Application of Tonsil-Derived Mesenchymal Stem Cells in Tissue Regeneration: Concise Review

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ABSTRACT

Since the discovery of stem cells and multipotency characteristics of mesenchymal stem cells (MSCs), there has been tremendous development in regenerative medicine. MSCs derived from bone marrow have been widely used in various research applications, yet there are limitations such as invasiveness of obtaining samples, low yield and proliferation rate, and questions regarding their practicality in clinical applications. Some have suggested that MSCs from other sources, specifically those derived from palatine tonsil tissues, that is, tonsil-derived MSCs (TMSCs), could be considered as a new potential therapeutic tool in regenerative medicine due to their superior proliferation rate and differentiation capabilities with low immunogenicity and ease of obtaining. Several studies have determined that TMSCs have differentiation potential not only into the mesodermal lineage but also into the endodermal as well as ectodermal lineages, expanding their potential usage and placing them as an appealing option to consider for future studies in regenerative medicine. In this review, the differentiation capacities of TMSCs and their therapeutic competencies from past studies are addressed. STEM CELLS 2019;37:1252–1260

SIGNIFICANCE STATEMENT

Mesenchymal stem cells (MSCs) are considered as a great candidate for tissue engineering in regenerative medicine. Tonsil-derived MSCs (TMSCs) could be an attractive option for clinical applications because of their noninvasiveness of tissue collection, relatively high proliferation rate, and low allogenicity. This review addresses potential differentiation capabilities of TMSCs into mesodermal, endodermal, and ectodermal lineages reported from previous in vitro and in vivo studies as well as their potential applications for treating various human diseases.

INTRODUCTION

The pluripotency/multipotency characteristics of stem cells make them an ideal material to be used in regenerative medicine. Embryonic stem cells (ESCs) are probably a better option to consider due to their pluripotency; however, the critical dilemma of ethical and political concerns associated with manipulation and destruction of human embryos nullifies their usefulness [1]. In 2006, Yamanaka and his colleagues at Kyoto University demonstrated a new technique to generate "induced pluripotent stem cells" (iPSCs) from adult fibroblasts by supplementing them with *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4* [2]. This protocol has subsequently been developed by many others in various somatic cells to create iPSCs, contributing tremendous achievement in stem cell engineering. This methodology avoids the issues associated with ESCs for various research applications. Nevertheless, iPSCs face a few critical issues including random insertion of retroviral vectors, difficulty of establishing stable iPSC lines, and potential oncogenicity of the pluripotency inducible factors [3].

Mesenchymal stem cells (MSCs) are considered as another option for regenerating tissues because of their easy accessibility and fast self-renewal capacity with fewer ethical concerns for clinical applications in medicine [4, 5]. Bone marrow (BM) has been the primary site for obtaining MSCs [6], but MSCs can also be isolated from other tissue sites including adipose [7], muscle [8], skin [9], liver [10], umbilical cord [11], and tonsil [4] as well as blood [12] and urine samples [13]. There are established common MSC markers, including CD73, CD90, and CD105. Nevertheless, these markers are not necessarily found in all MSCs as they could vary depending on origin and time of in vitro culture [14], and their proliferation and/or differentiation potentials can also be different [4, 15].
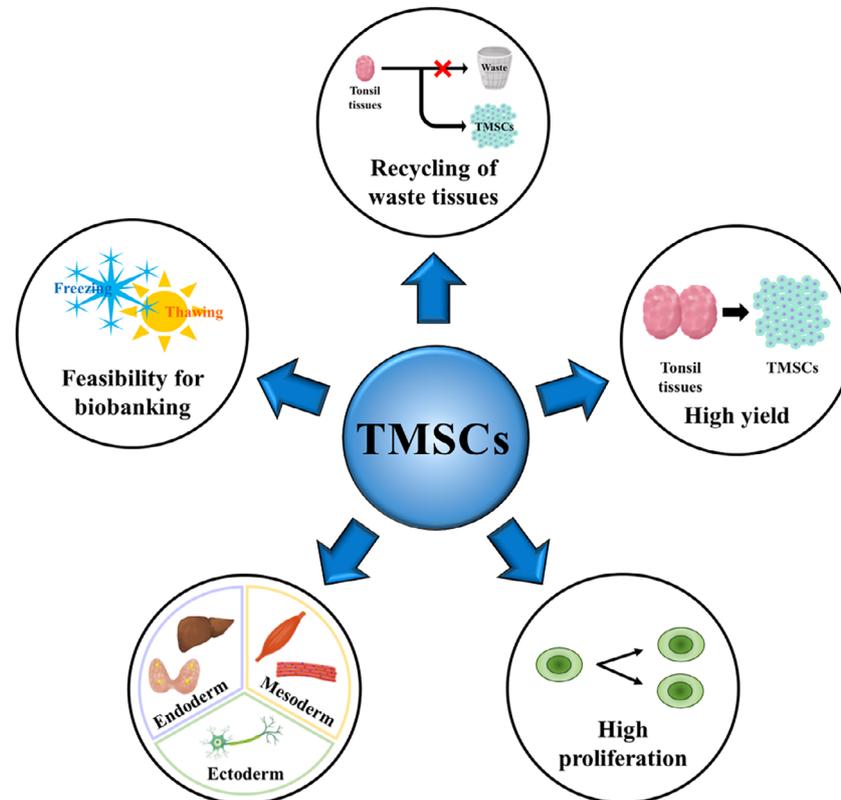
Figure 1. Phenotypical characteristics of TMSCs. TMSCs are isolated from waste tonsil tissues of tonsillectomy, which means there is no need for an unnecessary surgery and therefore describing "noninvasiveness." Compared with mesenchymal stem cells from other origins, TMSCs have relatively higher yield and proliferation (i.e., shorter doubling time) to further expand TMSCs in large number. TMSCs have differentiation potentials into not only mesodermal lineage (bone, cartilage, fat, muscle, and tendon) but also endodermal (PTH/insulin-releasing cells, hepatocytes) and ectodermal lineages (neuron-like or glial cells). The proliferation rate and differentiation potentials are stable after cryopreservation, allowing them for successful biobanking of potential clinical applications. All references are provided in the context. Abbreviation: TMSCs, tonsil-derived mesenchymal stem cells.

