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Current advances in stem cell-based therapies for hair regeneration

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ABSTRACT

Alopecia is resulted from various factors that can decrease the regeneration capability of hair follicles and affect hair cycles. This process can be devastating physically and psychologically. Nevertheless, the available treatment strategies are limited, and the therapeutic outcomes are not satisfactory. According to the possible pathogenesis of nonscarring alopecia, especially androgenetic alopecia, recovering or replenishing the signals responsible for hair follicle stem cells activation is a promising strategy for hair regeneration. Recently, stem cell-based therapies, especially those based on the stem cell-derived conditioned medium (CM), which is secreted by stem cells and is rich in paracrine factors, have been widely explored as the hair regenerative medicine. Several studies have focused on altering the composition and up-regulating the amount of secretome of the stem cells, thereby enhancing its therapeutic effects. Besides, stem cell-derived exosomes, which are present in the CM as message entities, are also promising for hair regrowth. In this review, the up-to-date progress of research efforts focused on stem cell-based therapies for hair regeneration will be discussed, including their therapeutic potentials with respective merits and demerits, as well as the possible mechanisms.

1. Introduction

Attributed to hereditary factors, emotional stress and psychiatric disorders, alopecia is highly prevalent in current society, resulting in devastating physical and psychological sequelae (Mohammadi et al., 2016; Zhang et al., 2014). Considering the role of stem cells in the pathogenesis, alopecia can be divided into two types: nonscarring alopecia and scarring alopecia (Al-Refu, 2012). In nonscarring alopecia, the progenitor cells are destructed, while the hair follicle stem cells (HFSCs) are preserved, which is why this kind of alopecia can be reversible (Mohammadi et al., 2016; Owczarczyk-Saczonek et al., 2018). Androgenetic alopecia (AGA) accounts for the majority of the nonscarring alopecia cases, affecting up to 80% of Caucasian men by the age of 80 and nearly 40% of Caucasian women by the age of 70 (Al-Refu, 2012; Gentile et al., 2017a; Sorbellini et al., 2018).

Currently, there are three therapeutic mechanisms related to the regeneration of hair follicles (HFs) with the employment of stem cells, reversing the pathogenesis of hair loss (particularly in AGA), regenerating HFs with "bulge", and neogenesis of HFs from a stem cell culture (Asakawa et al., 2012; Balañá et al., 2015; Gentile et al., 2017b). The action of reversing the pathological mechanism of AGA may primarily be based on the secreted bioactive factors, including growth factors and cytokines with which to induct HFSCs in their native niche. That is, stem cells can secret these factors to trigger host-site damage repair cascades *via* paracrine effects (Bak et al., 2018; Inukai et al., 2013; Katagiri et al., 2013). Therefore, the wide array of substances that are secreted by stem cells, designated as secretome, have gained increasing attention for their critical roles in the regulation of multiple physiological processes. The secretome consists of all the factors that are secreted into the extracellular space, including proteins, extracellular vesicles (EVs) and nucleic acids (Beer et al., 2017; Vizoso et al., 2017). The nutrient medium containing abundant secretome where stem cells are cultured has been termed as "conditioned medium (CM)" (Kim et al., 2013). It has been demonstrated that the stem cell-derived CM exerts positive effects on hair regeneration (Fukuoka and Suga, 2015). Moreover, to enhance the therapeutic efficacy, a wealth of researches focusing on altering the composition and upregulating the amount of secretome have emerged, such as through hypoxic incitement and gene engineering.

In addition, exosomes are the smallest type of EVs, which deliver functional mRNAs, microRNAs, cytokines and transcription factors to target cells, and are capable of triggering regeneration (Chevillet et al., 2014; Liu et al., 2018; Maguire, 2013). As the potent cell-to-cell transporters, exosomes are also promising for hair regrowth.

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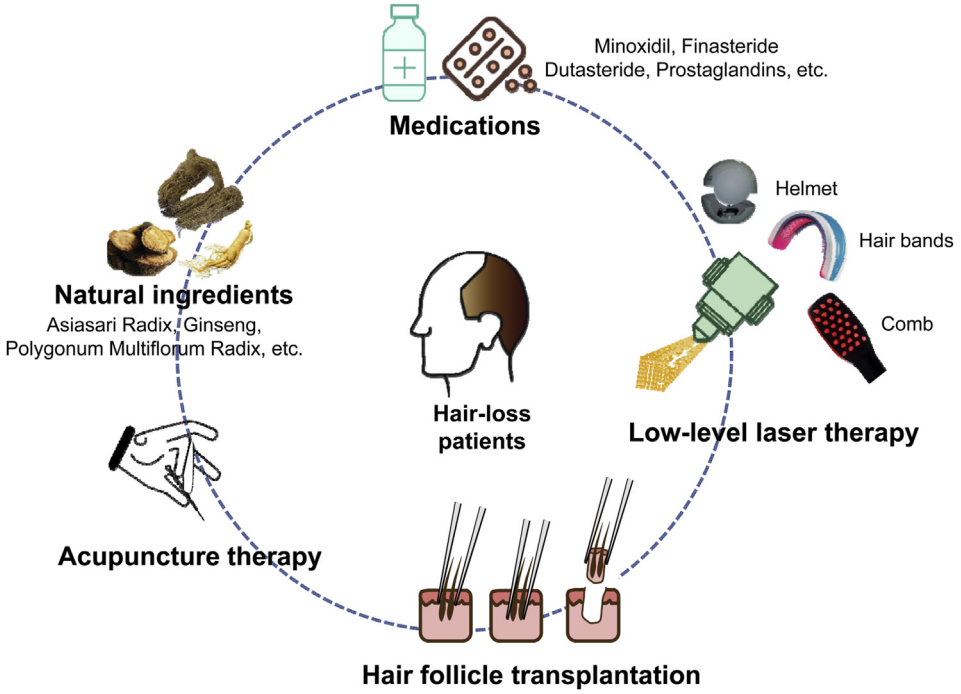

Fig. 1. Currently available treatments for hair loss.

Herein, we review the current research progress regarding stem cell-based therapies for hair regeneration, especially those based on the CM and exosomes, describing their therapeutic potentials and possible mechanisms. Based on the published research, the studies addressing the use of stem cell-based therapies in hair regeneration are limited. Also, there still remain several challenges in relation to the mass production and clinical application. Nevertheless, the approaches are promising since the outcomes from these studies are encouraging (Ramdasi and Tiwari, 2016).

2. Currently available and emerging treatments for hair loss

Although hair loss is highly prevalent in our society, effective treatments are lacking. Currently available treatments (Fig. 1), such as surgical procedures and medications, cannot meet the satisfaction of most patients due to the unfavorable outcomes, impermanent results or undesirable side effects. The HF transplantation that involved advanced surgical treatment is limited by the shortage of donor hair, the reduced viability of cells, and expensive and time-consuming nature of the procedure. Furthermore, the result is often temporary due to the progressive property of hair loss conditions (Mohammadi et al., 2016; Owczarczyk-Saczonek et al., 2018; Tully et al., 2010; Zhang et al., 2014).

