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Invited Review Article

Evaluating hair growth promoting effects of candidate substance: A review of research methods

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ABSTRACT

Androgenetic alopecia (AGA) is the most common form of hair loss disorder. As the prevalence of AGA rises, the demand for AGA treatments is rising accordingly, prompting research to identify therapeutic candidates to treat AGA. Because AGA is caused by crosstalk among multiple hair follicle (HF) cell components, understanding the effects of candidate molecules on HF cells is essential to determining therapeutic candidates for treatment. To date, research has centered on HF dermal papilla and outer root sheath cells and has indicated that the hair growth effects of candidate substances may be mediated via alterations in several signaling pathways and signature genes in these HF cells. In more integrative evaluations, the HF unit is used as an *ex vivo* organ culture model to verify the effects of therapeutic candidates. Animal models have also been used to evaluate the effects of candidate substances. The main outcomes used to evaluate the effects of candidate substances are 1) changes in HF growth rates *in vitro*, 2) anagen induction capabilities, and 3) the effects of androgen modulation. This article reviews a series of methods used to evaluate the hair growth-promoting effects of candidate substances, providing an overview of cell assays, organs, and animal models used in AGA research in order to facilitate AGA research moving forward.

1. Introduction

Androgenetic alopecia (AGA) is the most common hair loss disorder, and its prevalence is rising with increasing life expectancy. AGA not only affects one's appearance but also one's mental state, amplifying demands for treatment [1]. Topical minoxidil (for men and women), oral dutasteride and finasteride (for men) have been the only effective medications to date [2]. Consequently, high demand for novel and more effective treatments has prompted researchers from a variety of academic backgrounds to further investigate and identify new therapeutic candidates to treat AGA.

Although hair follicle (HF) sizes vary based on animal species and anatomic locations, the microscopic structures of hair follicles are similar. Hair follicles can be divided into two parts based on bulge, the insertion site of the arrector pili muscle: the upper part of the bulge is composed of the infundibulum and the isthmus, and the lower part of the bulge consists of the supra-bulbar area and the bulb (in which dermal papilla (DP) cells are embraced by epithelial cells). Every HF has cyclic physiology with three phases: anagen (growth), catagen (regression), and telogen (rest) [3]. The upper part of each follicle is permanent, irrespective of the hair cycle, but the lower part of the follicle regenerates cyclically and repeatedly.

The bulge and the DP cells contained therein are key components in HF hair growth (anagen phase) and regeneration (transition telogen phase to anagen phase) [4]. DP cells interact with surrounding epithelial cells, resulting in hair shaft elongation by proliferation and differentiation of the epithelium of the bulb in anagen phase. The resulting epithelium is arranged concentrically by cell type, as follows: the hair shaft (medulla and cortex), the inner root sheath, and the outer root sheath. The bulge supplies transit-amplifying cells and thereby plays an essential role in regenerating a new HF with its shaft in the transitional phase (from telogen to anagen) [5]. At the end of the telogen phase, DP cells

Abbreviations: AGA, androgenetic alopecia; DP, dermal papilla; HF, hair follicle; HFSC, hair follicle stem cell; ORS, outer root sheath.

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initiate the subsequent anagen phase by regulating HF stem cells in the bulge and secondary germ [6].

AGA is characterized by shortening of the anagen phase and prolongation of the telogen phase, resulting in HF miniaturization in androgen-sensitive scalp and conversion into vellus hair [7]. Thus, the majority of prospective therapeutic strategies for AGA treatment involve the modulation of hair cycle dynamics, specifically prolonging or inducing the anagen phase [8,9]. The present article focuses on the current methods used to evaluate the hair growth-promoting effects of candidate substances, providing a useful overview of published hair growth evaluation assays in cells, organs, and animal models (Table 1 and Fig. 1).

2. Cell studies

2.1. Individual cell model: DP cells

Although the hair shaft is mainly composed of keratinocytes (epithelium), DP cells in the bulb play a critical role in hair growth via dermal-epidermal interactions [4]. The number of DP cells increases during anagen phase of the human hair cycle [10]. In mice, DP cell numbers correlate with the sizes and shapes of the hair shafts in the follicles [11].

Surgical microdissection is a well-established method for isolating DP cells from human HFs [12]. After freely dissecting each follicle under a dissecting microscope, the bulb of each follicle is excised, and an opening is formed with a needle bevel; DP cells are removed via this opening using a light press with forceps. Once isolated, DP cultures display spindle-shaped cells in a monolayer, similar to fibroblast morphology. Human DP cells are also commercially available, enabling studies to continue despite difficulties in obtaining intact human HFs. Mouse vibrissa dermal papilla cells can be obtained in a similar fashion [13].