As mentioned earlier, BM-MSCs are the gold standard in MSC research applications. Nonetheless, invasiveness in obtaining the tissues as well as low yield and proliferation rate are the major challenges for BM-MSCs to be practically applied to a clinical setting [16]. To overcome these issues, MSCs from other alternative sources have been considered.

TONSIL-DERIVED MSCs (TMSCs)

TMSCs isolated from human palatine tonsil tissues, which are generally obtained from waste tissues from tonsillectomies, have been suggested an attractive MSC sources for clinical applications due to the completely noninvasive nature of tissue collection without needing for an unnecessary surgery, relatively high proliferation rate, and low immunogenicity [4, 5, 17] (Fig. 1). Detailed comparisons using RNA-seq analysis found 20 common surface markers uniformly expressed among MSCs derived from BM, adipose tissues, and palatine tonsil [18]; TMSCs retain the common MSC markers, such as CD90, but they also have their own distinctive markers, such as CD106 and CD166, most of which are closely related to adhesion, migration, and immunomodulation.

Tonsillectomy is a common practice to treat hyperplasia of tonsil tissues or complications related to respiratory functions [19]. Although this is mostly performed in young children,

donor age does not have a significant impact on the proliferation and mesodermal differentiation capacity of TMSCs [20]. More recently, Khatri et al. demonstrated that an optimal number of TMSCs for clinical applications also can be harvested from small samples of biopsied tonsillar tissues [21]. Compared with the BM-MSCs and adipose tissue-derived MSCs (AMSCs), TMSCs have the highest proliferation rate [18], and long-term passage as well as cryopreservation has no significant impact on cell proliferation (Fig. 1), even though the proliferation rate decreases after passage 15 [5, 20].