Minoxidil and finasteride, the only two medications available for the treatment of AGA that are approved by the Food and Drug Administration, are associated with variable efficacy and varying degrees of side effects (Falto-Aizpurua et al., 2014; Gupta and Charrette, 2015; Rossi et al., 2016; Shapiro and Kaufman, 2003). Minoxidil is a prodrug and can be converted by sulfotransferase into minoxidil sulfate, which is a potassium channel opener (Jahangir and Terzic, 2005; Roberts et al., 2014). The specific mechanism of inducing hair growth has not been elucidated, but may be related to its vasodilatory and pro-angiogenesis effects (Li et al., 2001). As the first-line pharmacologic recommendation for both male and female patients with AGA, minoxidil has been proved effective for slowing hair loss by plenty of randomized, double-blind, and case-control studies (Ej et al., 2016; Olsen et al., 2007, 2002). However, it does not work for all patients, with only 38.3% reporting improved hair regrowth (Olsen et al., 2007). Moreover, there is a risk of accelerated hair loss if the medication is discontinued after a prolonged use (Rossi et al., 2012; Zhang et al., 2014). The adverse events that occur with topical minoxidil include contact dermatitis, pruritus, dryness and facial hypertrichosis (Levy and Emer, 2013). Finasteride, a selective inhibitor of type II 5α-reductase, is effective in preventing androgen dependent miniaturization of HFs by blocking the conversion of testosterone to dihydrotestosterone (York et al., 2020). A daily dose of 1 mg is recommended for the treatment of AGA in men, and it should be continued for at least 12 months to determine its efficacy (Blumeyer et al., 2011). While finasteride halted hair loss in over 95% of men, only 66% achieved moderate hair regrowth and 5% showed marked hair regrowth (Kaufman et al., 1998; York et al., 2020). Additionally, the treatment needs to be continued indefinitely to maintain efficaciousness (Blumeyer et al., 2011). The most concerning side effects are sexual dysfunction, mood disorders and increased risk of prostate cancer in men (Traish et al., 2014). Besides, long-term use of finasteride may be associated with the development of insulin resistance, type 2 diabetes, non-alcoholic fatty liver diseases, dry eye disease and potential kidney dysfunction (Traish, 2020). As another hormone modulator, spironolactone competitively blocks androgen receptors and inhibits ovarian androgen production (Sinclair et al., 2005). For female patients, it is an off-label option to address alopecia, which is prescribed at 50–200 mg once daily (Harfmann and Bechtel, 2015). Also, spironolactone needs to be administrated continuously for 6 months to assess its full effects. It has been reported that only 44% women achieved hair regrowth after receiving the treatment of oral spironolactone (Sinclair et al., 2005). There may be some transient side effects of lethargy, nausea and menorrhagia, as well as lasting side effects of hyperkalemia, hypotension and teratogenicity (Levy and Emer, 2013). Therefore, alternative therapies with improved therapeutic efficacy and decreased adverse effects are needed.

With the advancement of regenerative medicine, stem cell-based therapies have opened new routes to cope up with the challenges posed by the conventional hair loss treatments. Stem cells are involved in the development and regeneration of tissues and organs, with the self-renewal ability and multi-lineage differentiation potential (Bacakova et al., 2018; Moore and Lemischka, 2006; Weissman, 2000). The

Table 1
Studies on the use of stem cell-derived conditioned medium in alopecia.

Cell type	Pre-treatments	Medium/vessel	CM processing	Mechanisms	Ref
Hu-ADSCs	–	Serum-free α-MEM	Centr.→ 0.22 μm	Promote the proliferation of DPCs through modulation of cell cycles and activate anagen phase of HFs	Won et al. (2010)
Hu-Dermal stem/progenitor cells	–	Serum-free DMEM	Centr. 1100 rpm, 5 min→ 0.22 μm→ Conc. 50x-3k Da	Activate ALP activity by secreting *Wnt*3a, maintain the characteristics of DPCs *in vitro* and promote early telogen-anagen conversion of HFs	Shim (2015)
Deer-antlerogenic-MSCs (DaMSCs)	–	NA	NA	Promote proliferation of DPCs by activating *Wnt* signaling pathways *in vitro* and the EVs secreted from DaMSCs act as mediators of paracrine actions	Seo et al. (2018)
KSCs	–	Serum free-Keratinocyte growth medium 2	Centr.1800 rpm, 10 min→ 0.22 μm→ Conc. 3k Da	A growth-factor cocktail induced proliferation of DPCs and ORSs by increasing the phosphorylation of *Akt* and *Erk1/2* and induced an earlier conversion of the hair cycle	Won et al. (2015)
Hu-ADSCs	hypoxia	serum-free DMEM/F12	Microfilter→ freeze-dry	Enhance the proliferation of DPCs by activating *Erk* and *Akt* signaling pathways, modulate the cell cycle of DPCs by upregulating Cyclin D_1 and CDK_2, and induce the HFs into anagen	Shin et al. (2015)
Hu-ADSCs	hypoxia	Serum-free DMEM/F12	Centr.300 g, 5 min→ 0.22 μm→ Conc. 3k Da	Hypoxic pretreatment increased the secretion of potent growth factors, thereby enhancing the ability of ADSC-CM to stimulate hair growth	Park et al. (2010)
ADSCs	UVB	Serum-free α-MEM	Centr.1800 rpm, 10 min→ 0.22 μm→ Conc. 3k Da	UVB preconditioning enhanced the generation of reactive oxygen species and the upregulation of Nox4 by ADSCs.	Jeong et al. (2013)
Mouse-preadipocytes	VD3	DMEM-0.1%FBS	NA	VD3 enhanced the production of VEGF *via* increasing *Erk1/2* phosphorylation, which promoted angiogenesis in the hair regeneration.	Jung et al. (2015)
Hu-ADSCs	LL-37	NA	NA	LL-37 treatment increased EGR1 expression and MAPK activation, thereby increasing the secretion of regenerative factors by ADSCs.	Yang et al. (2016)
Rat-BM-MSCs	*Wnt*1a-expressing virus	Serum-free DMEM	Centr.3k rpm, 10 min→ dialysis and Conc. 20x-7k Da in PEG20000	Accelerate the transition of HF into anagen, increase the number of HFs, promote the expression of proteins related to hair regeneration *in vivo*, restore the hair inductive capacity of DPCs impaired by DHT to *in vitro*	Dong et al. (2014)
Hu-UC-MSCs	*Wnt7*a-expressing virus	Serum-free DMEM	Centr.3000 rpm, 10 min, 4 °C→ dialysis and Conc. 20x-4k Da	Regenerate a thicker epidermis and more HFs	Dong et al. (2017a)
Hu-ADSCs	3 trichogenic factors-expressing lentivirus	Serum-free medium	0.2 μm→ Centr.	Prolong anagen phase of HFs	Choi et al. (2018b)
Hu-AF-MSCs	Nanog-expressing viruses	Serum-free High-glucose DMEM	Centr.500 g, 5min→ 0.2 μm	Accelerate the telogen-to-anagen transition of HFs and increase the density of HFs	Park et al. (2019)