It is first necessary to confirm whether the candidate substance affects DP cell viability or is toxic to DP cells. MTT assays are widely used to evaluate the cytotoxic effects of candidate substances on DP cells. Once it is determined that the candidate substance is not toxic to DP cells, studies can be performed to determine if the candidate substance acts on DP cells to promote hair growth. Intracellular molecules can be analyzed as a means of determining hair growth-promoting effects.

β-Catenin proteins in DP cells, which are mainly regulated by Wnt signals, are critical to HF regeneration and anagen phase maintenance. Disruption of β-catenin signaling prevents anagen induction [14]. Thus, it is important to assess intracellular β-catenin protein quantities and locations after treatment with the candidate substance. Cytoplasmic β-catenin translocated into the nucleus to activate the target signaling pathway (i.e., fibroblast growth factor (FGF), insulin-like growth factor (IGF)) [14]. During anagen phase, FGF7, FGF10, and IGF-1 mRNA expression levels increase in mouse DP cells, stimulating epithelial proliferation [15,16]. In short, activation of the Wnt pathway indicates hair growth promotion by a candidate substance. In addition, given that nuclear β-catenin also accumulates via phosphorylation of Akt [17], it appears that Akt signaling activation may be a screening tool for determining hair growth induction by a candidate substance.

The ERK signaling pathway also activates cell growth, and its effects on hair growth have been investigated. Vascular endothelial growth factor (VEGF) regulates the proliferation of DP cells via the ERK pathway [18]. In recent years, JAK-STAT signaling has been one of the most extensively investigated pathways in the field of hair research, with studies suggesting that inhibiting this pathway could induce normal anagen phase development in HFs by activating Wnt signaling pathways [19].

Table 1
Tools to evaluate the hair growth-promoting effects of candidate substances.

Tools	Grounds and checkpoints
Cell Studies	
Dermal papilla (DP) cells	DP cells are crucial mesenchymal components of HFs [4]
	DP numbers increase during the anagen phase in HFs [10]
	– Wnt and Akt signaling activation or JAK-STAT signaling inhibition [14,17,18,19]
	β-catenin cytoplasmic translocation [14]
	Fibroblast growth factor and insulin-like growth factor [14,15,16]
	– ERK signaling activation
	Vascular endothelial growth factor [18]
	– Increased DP markers
	Alkaline phosphatase, versican, and Sox2 [20]
	– Decreased signaling cytokine
	Transforming growth factor-β1 [24]
Outer root sheath (ORS) cells	ORS bulge area is the niche of HF stem cells [5]
	Activated HFSCs are required to regenerate new HFs [5,25]
	– Promotion of S/G2-M entry by cell cycle analysis [28]
	– Alterations in colony forming and proliferating efficiencies of K15+ cells [5,28]
DP cells and ORS cells	HFs are organs requiring reciprocal interactions between epidermal and dermal components [29]
Organ model studies	
Human hair follicle (HF) unit	Extracellular matrix is another component of the physiologic HF unit
	– HF bulb and matrix size and melanin distribution [37]
	– Immunofluorescence staining for DAPI/Ki67/TUNEL [37]
Rat vibrissae follicle unit	Cyclical activity of follicles [35]
	(compared to human HFs in which only anagen phase could be evaluated)
Full-thickness human scalp skin	Investigation in an *in vivo*-like situation [36]
Animal model studies	
Anagen phase induction models	Anagen induction ability is useful for evaluating hair growth effects [38]
	– Accelerated skin thickness and hair cycle score of C57BL/6 mouse or C3H mouse [39,40]
Androgen effect modulation models	The main pathogenesis of human AGA is associated with androgen
	– Sebum amounts and gland sizes in male fuzzy rats [44]
	– Hair growth patterns in Golden Syrian hamsters [45]
	– Delayed hair regeneration in Keratin 5-human androgen receptor transgenic mice [46]
	– Hair growth promoting effects in stump-tail macaque [47,48]