The potential stemness of TMSCs was first introduced by Janjanin and his colleagues [4]; they observed the expression of surface markers similar to those of BM-MSCs. The osteogenic, adipogenic, and chondrogenic capacities of TMSCs have also been validated in numerous studies [5, 22, 23] (Fig. 1). In addition to their mesodermal differentiation potential (bone, cartilage, and fat), it was verified that TMSCs have the capacity to differentiate into endodermal lineage, expressing markers of parathyroid cells [5, 24]. Subsequently, other studies also identified differentiation capacities of TMSCs into various cell types, including myocytes [23], insulin-releasing cells [25], neuron-like or glial cells [26, 27], tenocytes [28], and hepatocytes [29] (Fig. 2). Moreover, some studies also illustrated that TMSCs can contribute in part by releasing cytokines to regenerate skin tissues [30] as well as to alleviate inflammation [31].
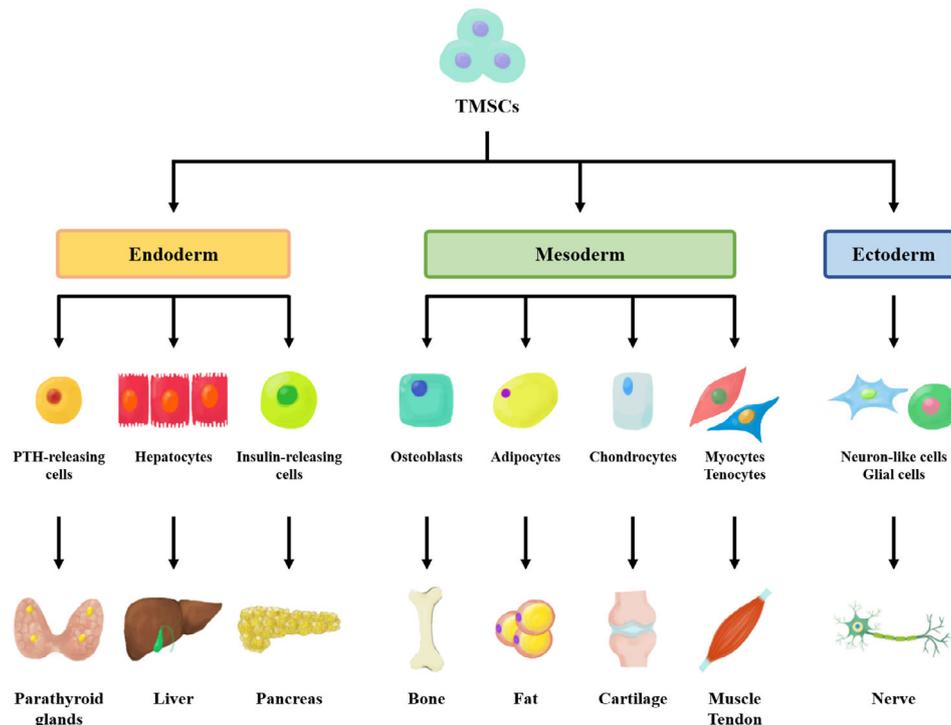
Figure 2. Schematic diagram of lineage differentiation of TMSCs. TMSCs have the potential to differentiate into endodermal (PTH/insulin-releasing cells, hepatocytes), mesodermal lineage (bone, cartilage, fat, muscle, and tendon), and ectodermal lineages (neuron-like or glial cells). All references are provided in the context. Abbreviations: PTH, parathyroid hormone; TMSCs, tonsil-derived mesenchymal stem cells.

Osteogenic Potential of TMSCs

Among various mesodermal differentiation capacities, osteogenesis is by far the most thoroughly studied functional phenotype of TMSCs [4, 5, 17, 26, 32–34]. TMSCs cultured under osteogenic differentiation factors for 21 days increased the gene and protein expression levels of osteocalcin (OC) and alkaline phosphatase (ALP) as well as matrix mineralization as visualized by Alizarin red S staining [4, 5, 26, 34]. However, the expression of both OC and ALP was lower in osteogenically differentiated TMSCs compared with those of osteogenically differentiated BM-MSCs [4]. It is intriguing to note that the osteogenic efficiency of TMSCs increases up to passage 10 and decreases thereafter [34]. The osteogenicity of TMSCs was shown to be further enhanced in the presence of other factors such as fiber growth factor 5 [17] and bone forming peptide 1 (BFP1) [33] by regulating cellular transcriptional pathways such as extracellular-signal-regulated kinase 1/2, and bone morphogenetic protein–mitogen-activated protein kinase, respectively. Recently, far-infrared irradiation was reported to induce osteogenicity of TMSCs [32]. Nonetheless, the mechanisms of action and their relation to osteogenesis are not fully understood at this point.

TMSCs have been tested in animal models of osteoporosis [35, 36], osteoradinecrosis (ORN) [37], or calvarial defect [33] and have demonstrated some promising outcomes as a feasible therapeutic treatment for bone-related disorders (Table 1). For example, tail injections of undifferentiated TMSCs or conditioned medium (CM) from TMSCs decreased the progression of osteoporosis in mice with BM depletion [36]. Similarly, the direct injection of undifferentiated TMSCs in proximal tibias of ovariecotmized (OVX) mice reduced the progression of osteoporosis [35]. It is interesting to note that TMSCs themselves appear to have not

only osteogenic but also wound healing properties even in the absence of induction from other osteogenic differentiation factors; the submucosal injection of undifferentiated TMSCs around the bone defect site decreased the risk of developing ORN caused by irradiation and tooth extraction in rats [37].