CM: conditioned medium, Hu: human, MSC: mesenchymal stem cell, ADSC: adipose-derived stem cell, DPCs: dermal papilla cells, AF: amniotic fluid, DMEM: Dulbecco's modified eagle medium, α-MEM: α-modified Eagle medium, FBS: fetal bovine serum, bFGF: basic fibroblast growth factor, ALP: alkaline phosphatase, *Wnt*: Wingless-type mouse mammary tumour virus integration site, KSC: keratinocyte stem/progenitor cell, NA: not available, HF: hair follicle, ORS: outer root sheath, Centr.: centrifugation, UVB: ultraviolet B, VD3: vitamin D3, VEGF: vascular endothelial growth factor, CDK: cyclin-dependent kinase, Nox: NADPH oxidase, Erk: extracellular regulated protein kinase, EGR1: early growth response 1, MAPK: mitogen-activated protein kinase, Conc.: concentrated, 0.22 μm: filter through a 0.22-μm syringe filter, BM: bone marrow, UC: umbilical Cord, EVs: extracellular vesicles, DHT: dihydrotestosterone.

transplantation of multipotent stem cells from adipose (Zanzottera et al., 2014), bone marrow (Iman Hamed et al., 2016), follicle (Gentile et al., 2017a), and umbilical cord blood (Yoo et al., 2010) could regenerate HFs in the skin; relevant articles in this regard have been reviewed by Owczarczyk-Saczonek et al. (2018). Advances in stem cell transplantation might possibly make HF regeneration a reality, with weak immunogenic potential and high multipotential differentiation (Falto-Aizpurua et al., 2014; Richardson et al., 2016). Nonetheless, the transplantation of pluripotent stem cells harbors a risk of tumorigenicity which is associated with self-renewal (Ben-David and Benvenisty, 2011; Vizoso et al., 2017). Also it attracts regulatory surveillance since stem cell transplantation for hair loss treatments is in its infancy and needs to be established (Ramdasi and Tiwari, 2016). In addition, the cost is high because of the short shelf life and requirement of specialized production, transportation and storage conditions (Gunawardena et al., 2019). Another emerging treatment is the injection of autogenous platelet-rich plasma (PRP) into the areas of alopecia. PRP is a concentrate of human platelets that contains a number of growth factors secreted by platelets (Giordano et al., 2017). It is widely studied in the treatment of AGA and alopecia areata, yet the outcomes are conflicting (Gentile and Garcovich, 2019; Jha et al., 2018; Singh, 2015). Due to lack of comparability between studies, it is difficult to interpret the efficacy of PRP (Badran and Sand, 2018; York et al., 2020). Apart from the stem cell transplantation and PRP therapy mentioned above, CM and exosome derived from stem cells have fueled the field of hair research in recent decades due to the demand for more safe, efficient and cost-effective therapies.

3. CM derived from stem cells for hair regeneration

The interactions of diffusible factors and morphogens with their cognate receptors constitute a complex network for the intercellular crosstalk in HFs, influencing the phases of HFs (Bernard, 2017). Stem cells are able to produce and release these diffusible factors, such as growth factors and cytokines, which can activate neighboring cells by paracrine effects (Lee et al., 2011; Liang et al., 2014; Zhang et al., 2014). It has been reported that up to 80% of the regenerative potential in transplanted stem cells is regulated through paracrine activities of paracrine factors (Chimenti et al., 2010; Maguire, 2013). Therefore, the secreted substances from stem cells have drawn wide attention for their potential use in hair regeneration. Moreover, some studies suggested that the cell-sourced secretome and vesicular elements in CM may work in concert to promote HF regrowth (Pawitan, 2014; Vizoso et al., 2017).

3.1. Growth factors and cytokines for hair regeneration

The factors that are secreted by stem cells and are present in CM, consist of vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), bone morphogenetic proteins (BMPs), interleukin-6 (IL-6), microphage colony-stimulating factor (M-CSF) and other cytokines, which are well-documented to be correlated with hair regrowth through diverse mechanisms (Kinnaird et al., 2004; Kruglikov and Scherer, 2016; Pawitan, 2014). For example, VEGF accelerates hair regeneration and augments the size of HFs and hair shafts by inducing perifollicular vascularization (Yano et al., 2001). IGF-1/IGF binding protein-1 complex and BMPs both act on dermal papilla cells (DPCs) to restore and maintain the hair-induction ability (Bak et al., 2018; Rendl et al., 2008). The paracrine hormone HGF can promote follicular growth potentially by increasing the expression of β-catenin (Qi et al., 2016). In addition, IL-6 and M-CSF are both involved in wound-induced hair regrowth (Talavera-Adame et al., 2017). Considering that the natural response related to hair regeneration is a complex process involving the activation and differentiation of HFSCs, a group of paracrine factors rather than one single paracrine factor in CM cocktail are responsible for triggering responses (Gunawardena et al., 2019). Further studies are required to identify and up-regulate the key growth factors and cytokines that are favorable for hair regeneration and growth (Ramdasi and Tiwari, 2016).

3.2. CM applications and potential mechanisms

Stem cell-derived CM is widely studied as hair regenerative medicine (Table 1). For instance, adipose-derived stem cell-CM (ADSC-CM) stimulates hair growth through the combined effects of diverse bioactive factors. These factors act in concert to promote the proliferation of DPCs *via* activation of both *Erk* and *Akt* signaling pathways, modulate the cell cycle of DPCs through upregulating the expression of Cyclin D_1 and CDK_2, and protect DPCs from damage caused by androgens and reactive oxygen species (Won et al., 2017, 2010).

On account of the cell-free state, a main advantage of CM would be its immunocompatibility with its recipients; thus, the donor-recipient match, which is a prerequisite in cell-based therapies, will not be required (Gunawardena et al., 2019). In addition, some safety concerns, such as tumorigenicity potentially associated with the stem cell transplantation, would not be considered (Bermudez et al., 2016, 2015; Eiro et al., 2014). Compared with stem cells, CM needs lower time and cost for production, with higher possibility of producing off the shelf and longer shelf life (Gunawardena et al., 2019). Furthermore, CM can achieve mass manufacture, freeze drying, and easy packaging, transportation, and storage, which is more economical and practical for clinical applications (Osugi et al., 2012).