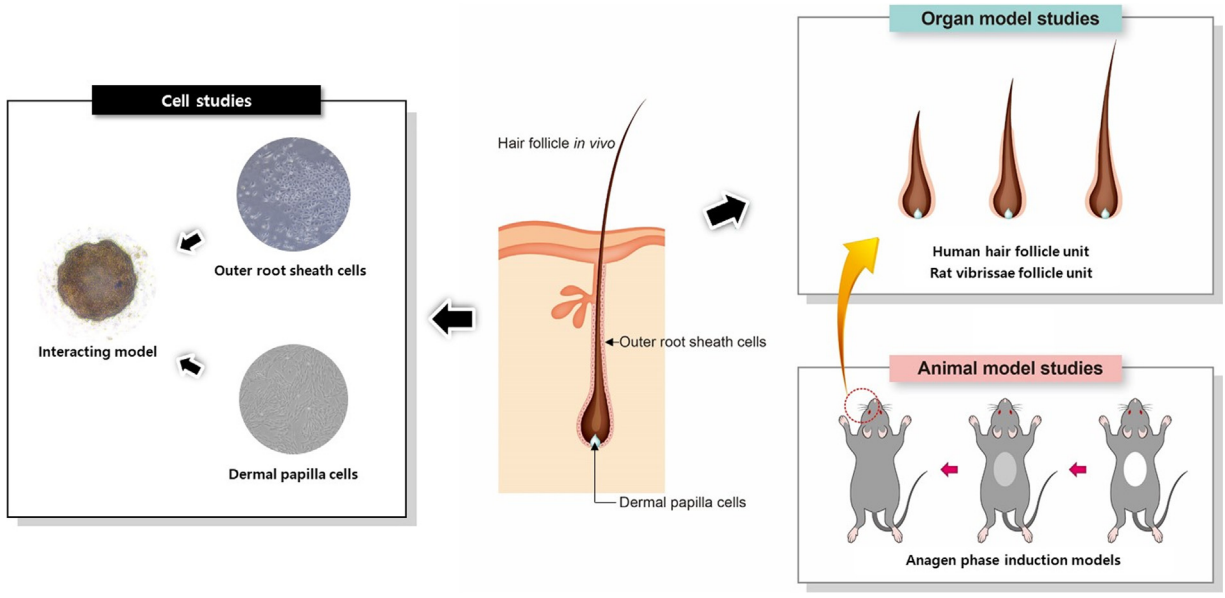

Fig. 1. A series of methods used to evaluate the hair growth-promoting effects of candidate substances: cell assays, organs, and animal models used in androgenetic alopecia research. In cell studies, dermal papilla cells and outer root sheath cells are used. For organ model studies, human hair follicle unit, as well as rat vibrissae follicle unit, are used. Anagen phase induction mouse models are widely used in animal studies.

Alkaline phosphatase, versican, and Sox2 are DP markers that may reflect hair growth properties [20]. The alkaline phosphatase activities of DP cells from mouse vibrissa follicles reach a maximum during the early anagen phase [21]. While versican expression is specific to DP cells in anagen phase HFs (not during the catagen or telogen phases) of humans and mice [22], versican expression is significantly decreased in the DP cells of vellus-like HFs (AGA affected hair) [22]. Sox2 expression is only detected during anagen phase [23]. In short, the increasing levels of alkaline phosphatase, versican, and Sox2 after treatment with a candidate substance indicate that the candidate substance modulates the hair cycle.

In contrast, transforming growth factor (TGF)-β1 derived from human DP cells inhibits hair growth by inducing keratinocyte apoptosis, as observed in androgenetic alopecia (AGA). Thus, evaluating the effects of TGF-β1 gene downregulation may provide a means of screening candidate substances for promoting hair growth [24].

2.2. Individual cell model: outer root sheath (ORS) cells

Anagen induction requires activation of hair follicle stem cells (HFSCs). Activated HFSCs provide transit amplifying cells, which produce downstream progeny cells in new, regenerative HFs [5,25]. Because HFSCs are located primarily in the bulges of ORS, ORS cells may be useful for screening the anagen inductive efficiencies of candidate substances for AGA treatment. ORS cells can be isolated from plucked HFs and cultured as previously described [26]. Anagen phase HFs should be used, as they contain visible bulbs and intact ORS. After removing the bulbs and infundibulum parts, selective trypsinization of the ORS yields single ORS cells. As with DP cells, the toxicity or proliferation effects of the candidate substance on ORS cells can be measured using the MTT or bromodeoxyuridine (BrdU) methods [27]. Cell cycle analysis can be used to evaluate anagen-prolonging effects [28]. The promotion of S/G2-M entry in ORS cells by a certain substance can be interpreted as that substance's anagen-prolonging effects. Among ORS cells, keratin 15 (K15+) cells are HFSCs [5]. Furthermore, changes in colony forming and the proliferating efficiency of cultured K15+ epithelial stem cells by a candidate substance indicates that substance's ability to modulate stem cells; and K15 mRNA expression in the cells provides another indicator [28].