In a few studies, various biomaterials have been applied together with osteogenically differentiated TMSCs to create a more effective therapeutic application. Fabrication of TMSCs using a scaffold consisting of polycaprolcotone biomaterial and BFP1 increased cell adhesion and osteogenic differentiation thereby enhancing the bone regeneration of the calvarial defect region of rabbits [33]. Other biomaterials made up with in situ crosslinkable gelatin-hydroxyphenyl propionic acid hydrogel (GHH) [38], methacrylated chondroitin sulfate [39], or inorganic mesocrystals [40] were also tested to fabricate the osteogenically differentiated TMSCs, and they promoted osteogenesis and bone mineralization in both in vitro and in vivo studies. In the case of GHH, injection of TMSCs fabricated with GHH to the dorsa of OVX mice improved signs of osteoporosis [38], suggesting possible applications of TMSCs in managing osteoporosis.

Adipogenic Potential of TMSCs

In the presence of adipogenic factors, TMSCs can differentiate into adipocytes, increasing the expression of adipocyte markers including lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor γ (PPARγ) [4, 5]. Accumulation of lipid droplets stained by Oil red O staining was observed with adipogenically differentiated TMSCs, but the adipogenic efficiency of TMSCs decreased in a passage dependent manner [34]. Additionally, exposure to far-infrared irradiation was shown to

Table 1. Experimental trials of TMSCs in animal models for the application of human diseases

Disease model	Treatments (animal model)	References
Osteoporosis	Direct injection of undifferentiated TMSCs at proximal tibias (OVX mice)	[35]
	Tail injection of osteogenically differentiated TMSCs or the CM from the TMSCs (SAMP6 mice)	[36]
	Intraperitoneal injection of undifferentiated TMSCs fabricated with GHH (OVX mice)	[38]
Osteoradinecrosis	Submucosa injection of undifferentiated TMSCs around defect region (rats with irradiation and tooth extraction)	[37]
Calvarial defect	Implantation of a polycaprolcotone-based scaffold containing osteogenically differentiated TMSCs and BFP1 at defect region (rabbits with surgically induced defect)	[33]
Obesity	Injection of CM from adipogenically differentiated TMSCs (SAMP6 mice)	[41]
Medial meniscus defect	Subcutaneous implantation of chondrogenically differentiated TMSCs fabricated with the riboflavin-induced photocrosslinked collagen-hyaluronic acid at defect region (mice with surgically induced defect)	[45]
Muscle injury	Intramuscular injection of TMSC-derived myocytes at defect region (mice with gastrocnemius myectomy)	[23]
Hypoparathyroidism	Subcutaneous injection of TMSC-derived PTH-secreting cells fabricated with Matrigel at nape (PTX rats)	[24]
	Subcutaneous injection of TMSC-derived PTH-secreting cells fabricated with GHH at nape (PTX rats)	[53]
	Subcutaneous injection of scaffold-free 3D spheroid of TMSC-derived PTH-secreting cells at the nape (PTX rats)	[54]
	Intramuscular injection of TMSC-derived PTH-secreting cells fabricated with plasma gel at dorsum (PTX rats)	[55]
Diabetes	Subcutaneous injection of TMSC-derived insulin-secreting cells fabricated with Matrigel (mice with streptozotocin-induced diabetes)	[25]
Sciatic nerve injury	Implantation of TMSC-derived Schwann-like cells fabricated with poly(ethylene glycol)-b-poly(l-alanine) gel on defect region (mice with transected right sciatic nerve)	[26]
Charcot–Marie–Tooth disease type 1	Intramuscular injection of TMSC-derived Schwann-like cells at the right thigh muscle near sciatic nerve (heterozygous Trembler-J mice)	[62]
Full-thickness skin wound	Implantation of engineered skin analogs (TMSCs replacing the function of dermal fibroblasts layer) onto defect region (immune-incompetent nu/nu rats with surgically induced defect)	[64]
	Direct administration of undifferentiated TMSCs onto wound site (mice with surgically induced defect)	[30]
Acute hepatitis	Intravenous injection of undifferentiated TMSCs (mice with concanavalin-A-induced acute liver damage)	[67]
Liver fibrosis	Tail vein injection of undifferentiated TMSCs (mice with CCl_4-indued liver damage)	[22]
	Tail vein injection of CM from undifferentiated TMSCs (mice with CCl_4-indued liver damage)	[68]
Inflammatory bowel disease	Multiple intraperitoneal injections of undifferentiated TMSCs (mice with DSS-induced colitis)	[31]

Abbreviations: BFP1, bone forming peptide 1; CM, conditioned media; DSS, dextran sulfate sodium; GHH, gelatin-hydroxyphenyl propionic acid hydrogel; OVX, ovariectomized; PTH, parathyroid hormone; PTX, parathyroidectomized; SAMP6, senescence-accelerated mouse prone 6; TMSCs, tonsil-derived mesenchymal stem cells.

dramatically decrease the adipogenic potential of TMSCs by activating Ca^{2+}-dependent protein phosphatase 2B [32].