There is no doubt that the utilization of CM as hair regenerative medicine is in its infancy. Inevitably, there are several challenges that need to be overcome. The type and level of paracrine factors may be variable, depending on the sources, age and the culture conditions of the stem cells (Maguire, 2013; Park et al., 2010). So, selecting an optimal source of stem cells is of paramount importance, and evaluating the levels of paracrine factors at different passages could help to understand the optimal growth stage of stem cells to obtain a specific set of factors (Gunawardena et al., 2019). Additionally, exploration of culture conditions, such as hypoxia/normoxia and monolayer/3D cultures is an indispensable aspect to raise the content of bioactive factors in CM (Bhang et al., 2014; Park et al., 2010). It is also essential to standardize the aforementioned parameters to obtain the CM with a consistent composition. With respect to the safety concern, contradictory results have been described. While it has been reported that bone marrow-derived mesenchymal stem cells (MSCs)-CM has an antitumor effects on non-small cell lung cancer cells, studies conducted have also reported that the treatment of bone marrow-derived MSC-CM achieved an equivalent effect of potentiating tumor growth that is similar to the effect of utilizing MSCs *in vitro* (Zhu et al., 2011). Moreover, cells cultured in xeno-free media are recommended to avoid the transmission of pathogens and other harmful agents carried by the addition of serum containing media, which further ensures the safety of CM in clinical usage (Spees et al., 2004). Furthermore, the short half-lives and consumption of the paracrine factors *in vivo* upon administration may require large and frequent dosages (Khosravi et al., 2007; Teixeira et al., 2016).

3.3. Strategies to up-regulate the therapeutic effects of CM for hair regeneration

Although previous studies have demonstrated that CM has the potential to promote hair regeneration, unmet needs exist for enhancing its therapeutic efficacy due to the low concentration of paracrine factors (Choi et al., 2018b; Gunawardena et al., 2019). Besides, there are both anti-inflammatory and pro-inflammatory cytokines, pro-angiogenic and anti-angiogenic factors in CM, indicating that various factors present in CM may represent a balanced cocktail. The balance may determine the final effect. Accordingly, different types of strategies, such as environmental stimulus, biomacromolecules preconditioning, and gene

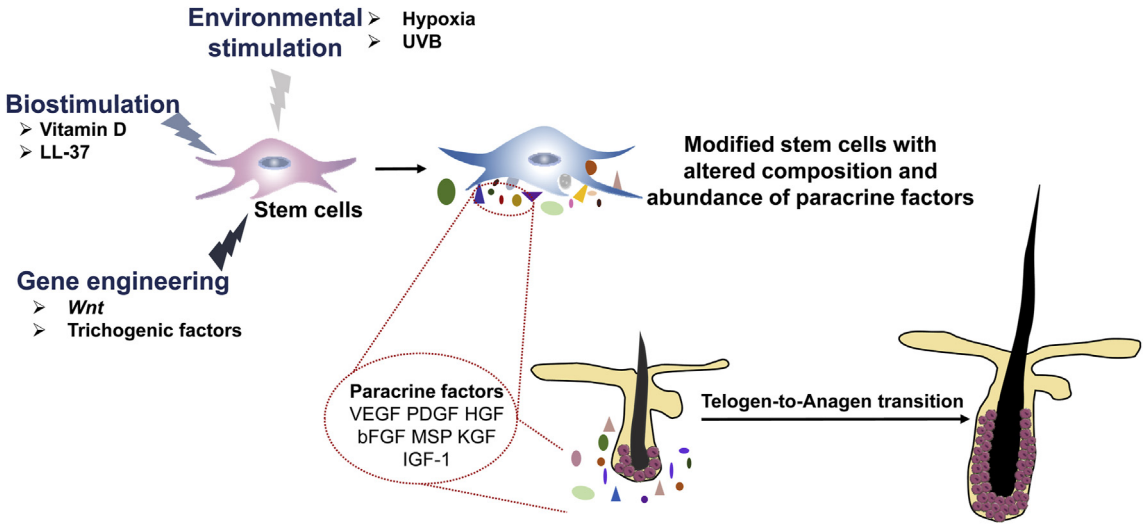

Fig. 2. Strategies to up-regulate the therapeutic effects of CM on hair regeneration.

