation of quiescent HFSCs [110]. As well as increase the proliferative capacity of HFs, especially the keratinocytes. Thus, it is evident that PBM if applied to HF organoids would generate a similar positive effect and could possibly assist in solving the current challenges experienced in the large-scale production of HF organoids for clinical applications.

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List of abbreviations

3D	Three-dimensional
ADSC	Adipose-derived stem cell
AGA	Androgenic alopecia
AR gene	Androgen *receptor* gene
ATP	adenosine triphosphate
CM	conditioned media
DHT	Dihydrotestosterone
DP	Dermal papilla cells
ECM	Extracellular matrix
EDA2R gene	Ectodysplasin A2 gene
EMI/s	Epithelial mesenchymal interaction/s
FDA	Food and drug administration
FGF	Fibroblast growth factor
FUE	Follicular unit extraction
FUT	Follicular unit transplant
HF/s	Hair follicle/s
IL-1β	Interleukin 1-β
LEF	Lymphoid enhancer factor
LLLT	Low-level light therapy
NF-kβ	Nuclear factor kapa-β
NO	Nitric oxide
ORS	Outer root sheath
PBM	Photobiomodulation
PRP	Platelet rich plasma
ROS	Reactive oxygen species
SVF	Stromal vascular fraction
TCF	T cell factor
TGF-β1	Transforming growth factor-β1
TNF-α	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor
WNT	Wingless-related integration site

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