In contrast to the osteogenic TMSCs, there are few studies looking at possible applications of adipogenically differentiated TMSCs, probably due to the lack of translational and clinical applications. However, in one study of interest, CM from adipogenically differentiated TMSCs was injected into mice and produced a decrease in body fat mass and subsequent weight loss [41]. The same study also determined that injected TMSCs probably stimulated adipose tissues of mice to increase the production of adiponectin, which triggers redistribution of body fat [42], eventually leading to weight loss. Based on these findings, the study pointed out a plausible therapeutic use of CM from TMSCs for treating obesity-related disorders.

Chondrogenic Potential of TMSCs

In early studies, the chondrogenic potential of TMSCs was examined to provide evidence of their mesodermal differentiation capacity [4, 5]. Upon culturing TMSCs in chondrogenic media, the expression of the chondrogenic markers aggrecan (AGN) and collagen type II (COL2) as well as the accumulation of sulfated glycosaminoglycan-rich matrix visualized by Alcian blue staining increased, yet COL2 expression was lower in the chondrogenically differentiated TMSCs compared with those of chondrogenically differentiated BM-MSCs. Later, other groups tested 2-dimensional (2D)/3D hybrid systems to enhance the chondrogenic differentiation efficiency of TMSCs [43, 44]. These studies revealed significant increases in the chondrogenic markers COL2 and SOX9, demonstrating the improved biofunctionality of

chondrogenically differentiated TMSCs by the 2D/3D hybrid cell culture system. Another group reported that supplementation of CM from meniscus tissue followed by transforming growth factor-β3 (TGF-β3) resulted in the induced expression of fibrocartilage markers such as COL2, AGN, SOX9, and COL1 by the TMSCs [45]. Moreover, the chondrogenically differentiated TMSCs, when fabricated with the riboflavin-induced photocrosslinked collagen-hyaluronic acid, demonstrated complete regeneration of the damaged regions of rabbit medial meniscus. Considering the outcomes of these previous studies, TMSCs could be a promising MSC source for cartilage regenerative medicine.

Skeletomyogenic Potential of TMSCs

Previous studies reported that TMSCs also have a skeletomyogenic potential in the presence of TGF-β, nonessential amino acids, and insulin-transferrin-selenium (ITS). Skeletomyogenically differentiated TMSCs showed increased expression of skeletal myoblast and myocyte markers such as α-actinin, troponin I type 1, and myogenin [23, 46]. Transplantation of the skeletomyogenically differentiated TMSCs also significantly recovered damaged gastrocnemius (lower legs) muscles and showed restored locomotion of mice after 8-weeks post-transplantation during gait assessment analyses [23]. The recovery of these muscle functions was probably mediated not only by skeletomyogenically differentiated TMSCs but also by other factors secreted from TMSCs to promote the regeneration of neighboring muscle cells. In support of this statement, a study reported that TMSCs secrete interleukin (IL)-1 receptor antagonist (IL-1Ra) to antagonize the activity of IL-1β [47]. The blockage of IL-1β activity reduces inflammation from injury at the damaged muscle sites, eventually promoting regeneration of skeletomyocytes [48]. These studies indicate that TMSCs have the potential to assist in muscle recovery directly and/or indirectly, and therefore, could be useful in treating skeletal muscle injuries.

Tenogenic Potential of TMSCs

In addition to their skeletomyogenic potential, TMSCs also have a capacity to differentiate into tenocytes. Under exposure to TGF-β3 for 3 days, TMSCs increased gene expression of tenocytic markers including COL1, tenomodulin, and scleraxis [28]. However, the expression of these genes declined dramatically by day 7 of TGF-β3 treatment, returning back to the similar levels as those of the TMSCs without TGF-β3 by 10 days of the treatment. Regardless of the declined tenocytic markers observed after 7 days of TGF-β3 exposure from gene expression analyses, protein expression of these markers analyzed by immunofluorescence, immunohistochemistry, and Western blotting was maintained at significantly higher levels compared with those without TGF-β3 at days 7 and 10 of the treatment [28]. This study provides evidence that, under these differentiation conditions, TMSCs have the ability to differentiate into tenocyte-like cells.

Differentiation Potential of TMSCs into Parathyroid Hormone-Releasing Cells

MSCs are considered to be restricted to a tri-lineage differentiation potential into bone, fat, and cartilage [49]. Nonetheless, there is enough evidence indicating that MSCs possess the capacity to differentiate into endodermal and/or ectodermal lineage in response to inductive extracellular microenvironments [50, 51]. For example, Moriscot et al. demonstrated the differentiation potentials of BM-MSCs into endodermal lineage, expressing markers of pancreatic beta cells [51].