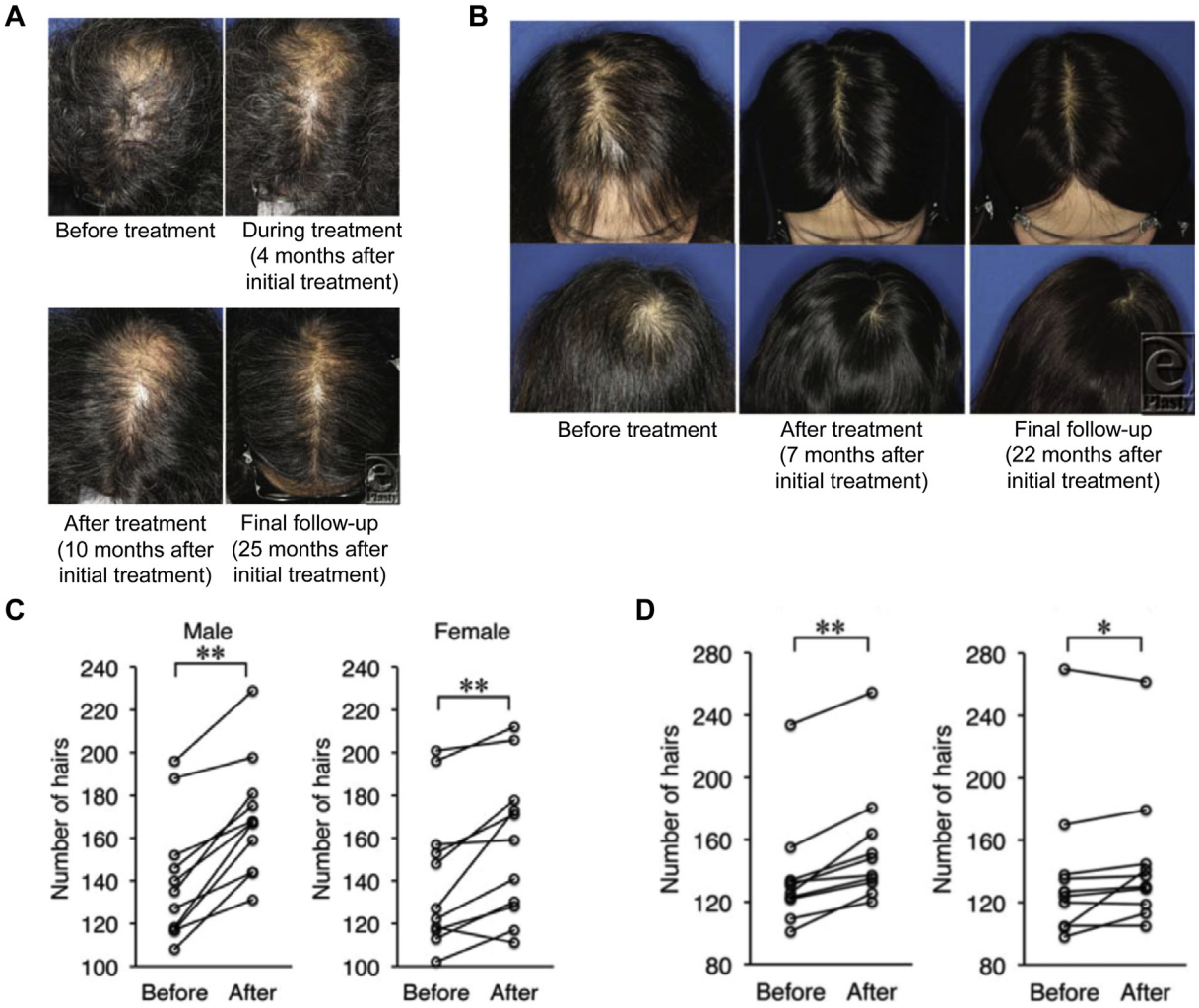

Fig. 3. The effects of AAPE® (ADSC-CM cultured under hypoxic conditions) on alopecia. (A) Photographs of a male patient before treatment, during treatment (4 months after the initial treatment), after treatment (10 months after the initial treatment), and the final follow-up (25 months after the initial treatment). (B) Photographs of a female patient before treatment, after treatment (7 months after the initial treatment), and the final follow-up (22 months after the initial treatment). (C) Changes in the number of hairs. $^{**}P < 0.01$. (D) Changes in the number of hairs in the half-side comparison study, left: AAPE®, right: placebo, half-side comparison study: patients received AAPE® on the left side of the scalp and placebo treatment (saline injection) on the right of the scalp. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Fukuoka and Suga, 2015).

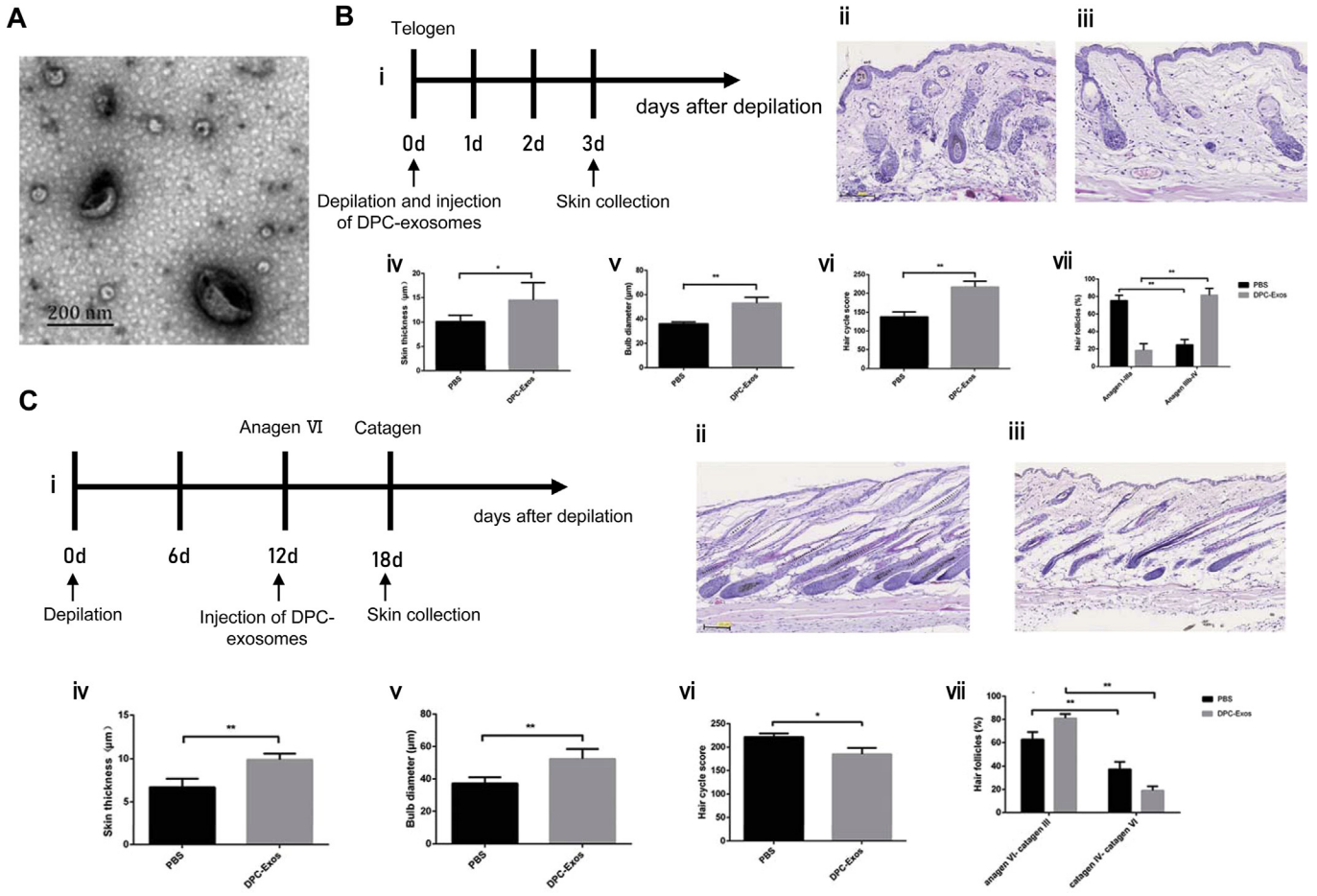

Fig. 4. Treatment with exosomes promotes hair regeneration in mice. (A) Transmission electron microscopy of cup-shaped exosomes (Jiang and Gao, 2017). (Reprinted from Elsevier, with permission). (B) Treatment with dermal papilla cell (DPC)-exosomes accelerates the entry of telogen into anagen. (i) The time course for the transition from telogen to anagen of hair follicles (HFs) in mice (ii) Hematoxylin & eosin (H&E) staining of the DPC-exosomes treated skin: HFs were in anagen, with more hair bulbs present in the subcutis and enlarged HF bulge surrounded with increased melanin (iii) H&E staining of PBS treated skin: HFs were in earlier anagen, with most bulbs located at the dermis-subcutis border (iv) skin thickness (v) bulb diameter (vi) hair cycle scores (vii) percentage of hair follicles in different phases. Scale bar: 100 μm. Data are expressed as mean ± S.D. (n = 6 per group). *P < 0.05, **P < 0.01 (Zhou et al., 2018). (C) Treatment with DPC-exosomes delays entry into catagen. (i) The time course for the transition from anagen to catagen of HFs in mice (ii) H&E staining of the DPC-exosomes treated skin: HFs were in anagen VI, with enlarged hair bulbs (iii) H&E staining of PBS treated skin: HFs were in catagen, with narrower hair bulbs (iv) skin thickness (v) bulb diameter (vi) hair cycle scores (vii) percentage of hair follicles in different phases. Scale bar: 100 μm. Data are expressed as mean ± S.D. (n = 6 per group). *P < 0.05, **P < 0.01 (Zhou et al., 2018). (Reprinted from Elsevier, with permission).

Table 2
Studies that involved the use of stem cell-derived EVs for hair regeneration.