Because a candidate substance should target vellus HFs rather than normal HFs, a more appropriate target *in vitro* experimental cell line may be the vellus HF-derived keratinocytes, rather than ORS cells from normal HFs. In fact, vellus HF-derived keratinocytes manifest morphological and differentiation characters that are distinct from normal epidermal keratinocytes [29].

2.3. Interacting model: DP cells and ORS cells

The HF is a complex mini organ that requires epithelial–mesenchymal interactions to function properly [29]. Given the importance of reciprocal interactions between epidermal and dermal components in hair growth, a model incorporating the interactions between DP cells and ORS cells would represent a more applicable "physiologic" *in vivo* state than that assumed by individual cell research models that use separate cell populations, such as those discussed above. Indeed, ORS cell proliferation rates were significantly accelerated in the two-chamber culture model using microporous-membrane-separated human DP cells, and the effect was significantly enhanced by cell-cell adjacency, suggesting that two cell groups are involved in proliferation [30]. In a recent paper indirectly employing an interacting model between DP cells and ORS cells [27], ORS cells were cultured in DP cell-conditioned media to evaluate the paracrine effects of candidate-substance-treated DP cells on ORS cells.

Three-dimensional human folliculoid microspheres *in vitro*, composed of human DP cells and ORS cells within an extracellular matrix, may provide an interacting model that effectively mimics conditions *in vivo* [31], thereby providing a simplified screening system. In this system, certain known hair growth-modulating molecules (i.e., calcitriol, cyclosporin A) alter the folliculoid microspheres, protein and gene expression associated with hair growth. A similar three-dimensional model, consisting of human

DP cell spheroids encapsulated by silk gelatin hydrogel and HFSCs and keratinocytes [32], has been used to simulate the expression of various DP cell genes in the spheroids of HFs *in vitro*.

3. Organ model studies

A hair follicle is not merely a simple aggregation of DP and ORS cells within an extracellular matrix but a mini-organ whose cycles are regulated by well-orchestrated components. Thus, it is more suitable to evaluate HF components simultaneously when assessing the hair growth-promoting effects of a candidate substance.

An intact human HF or rat vibrissae unit provides an appropriate organ culture model *ex vivo*. Philpott, et al. were the first to report an organ culture method using human HF units [33]. Using a dissecting microscope, intact HFs are obtained by pulling hair bulbs from a subcutaneous fat layer separated from human scalp tissue. This enables the harvesting of anagen phase human HFs, which can be maintained in Williams E medium with supplements. The hair shaft growth rate *in vitro* is around 0.3 mm per day, similar to growth rates measured for human scalp *in vivo*. Studies should employ follicles in the precisely same hair cycle, which could be determined by bleaching methods [34], in order to ensure more concise and consistent HF organ cultures. HF elongation *in vitro* generally correlates with human hair growth *in vivo*, though this type of assessment may only be applicable for HFs in anagen phase. Rat vibrissae follicles may provide an organ culture unit model enabling the observation of cyclical activity in follicles of all phases [35]. In addition, an organ culture method for full-thickness human scalp skin has been established [36]. This method confirmed human HF unit in skin tissue could survive for more than two weeks in serum-free culture condition, which means that it could be a tool for investigating hair growth modulating effect of selected substance under *in vivo*-like situation.

In order to conclude a candidate substance exerts hair growth-promoting effects, it should be considered whether the anagen duration of culture HF organ *in vitro* is prolonged by the substance treatment. Comprehensive criteria set, which is based on morphological and morphometric data in microscopy or immunofluorescence, has been suggested for categorizing the *in vitro* cultured HFs into anagen or catagen phase objectively [37]. In brief, the HFs in anagen phase, compared to those in catagen phase, show 1) larger sized HF bulb and matrix, 2) higher melanin in central hair matrix 3) with more evenly distributed melanocytes. Immunofluorescence staining for DAPI/Ki67/TUNEL resulted that HF blub in anagen phase exhibited more DAPI + cells, higher percentage of Ki67+ cells, and lower number of TUNEL + cells, compared to in the catagen phase.