The protocol to differentiate human ESCs into parathyroid hormone (PTH)-releasing cells, expressing definitive markers of parathyroid cells such as PTH and glial cell missing homolog 2 (GCM2), was initially reported by D'Amour et al. [50] and was further modified by Bingham and his colleagues [52]. TMSCs under the protocol established by Bingham et al. require supplementation of activin A and sonic hedgehog for 7 [24] or 21 days [5] and were also shown to differentiate into PTH-releasing cells. These differentiated TMSCs exhibited gene expression of parathyroid cell markers, including GCM2, PTH, calcium sensing receptor (CaSR), and chemokine ligand 21 [5] as well as protein expression of secretory PTH and CaSR [24]. It was also found that the TMSCs that were differentiated into PTH-releasing cells have the capacity to regulate PTH and CaSR, and the secreted PTH shows stronger potency in inducing osteogenic activity of preosteoblastic cells, MC3T3-E1, compared with recombinant PTH [24]. When the TMSC-derived PTH-releasing cells were fabricated with either Matrigel or GHH and were implanted at nape of parathyroidectomized (PTX) rats, vascularization around the implanted sites was observed. The PTX rats receiving these implants also showed higher levels of serum PTH, ionized Ca^{2+}, and survival rates compared with those groups receiving no implants or implants containing nondifferentiated control TMSCs [24, 53]. Additionally, the subcutaneous injection of TMSCs differentiated into PTH-releasing cells in the form of a scaffold-free 3D spheroid showed similar results as it was observed from the studies with scaffolding materials [54]. More recently, it was shown that the intramuscular injection of the PTH-releasing cells derived from TMSCs fabricated with autologous plasma to the rat dorsum resulted in elevation of serum PTH and chromogranin A in PTX rats [55]. These studies propose that TMSCs differentiated into PTH-releasing cells may provide a practical therapeutic treatment for hypoparathyroidism.

Differentiation Potential of TMSCs into Insulin-Releasing Cells

The generation of insulin-releasing cells has been extensively studied with ESCs [56, 57]; however, the differentiation capabilities of MSCs such as BM-MSCs and AMSCs into insulin-releasing cells were also reported in a few studies [58, 59].

Similar to the other MSCs mentioned earlier, TMSCs also have the ability to differentiate into insulin-releasing cells. It was confirmed that TMSCs stimulated with ITS resulted in relatively higher gene expression of pancreatic cell markers including pancreatic and duodenal homeobox 1 (PDX1) and insulin compared with the TMSCs supplemented with nicotinamide and β-mercaptoethanol [25]. Additionally, TMSCs differentiated into insulin-releasing cells with ITS had a better responsiveness to high blood glucose, secreting insulin, compared with the TMSCs differentiated with nicotinamide and β-mercaptoethanol [25].

Ironically, under the same ITS differentiation conditions, insulin secretion from the differentiated TMSCs was lower than that of the differentiated AMSCs despite the higher total insulin content in the former. This dichotomy can be attributed from impaired insulin exocytosis in the TMSCs differentiated into insulin-releasing cells due to diminished expressions of synaptotagmin isoforms, which play an important role in efficient insulin exocytosis [60]. Nevertheless, this study demonstrates

that the TMSCs produced with ITS protocol could retain similar functionality as the pancreatic islet cells that regulate blood glucose. In fact, the implantation of TMSCs differentiated with the ITS protocol partially regulated the blood glucose of the mice with streptozotocin-induced diabetes [25], indicating potential applications of TMSCs as another novel cell therapy for diabetes mellitus.

Differentiation Capacity of TMSCs into Neuron-Like or Glial Cells

BM-MSCs were shown to differentiate into neural cells that could terminally differentiate into either neuronal or glial subtypes depending on extracellular cues [61]. Neurons have a major function in transmitting signals, whereas glial cells assist in homeostasis of neuronal functions.

Similar to BM-MSCs, TMSCs have a potential to differentiate into either neuron-like [27] or glial cells [26, 62]. In a 3D hybrid thermogel that fabricates growth factor-releasing microspheres, TMSCs expressed phenotypes similar to immature neurons, exhibiting multipolar elongation with weak expression of neuronal markers such as neuronal growth factor, nuclear receptor-related protein, and neuron-specific enolase [27]. This study showed that TMSCs may have a potential to differentiate into neuron-like cells. However, the study only demonstrated weak expression of neuronal markers, typically for neural stem cells and immature neurons. Therefore, the protocol needs to be further optimized to generate fully functional mature neurons, and its functionality should be analyzed in more detail in future studies.