Donor	EVs	Diameter (nm)	References
Ginseng root	Exosome-like vesicles	20–500	(Choi et al. (2018))
Hu-newborn foreskin SCs	Exosomes	20–60	Sahin et al. (2018)
MSCs	Exosomes	NA	Levi et al. (2013)
Hu-DPCs	Exosomes	50–150	Zhou et al. (2018)
Hu-BM-MSCs	Exosomes	~95	Yang et al. (2019)
Deer antlerogenic MSCs	EVs	~120	Seo et al. (2018)
Mouse-BM-MSCs	EVs	30–250	Rajendran et al. (2017)

Hu: human, MSCs: mesenchymal stem cells, SCs: stem cells, EVs: extracellular vesicles, BM: bone marrow, DPCs: dermal papilla cells.

engineering have been employed to change the composition and abundance of paracrine factors, thereby influencing the regulatory effects of CM on HFs (Fig. 2).

3.3.1. Environmental stimulation

Since stem cells are usually thought to reside in the hypoxic areas of the body, hypoxia serves an essential role in maintaining the stem cell niche (Haque et al., 2013; Hawkins and Sharp, 2013; Vizoso et al., 2017). It has been demonstrated that culturing stem cells, especially ADSCs, under hypoxic conditions would up-regulate the secretion of most growth factors and maintain the undifferentiated phenotype for self-renewal (Pawitan, 2014). There is a commercially available product, AAPE® (Prostemics, Seoul, Korea), containing proteins from ADSC-CM cultured under hypoxic conditions. Intradermal injection of AAPE® could significantly increase the number and thickness of hairs in both male and female alopecia patients (Fig. 3A–C) (Fukuoka and Suga, 2015). Notably, the treatment on one side could affect the other side in the half-side comparison study, which perhaps could be attributed to the local circulation (Fig. 3D). However, the pain during and after injection is a common complication, resulting in reduced patient compliance. Shin et al. (2015) tactfully resolved this issue with the assistance of micro-needle roller; they pre-treated the bald scalp with micro-needle roller prior to the topical application of ADSC-CM to enhance the transdermal delivery efficacy.

Moreover, ultraviolet B (UVB) radiation is another pre-treatment

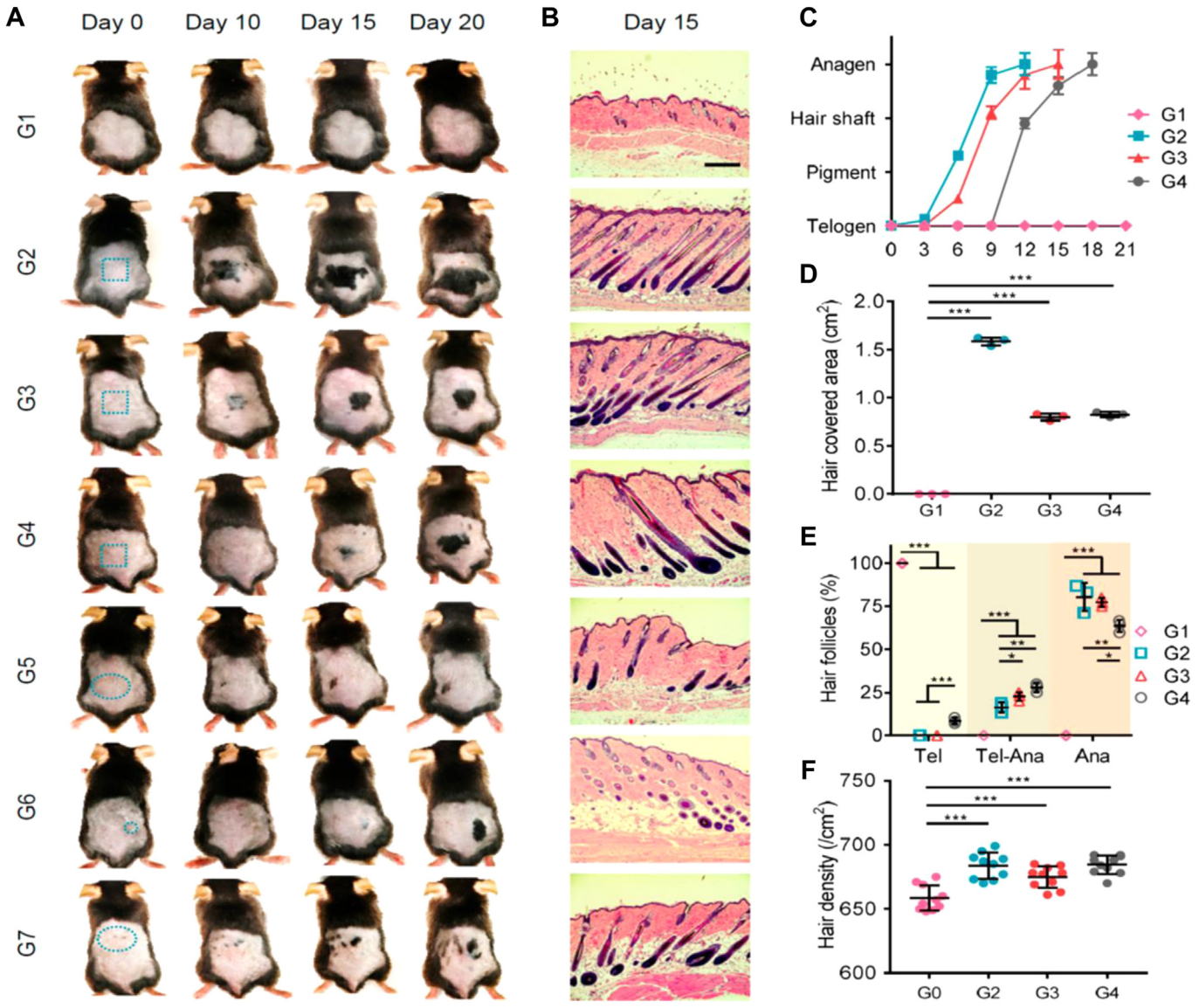

Fig. 5. Treatments with mesenchymal stem cell-exosomes promote hair regeneration in the treated area. (A) Photographs of mice treated with microneedles which were loaded with exosomes and a small molecular drug UK5099 (G2), microneedles which were loaded with UK5099 (G3), microneedles which were loaded with exosomes (G4), topical application of UK5099 (G5), subcutaneous injection of exosomes (G6), and topical application of minoxidil (G7). The untreated mice were set as the control (G1): As compared with G1, both the administration of exosomes-loaded microneedles (G4) and subcutaneous injection of exosomes (G6) exhibited therapeutic effects. The treated skin area was outlined by the blue dotted line. (B) Hematoxylin & eosin staining of the skin at day 15 post administration of G1-G7. Scale bar: 300 μm. (C) Transition of the hair phase with the treatment of G1-G4. Data are expressed as mean ± S.D. (n = 3). (D) The covered area by regrowed hair in the skin with the treatment of G1-G4. Data are expressed as mean ± S.D. (n = 3). (E) Quantification analysis of the hair follicle cycle in mice with the treatment of G1-G4. (F) Hair density of mice with the treatment of G2-G4 compared to wild-type mice (G0). Data are expressed as mean ± S.D. (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Yang et al., 2019). (Reprinted with permission from (ACS Nano, 2019, 13, 4, 4354–4360). Copyright (2019) American Chemical Society). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

method for stem cells (Jeong et al., 2013). Similar to hypoxia, UVB mediates the generation of reactive oxygen species. According to previous reports, the production of reactive oxygen species correlates with the proliferation, migration, and paracrine activities of ADSCs (Kim et al., 2011). Thus, it is well-reasoned that the treatment of low-dose UVB (10 or 20 mJ/cm^2) could up-regulate the expression of ADSC-derived growth factors, and the corresponding CM was able to promote the proliferation of DPCs and outer root sheath cells. Furthermore, subcutaneous injection of such CM was capable of inducing the transition of HFs from telogen to anagen *in vivo* (Jeong et al., 2013).

