Aesth Plast Surg (2019) 43:1381–1386
https://doi.org/10.1007/s00266-019-01439-w

ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

Effect of the Stromal Vascular Fraction on Changes in Melanin Formation in B16 Cells Treated by IBMX

Li-hong Peng[1,2] · **Qin Li**[3]

Received: 12 April 2019 / Accepted: 18 June 2019 / Published online: 1 August 2019

Abstract
Objective To investigate the effect of the stromal vascular fraction (SVF) on changes in melanin formation and tyrosinase activity in B16 cells treated by 3-isobutyl-1 methylxanthine (IBMX) and to explore the mechanism of SVF-mediated inhibition of pigmentation.
Methods We co-cultured extracted SVFs and B16 cells treated with IBMX in a certain proportion, and the marker molecule HMB-45 was detected by immunochemistry. Melanin content was determined by NaOH lysis. Activity of tyrosinase was measured by the DOPA oxidation method.
Results HMB-45 was commonly expressed in B16 cells induced by IBMX. After the addition of SVFs, the expression of HMB-45 decreased significantly and positively correlated with increases in SVFs. After the induction of B16 cells by IBMX, melanin content increased significantly. However, melanin decreased after SVF and B16 co-culturing; the effect was more substantial with the increase and decrease in SVFs, and the activity of tyrosinase decreased.
Conclusion SVFs inhibit the production of melanin and reduce the activity of tyrosinase, possibly providing a new breakthrough for the treatment of pigment disorders.

✉ Qin Li
gzzxwk@163.com

[1] Southern Medical University, Guangzhou, China

[2] Department of Plastic Surgery and Burn Center, Second Affiliated Hospital, Shantou University Medical College, Shantou, China

[3] Guangzhou School of Clinical Medicine, Southern Medical University (Guangzhou General Hospital of Guangzhou Military Region), Guangzhou, China

No Level Assigned This journal requires that authors assign a level of evidence to each article. For a full description of these Evidence-Based Medicine ratings, please refer to the Table of Contents or the online Instructions to Authors www.springer.com/00266.

Keywords Stromal vascular fraction (SVF) · Tyrosinase · Melanin · B16 cells · IBMX

Introduction

The synthesis of melanin is a complex process, and understanding the activity of tyrosinase and the expression of related proteins has always been the focus of studies on pigment metabolism. The amount of melanin synthesis is directly proportional to the activity of tyrosinase [1]. Previous studies have shown that 3-isobutyl-1 methylxanthine (IBMX) promotes the activity of tyrosinase [2, 3] and activates the generation of melanin.

The adipogenic stromal vascular fraction (SVF) is a composite cell component rich in adipogenic stem cells. After fat tissue is digested by collagenase and centrifuged, SVFs can be obtained. SFV is a complex cellular component rich in fat-derived stem cells [4], and it has been shown to be capable of promoting tissue regeneration, healing and vascularization [5]. To date, there have been no reports that SVFs inhibit the production of melanin and whiten the skin. In this study, we co-cultured mouse melanoma cell B16 cells treated with IBMX and SVFs to observe the changes in melanogenesis and tyrosinase activity.

Materials and Methods

Materials

C57 mice were purchased from the Animal Experimental Center of Medical School of Shantou University School of Medicine, and type I collagenase was purchased from Shanghai Qiaoyuan Biological Pharmaceutical Co., LTD. Melanoma B16 cells were purchased from the Institute of Cell Biology, Chinese Academy of Sciences. IBMX and L-dopa were purchased from Sigma USA, and HMB-45 was purchased from Beijing Zsbio Commerce Store Co., LTD.

Experimental Group

The experiment was divided into the blank control group and SVFs groups at various concentrations.

Method

SVFs Segregation

We washed the subcutaneous fat of the mice with green streptomycin resistance in PBS three times with sterilized scissors. We then added PBS to remove the upper oil fraction at 37 °C and added 0.25% type I collagen enzyme on a 250 RPM thermostatic table for digestion 20–30 min; the hybrid cell group SVFs were counted with a cell counting metre.

Drug Administration of B16 Cells and Co-culture of SVFs and B16 Cells

To press with DMEM sugar medium, B16 cells were inoculated at 1 x 10^5/ml on cell culture plates and were placed in 37 °C, 5% CO_2 incubator until adherence. After the cells were treated with IBMX (75 μM), SVFs were co-cultured with B16 at concentrations of 1:1 and 2:1.

Immunohistochemical Analysis of HMB-45

B16 cells were seeded on the slides and cultured, washed with PBS three times, fixed with 4% paraformaldehyde for 1 h and then permeabilized with 1% Triton X 100 for 15 min. This procedure was followed by a 1-h incubation with donkey serum at 37 °C for 1 h. The slides were incubated with diluted HMB-45 antibody (Abcam, 1:100) at 4 °C overnight. Subsequently, secondary antibody was added, and the slides were incubated for 1 h at room temperature. The slides were incubated with a streptavidin-HRP conjugate complex and then counterstained in haematoxylin.