Other studies reported that TMSCs have a high potential to differentiate into glial cells, promoting regeneration of surrounding nerves. For example, Jung et al. reported that TMSCs have a capacity to differentiate into Schwann cell-like cells when stimulated with basic fibroblast growth factor, epidermal growth factor, platelet-derived growth factor-AA, and heregulin-β1 [26]. Myelination by the TMSCs differentiated into Schwann cell-like cells was observed around the axons of cocultured mouse dorsal root ganglion. Moreover, the implantation of the differentiated TMSCs resulted in regeneration of nerves and recovery of neuromuscular function in mice models with sciatic nerve injuries and improvement of neuromuscular function in a mouse model of Charcot–Marie–Tooth disease type 1 [26, 62].

Capability of TMSCs for Skin Regeneration

To the best of our knowledge, there are no previous reports indicating the direct differentiation capacity of TMSCs into the skin cell lineage, yet a few mentioned TMSCs as an applicable tool to regenerate skin tissues. For example, Böttcher-Haberzeth et al. discuss TMSCs as an alternative source for replacing dermal fibroblasts, which have an important role in maintaining function and esthetics of skin analogs for a transplant [63]. The study reported that TMSCs completely mimicked the functional and structural integrity of the original dermal fibroblast in pigmented dermoepidermal skin analogs. Histologically, it was observed that the skin analogs with TMSCs had identical epidermal stratification, pigmentation, and cornification as dermal fibroblasts. Melanin production was maintained at the same level for both types of skin analogs. For this reason, the authors indicated TMSCs as an excellent replacement of dermal fibroblasts in designing skin analogs for transplantation [64] in cases where sufficient amounts of dermal fibroblasts are not obtainable.

Another group also investigated potential regenerative effect of TMSCs at wound sites [30]. The application of TMSCs at cutaneous surgical defect regions improved both epidermal and dermal regeneration while attenuating inflammation by restricting T-cell specific proliferation and M1 polarization of macrophages. TMSCs also promote proliferation of fibroblasts and keratinocytes while inhibiting differentiation of fibroblasts into myofibroblasts, and therefore minimizing scar formation during wound healing of skin tissues [30].

Differentiation Potential of TMSCs into Hepatocytes

The protocol for differentiating BM-MSCs into hepatocytes generally includes two steps: initial 7 days of culturing in hepatogenic media consisting of fibroblast growth factor, hepatocyte growth factor, and nicotinamide followed by 14 additional days of culturing in media supplemented with oncostatin M, dexamethasone, and ITS [65]. A similar but slightly modified two-step protocol was used to differentiate TMSCs into hepatocyte-like cells: 7 days in hepatogenic media containing insulin-like growth factor, hepatocyte growth factor, and dexamethasone followed by 14 days in media containing oncostatin M [22]. Under the described hepatogenic differentiation condition, TMSCs showed increased expression of albumin and hepatocyte nuclear factor 4α (HNF4α) [22]. In another study, TMSC cultured for 28 days in 3D matrices of thermogel supplemented with hepatogenic growth factors showed increases in typical hepatic genes, such as albumin, cytokeratin 18, and HNF4α [66]. This study further reported that these hepatogenically differentiated TMSCs also induced protein expressions of hepatic markers, albumin, and α-fetoprotein, as well as the metabolic activity of hepatocytes, as evidenced by the increases in uptake of cardiogreen and expression of low-density lipoprotein. Later, Hong et al. modified the protocol to make an injectable system for a more practical application of the hepatogenically differentiated TMSCs [29]; these cells exhibited more than 50% of the hepatic functions compared with HepG2 cells.

Despite the studies reporting hepatogenicity of TMSCs, there is no report of applying hepatogenically differentiated TMSCs in an animal model. Instead, a few studies have addressed the direct application of undifferentiated TMSCs in in vivo animal models for treating liver diseases (Table 1). For example, the intravenous administration of TMSCs significantly reduced concanavalin A-induced hepatic toxicity but not acetaminophen-induced liver injury, which is mediated in part by decreased levels of IL-2 and tumor necrosis factor α by galectin-1 [67]. In another liver disease model, injection of undifferentiated TMSCs decreased liver fibrosis induced by CCl_4 in mice [22, 68]. These studies further demonstrated that the alleviation of the liver fibrosis by the TMSCs is mediated through autophagy activation to reduce the expression of TGF-β, a known activator for the progression of liver fibrosis [22] as well as the induction of IL-1Ra by downregulating inflammatory response [68]. Taken together, these studies addressed the role for an immunoregulatory capacity of T-MSCs in contributing to alleviation of liver diseases.