3.3.2. *Biostimulation*

A growing body of evidence suggests that appropriate stimulation on stem cells by biomolecules could make the resulting CM more conducive to hair regeneration. Vitamin D is synthesized in epidermal keratinocytes when exposed to UVB (Bouillon et al., 2008). As a hair-growth associator, vitamin D analog is able to up-regulate the expression of transforming growth factor-β2 and the activity of alkaline phosphatase in human DPCs, both of which are the indices for hair-inductive capacity, and promote the differentiation of stem cell populations into DPCs (Doi et al., 2012; Inoue et al., 2009; Jung et al., 2015). It has been reported that vitamin D3 pre-activation could augment the content of VEGF in preadipocyte CM, which improved the hair regeneration effects by regulating angiogenesis (Jung et al., 2015). Alternatively, pre-treatment with vitamin C could also up-regulate the expression of HGF, IGF binding protein-6, VEGF, basic fibroblast growth factor and keratinocyte growth factor in ADSCs, thereby inducing the hair-regenerative potential of ADSC-CM *in vitro* (Kim et al.,

Table 3
Currently reported clinical trials with CM derived from stem cells for alopecia.

Type of MSCs	Treatment	Conditions	CT phases	Subjects	NCT number or Ref
Umbilical cord blood-derived MSCs	Apply 5% CM directly on hair and scalp twice a day for 24 weeks	AGA	NA	Female and male, 18–60 years old, with AGA	NCT03676400
Hypoxia-induced multipotent cells	Intradermally inject 2 ml HSC separated by 6 weeks (total of 4 ml)	FPHL	I	Female, 40–70 years old, with FPHL	NCT03662854
Hypoxia and bioreactor-induced dermal cells	Intradermally inject 0.8 ml HSC for 12 weeks	AGA	I/II	56 participants, male, 21–65 years old, with AGA	NCT01501617
Hypoxia-induced Hu-ADSCs	Weekly apply for 12 consecutive weeks with a micro-needle roller	FPHL	NA	27 female patients	Shin et al. (2015)
Hypoxia-induced Hu-ADSCs	Six sessions of intradermal injection with ~3–4 ml (0.02 ml/cm^2)	alopecia	NA	22 patients (11 men and 11 women) + 10 patients (8 men and 2 women)	Fukuoka and Suga (2015)
Hypoxia-induced Hu-ADSCs	Four weekly sessions of intradermal injection with ~3–4 ml (~0.02–0.05 ml/cm^2)	AGA	NA	25 patients (13 men and 12 women)	Fukuoka et al. (2012)
Hypoxia-induced Hu-ADSCs	Six sessions of intradermal injection (1 vial of AAPE® in each session)	AGA or FPHL	NA	40 patients (21 men and 19 women)	Narita et al. (2019)

MSC: mesenchymal stem cell, ADSC: adipose-derived stem cell, HSC: hair stimulating complex, FPHL: female pattern hair loss, AGA: androgenetic alopecia, Hu: human, CM: conditioned medium, NA: not available, AAPE: a commercialized ADSC-CM product cultured under hypoxic conditions.

2014). Similarly, the treatment with CM of ADSCs pre-activated with LL-37 (a naturally occurring 37-animo acid sequence) manifested a strong effect on hair growth promotion *in vivo* through enhanced paracrine effects of various growth factors (Yang et al., 2016).

3.3.3. Gene engineering

Based on our previous work, genetic modification of stem cells holds promise for enhancing the expression of distinct growth factors (Jiang et al., 2019; Li et al., 2015b). The CM derived from gene-engineered stem cells with altered composites has generated a lot of interest for enhanced therapeutic effects in hair loss. Wingless-type mouse mammary tumor virus integration site (*Wnt*) signaling pathway plays a vital role in regulating hair morphogenesis and regeneration (Dey-Rao and Sinha, 2017; Li et al., 2015a; Reddy et al., 2001). *Wnt7a* correlates with the increased number of HFs in the wound site (Ito et al., 2007) and *Wnt10b* regulates the telogen-to-anagen transition of HFs (Li et al., 2013). It has been reported that intradermal injection of CM obtained from retroviral-mediated *Wnt1a*-overexpressing bone marrow MSCs could induce hair regrowth by actively maintaining and facilitating the capacity of DPCs to induce hair cycling (Dong et al., 2014, Dong et al., 2017b). Similarly, *Wnt7a*-MSC-CM is also capable of inducing regeneration of more HFs *via* cellular communication as compared to normal MSC-CM (Dong et al., 2017a).

Apart from modifying the expression of *Wnt* proteins, there are also studies about introducing genes of trichogenic factors to stem cells. Choi et al. (2018b)introduced genes of three trichogenic factors, PDGF-A, SOX2, and β-catenin, to ADSCs and found that the derived CM could increase the number of vibrissal HFs in anagen.

4. Exosomes and their applications

Since the HF is a compartmentalized organ, it is expected that the cell-cell communications in HFs could be mediated by a class of message entities like EVs, especially exosomes (Bernard, 2017; Braicu et al., 2015). Composed of exosomes and microvesicles, EVs (30–1000 nm in diameter) can be secreted by most cell types into the CM (Gangadaran et al., 2017; Kalimuthu et al., 2016; Rajendran et al., 2017). Exosomes are a specific class of phospholipid bilayer EVs, with a diameter of 40–120 nm and a sucrose density of 1.13–1.19 g/ml (Fig. 4A) (Jiang and Gao, 2017; Liu et al., 2013; Raposo and Stoorvogel, 2013). Exosomes contain functional DNAs, RNAs, proteins and lipids that have regulatory effects on the recipient cells (Hong et al., 2009; Valadi et al., 2007). It has been identified that exosomes are able to carry hydrophobic *Wnt* proteins on their surface to induce the activation of β-catenin over a distance, which is a key signaling pathway involved in the regulation of hair morphogenesis and regeneration (Dey-Rao and Sinha, 2017; Gross et al., 2012; Li et al., 2015a; Reddy et al., 2001).