4. Animal model studies

4.1. Anagen phase induction models

Assessing the HF follicles anagen induction abilities is useful for evaluating the hair growth effects of a hair-promoting candidate substance [38]. Mice with pigmented hair, such as C57BL/6 [39] and C3H [40], at 7 weeks old (telogen-phase starting point) are commonly used to evaluate the anagen induction abilities of candidate substances *in vivo*. Two important advantages of using these mouse models to evaluate anagen induction ability are as follows: 1) the mouse back hair cycle is synchronized and relatively short, and 2) in the adult mouse, cutaneous melanocytes are located exclusively in HFs (except in footpads, tails, and ears), that is, not in inter-follicular epidermis cells [41]. The method, in short, involves shaving the dorsal skin of a 7-week-old mouse and exposing the non-pigmented back skin, which is exposed and maintained for 5 weeks without any intervention. The exposed skin becomes darker due to the pigmentation of incoming hair, and HFs are regenerated via telogen to anagen phase transitions (around 12-week-old) [42]. When an effective candidate substance has been used, hair re-growth (manifested by darkening back skin) should occur in the experimental group sooner than in the control group.

Quantitatively, skin thickness and hair cycle score can be assessed by histologic examination [42,43]. Mouse skin thickness depends on the hair cycle, peaking in the anagen phase and becoming thinner until the telogen phase is reached. By comparing the skin thicknesses of experimental versus control groups, investigators can evaluate anagen inductive activity. The hair cycle is assessed using an arbitrary scoring system in numerical order [42]. In brief, HFs are assigned values based on their current subdivided hair cycle phases, and each anagen or catagen subdivided phase is assigned a numerical value: anagen I = 1, anagen II = 2, and so on; catagen I = 1, catagen II = 2, and so on). Averaging that value by the number of HFs in each subdivided phase yields a hair cycle score between 1 and 6 for anagen HFs and between 1 and 8 for catagen HFs. The hair cycle score reflects changes in HF cycling.

4.2. Androgen effect modulation models

Given that the main pathogenesis of AGA in humans is associated with testosterone (which is converted to 5α-dihydrotestosterone by 5α-reductase and binds to androgen receptors), evaluating testosterone pathway modulating capacities of candidate substances provides an important means of assessing their effectiveness at improving hair growth. Male fuzzy rats secrete sebum and exhibit androgen-dependent hyperplasia in their sebaceous glands [44]. Using this model, researchers can determine the amount of sebum secreted and sebaceous gland sizes to assess the ability of the candidate substance to induce androgen effect modulations. In the Golden Syrian hamster (*Mesocricetus auratus*), another animal model used to evaluate androgen effect modulations, androgen has opposite effects on hair growth depending on anatomical location. For instance, androgen has been found to stimulate hair growth on the flank but to suppress hair growth on the surrounding skin [45]. Crabtree et al. introduced an AGA murine model based on keratin 5-human androgen receptor transgenic mice. Exposing high levels of 5-alpha dihydrotestosterone to this model results in delayed hair regeneration, similar to AGA in humans [46].

Non-human primate species manifested AGA; chimpanzees (*Pan troglodytes*), stump-tailed macaques (*Macaca arctoides*), and bald uakari (*Cacajao rubicundus*) [47]. Among them, stump-tailed macaques have been used to evaluate the hair growth effects as a biological model for human AGA. This species exhibits hereditary spontaneous hair thinning over the frontal scalp, which is similar to AGA in humans [47,48].

5. Conclusion

Demands for AGA treatments are increasing, prompting research to develop hair growth-promoting agents. AGA results from crosstalk among multiple HF components. Every part of the HF plays a unique role in regulating hair growth. To date, cellular research has focused on DP and ORS cells. Evaluating the cellular effects of candidate molecules is one of the most crucial components in assessing their potential for inducing hair growth. Furthermore, pharmacological outcomes obtained in HFs *in vitro* and in animal models provide the primary means of assessing the physiological effects of candidate substances.

Promising results in the laboratory do not necessarily equate to the promotion of hair growth in humans with AGA. Clinical human

trials provide the most definitive and meaningful test system. However, a target substance cannot be administered directly to humans without a clear understanding of its benefits and side effects. An ideal screening model for testing hair growth-promoting agents in HF has yet to be developed. Meanwhile, new tools for screening candidate substances have been under development, such as a skin organoids model with *de novo* HFs, which may be used as an organ-on-a-chip model [49]. In addition, *in silico* methods employing computer systems have been used in research aimed at discovering active molecules for specific target organs [50]. More reliable and simple assay methods are expected in the future. The present review of assay methods used to assess hair growth effects should prove useful to researchers with varied backgrounds who wish to evaluate candidate substances for treating AGA.

Conflicts of interest

The authors have no conflict of interest to declare.

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