Immunomodulatory Capacity of TMSCs for Alleviating Allogenicity

In addition to differentiation, immunomodulatory property is another aspect to consider in designing an effective therapeutic tool. An allogenic response is inevitable in stem cell therapy because these cells are generally isolated from healthy donors

and given to patients with specific needs [69]. MSCs have the ability to modulate immune responses through surface molecules and soluble mediators, which then assist the cells in avoiding allogenic reactions for successful implantation. TMSCs share great number of surface markers with MSCs derived from other tissues, yet they also express their own specific surface markers unique from other MSCs [18]. Some of these distinctive markers include CD106 (VCAM-1) and CD274 (PD-L1), and have functions in adhesion and migration as well as immunomodulation [70, 71]. In fact, there are numerous studies describing the immunomodulatory properties of TMSCs, most of which are involved in the general downregulation of inflammatory responses [72–77].

PD-L1 of TMSCs prevents the differentiation of Th17, which is important for the inflammatory response of neutrophils and inflammatory disease [72, 73]. TMSCs can also hinder dendritic cell (DC) maturation and CD4+ T-cell differentiation [74], while increasing IL-10-producing regulatory B-cells to downregulate the inflammatory response [75]. At the same time, the inflammation may provide conditions for TMSCs to differentiate into follicular DCs [76, 77], thereby inducing peripheral immune tolerance by interfering with the priming of alloreactive T-cells [78]. In a dextran sulfate sodium (DSS)-induced colitis mice model, multiple intraperitoneal injections of TMSCs were shown to reduce gene expression of the inflammatory cytokines *IL-1β* and *IL-6* consequently resolving the signs associated with the DSS-induced colitis as evidenced from reduced disease activity index, rectal bleeding, and diarrhea [31]. All of these immunomodulatory effects could be attributed to the downregulation of possible alloreactivity of TMSCs for successful implantation.

CONCLUSION AND PERSPECTIVES

Until now, many studies have addressed the differentiation capacities of TMSCs and their practical applications in disease animal models (Table 1). Mesodermal differentiation (bone, fat, cartilage, muscle, and tendon) is the common lineage for TMSCs to enter into [4], yet there appears to be sufficient evidence suggesting that TMSCs could also differentiate into endodermal (PTH/insulin-releasing cells, hepatocytes) [5, 25, 29] and ectodermal lineages (neuron-like or glial cells) [26, 27, 64] under appropriate microenvironments (Fig. 2). Mesodermal differentiation capacities and MSC-specific markers are unlikely affected by donor age, passage, and cryopreservation [5, 20]. TMSCs retain higher proliferation rate compared with BM-MSCs and AMSCs, probably due to the fact that TMSCs are generally obtained from younger donors, with an average age of less than 10 years old. Most importantly, the unique surface markers and soluble mediators makes TMSCs as an attractive MSC candidate to be used in a practical situations due to both their ease obtaining and of expanding [5, 47]. Taken together, the phenotypical and

functional characteristics of TMSCs suggest that they could potentially be designated as a great source of MSCs for future stem cell engineering of regenerative medicine.

Since the complications of ESC regulation arose for research applications [79], the demands in MSCs for research and clinical applications have exponentially been accelerated [80]. Olsen et al. estimated that approved MSC-based products in the market will reach 86 products by the year 2040. However, little progress has been made over the last few decades regardless of tremendous expense and effort spent in MSC research. According to the database found in http://clinicaltrials.gov, there are 265 MSC-related clinical studies that are completed world widely. Ironically, the clinical studies that are either suspended or of unknown status are estimated to be 248 cases, almost equal in number to the completed studies. Indeed, only a few MSC products, such as Cartistem (Medipost, South Korea), HeartSheet (Terumo, Japan), and Prochymal (Osiris, Canada) have been commercialized for clinical of regenerative medicine under the approval of regulatory agencies [80]. Regarding TMSCs, no clinical trials have been started, but a few preclinical trials are currently ongoing in attempts to recovering neural, muscle, and parathyroid functions. Considering that it has only been just over a decade since the discovery of TMSCs, this is a rapid transition. However, a clear mode of action underlying the in vitro and in vivo functionality of TMSCs must be investigated through basic research prior to entering into the next steps to achieve successful translational and clinical applications.

AUTHOR CONTRIBUTIONS

S.Y.O., I.J.: conception and design, manuscript writing, final approval of manuscript; Y.M.C., H.Y.K.: conception and design, manuscript writing; Y.S.P., S.C.J., J.W.P., S.Y.W. K.H.R., H.S.K.: manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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