Table 2 lists the published studies about employing EVs, including exosomes, for hair regeneration. Exosomes that are derived from MSCs are frequently used in current hair regrowth experiments (Fig. 4 and 5). A patented study demonstrated that a pharmaceutical composition comprising MSC-derived exosomes may be capable of promoting hair growth (Levi et al., 2013). Increasing pieces of evidence indicate that the regulatory function of DPCs with mesenchymal-like phenotype on HF regeneration mainly depends on the paracrine mechanism (Al-Nuaimi et al., 2014; Mohammadi et al., 2016; Won et al., 2012). Exosomes are small vesicles that are important components of paracrine signaling (Zhou et al., 2018). Accordingly, Zhou et al. (2018) employed exosomes derived from DPCs to regulate the growth and development of HFs (Fig. 4B and C).

Noticeably, exosomes encapsulate the therapeutically relevant molecules (proteins and nucleic acids) in vesicles, protecting them from degradation, which is different from the cytokines, growth factors and nucleic acids in CM that are rapidly degraded (Basu and Ludlow, 2016; Vizoso et al., 2017). In this sense, the durability of exosomes makes it possible to obtain abundant exosomes through simply extending the

Table 4
Comparison of stem cell, CM and exosomes for hair regeneration.

Therapeutic strategies	Advantages	Limitations
Stem cell transplantation	Good efficacy Weak immunogenic potential (Falto-Aizpurua et al., 2014; Richardson et al., 2016) High multipotential differentiation (Falto-Aizpurua et al., 2014; Richardson et al., 2016)	Safety considerations: tumorigenicity and transmission of infection (Ben-David and Benvenisty, 2011; Vizoso et al., 2017) Tight regulations (Ramdasi and Tiwari, 2016) Short shelf life (Gunawardena et al., 2019) High cost: strict production, transport and storage conditions (Gunawardena et al., 2019)
CM	Immunocompatibility (Gunawardena et al., 2019) Improved safety compared with stem cell transplantation (Bermudez et al., 2016, 2015; Eiro et al., 2014) Feasibility of mass production (Osugi et al., 2012) Low cost (Gunawardena et al., 2019) More practical for clinical application (Pawitan, 2014)	Low concentration of paracrine factors with limited therapeutic efficacy (Maguire, 2013) Difficulty in obtaining the CM with a consistent composition (Gunawardena et al., 2019) Short half-lives of paracrine factors (Khosravi et al., 2007) Require frequent administrations with large doses (Bhang et al., 2014)
Exosomes	Protect ABIs from degradation (Basu and Ludlow, 2016) More stable for large-scale production (Basu and Ludlow, 2016) Retain the ability of homing, and being internalized by the targeted cells (Altaner, 2015; Altanerova et al., 2016; Vizoso et al., 2017)	Lack of effective isolation method (Jiang and Gao, 2017) Lack of guidelines for large-scale manufacturing (Jiang and Gao, 2017) Potential biological safety issues (Lai et al., 2014; Stenqvist et al., 2013; Valadi et al., 2007)

ABI: active biological ingredient, CM: conditioned medium.

culture of the producer cell line. This is not the case for soluble elements in CM, which are vulnerable to degradation while in extended culture (Basu and Ludlow, 2016). Moreover, exosomes can trigger tissue-specific responses by guiding informational molecules to the target cells (Santangelo et al., 2017). Consequently, it is promising to modify stem cells to generate exosomes possessing rich cargos of the mRNAs, microRNAs and proteins relevant to hair regrowth, therefore retaining the ability of homing to HFs, and being internalized by the target cells (Altaner, 2015; Altanerova et al., 2016; Vizoso et al., 2017). Exosomes are present in CM, but in general, the content is not high. Hence, there is an urgent need to develop more effective methods to isolate exosomes from CM, which may balance both efficiency and purity. Apart from that, the administration of exosomes harbors the potential risks of the uncontrolled transfer of genetic information, immune responses as well as biodistribution (Lai et al., 2014; Stenqvist et al., 2013; Valadi et al., 2007). Thus, a comprehensive understanding of the correlations between the dosage, biodistribution and elimination dynamics for exosomes is critical to reduce the potential risks (Basu and Ludlow, 2016).

5. Clinical trials of CM-based therapy in alopecia

A few of human clinical studies on the application of stem cells-derived CM for the treatment of alopecia have been carried out (Table 3). However, there are no clinical studies regarding the use of exosomes or EVs for the treatment of hair loss. Besides intradermal injection, one clinical trial (NCT03676400) employed CM of human umbilical cord blood-derived MSCs to alleviate hair loss, which was topically applied on the hair and scalp by the subjects themselves, increasing convenience and patient compliance. The efficacy evaluation indices were particularly comprehensive, including the total hair density, telogen hair density, anagen hair density, hair growth speed, hair diameter and visual assessment before and after treatment.

AAPE®, as previously mentioned, is the commercialized ADSC-CM freeze-dried powder that has been widely used in clinical studies to treat hair loss (Fukuoka et al., 2017, 2012; Fukuoka and Suga, 2015). Notably, the ADSCs are cultured under hypoxia for 2 weeks to collect the CM. Clinical studies (Table 3) have shown the satisfactory efficacy of AAPE® in hair regeneration.

There are three points worth noticing from these clinical studies. First, hypoxic pre-treatment is a promising method to induce stem cells to secret more growth factors and cytokines that are conducive to hair regeneration. Second, most clinical applications of CM are intradermal injection and require long treatment sessions. Although there are no severe adverse effects observed during treatment, it can affect patient compliance to a large extent. Subsequent studies can combine CM with a more patiently compliant delivery system without compromising the efficacy. In addition, it is necessary to follow up on patients after the completion of the treatment. Overall, more randomized, controlled, double-blind studies with large sample size, objective evaluation methodologies and long follow-up periods, as well as improved delivery systems are needed to make it possible to have more products with clear-cut benefits in the treatment of alopecia.

6. Discussion

Recent advances in regenerative medicine have raised new hopes and paved the way for the development of new therapies against hair loss. The merits and demerits of stem cells, CM and exosomes as regenerative medicine are separately shown in Table 4. While the use of stem cells in hair regeneration has got high expectations, concerns about its biosafety have hindered the clinical applications.

To overcome these challenges, induction of HFSCs in their native niche by CM, and/or exosomes to stimulate the regeneration process, is a promising cell-free approach. As the hair regenerative medicine, the complex composition of CM, and/or exosomes is likely to hamper the regulatory approval even if their certain therapeutic efficacy has been determined. More randomized, controlled double-blind studies are required to confirm the role of CM and/or exosomes in the stimulation of hair regrowth and to define the mechanism of actions. There is a tendency to enhance the secretion of bioactive factors with effective methods for better therapeutic effect. To increase the likelihood of clinical translation, it is inevitable to standardize the cultivation of stem cells, the collection, preservation and validation of CM, as well as the isolation of exosomes. In addition, the combination of CM and/or exosomes with a non-invasive delivery system, without compromising the efficacy will lead to convenience and patient compliance.

Authors' contributions

An-Ran Yuan: Writing - original draft, preparation, Writing - review & editing. Qiong Bian: Writing - original draft, preparation, Writing - review & editing. Jian-Qing Gao: Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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