Western Blot

Cells protein lysates were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and incubated with specific antibodies. The membranes were washed with TBST three times, followed by a 1-h incubation with the appropriate secondary antibodies. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad). β-actin antibody was used as control.

The Activity of Tyrosinase

Tyrosinase activity was estimated by measuring the rate of oxidation of L-DOPA (Takahashi and Parsons, 1992). A total of 2 × 10^5 cells were suspended in 50 μl cold M/15 phosphate buffer, pH 6.8, containing 1% (w/v) Triton X-100. After pipetting and vortexing to lyse the cells, the extracts were clarified by centrifugation at 10000 rpm for 5 minutes. L-DOPA (2 mg/ml) was prepared in phosphate buffer as above without Triton X-100 (assay buffer). Samples (40 μl) of cell lysate were added to the wells of 96-well plates, and the assay was started by the addition of 100 μl L-DOPA solution at 37 °C. Control wells contained 40 μl lysis buffer or boiled cell lysate. Absorbance at 490 nm was read every minute for at least 20 minutes at 37 °C on a microplate reader fitted with a temperature control mechanism. One unit of tyrosinase activity was arbitrarily defined as a rate of increase of 1 absorbance unit per h per 106 cells in the initial linear region of a plot of absorbance against time [6].

Melanin Assay

A total of 2 × 10^5 cells were solubilized in 100 μl 1 M NaOH and diluted with 400 μl distilled water. Absorbance at 475 nm was compared with a standard curve of synthetic melanin (Sigma) prepared in a final NaOH concentration of 0.2 M [6].

Results

(1) HMB-45 is a product of the SILV gene locus and membrane-bound melanosomal protein [7]. HMB-45 is a marker of melanocyte activation and an important melanogenesis-related protein. HMB-45 was involved in the transition from stage I to stage II melanosome maturation and stabilization of melanin intermediates [8]. A part of normal melanocytes, subsets of

melanocytic nevi, and most metastatic melanomas typically express HMB-45 [9, 10]. HMB-45 is more commonly expressed in B16 cells, and IBMX-induced HMB-45 expression becomes stronger. However, under conditions of co-culture of SVFs and B16 cells, the expression of HMB-45 decreased significantly and positively correlated with the increase in SVFs (Fig. 1).

(2) After processing B16 with IBMX, there was a marked increase in melanin that reduced after co-culture with SVFs. With the increase in SVFs, the reduction effect was more substantial (Fig. 2).

(3) The expression of tyrosinase showed no obvious change in cell processing induced by various drugs (Fig. 3).

(4) SVFs inhibited the activity of B16 tyrosinase, and with the increase in dosage, the inhibitory effect was more substantial (Fig. 4).

(5) SVFs showed a significant inhibitory effect on tyrosinase activity of B16 induced by IBMX, compared to that of the control group ($P < 0.05$) (Table 1).

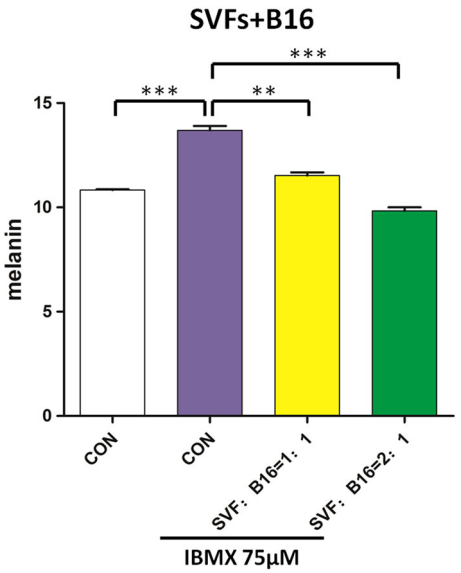

Fig. 2 Melanin assay of B16 cells (con), B16 cells induced by IBMX, co-culture of SVFs and B16 cells induced by IBMX. With the increase in SVFs, the melanin decreased. The data represent the mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

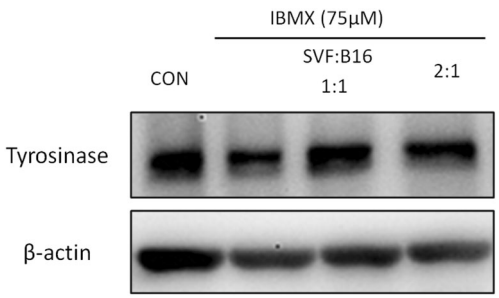

Fig. 3 The expression of tyrosinase of B16 cells (con), B16 cells induced by IBMX, co-culture of SVFs and B16 cells induced by IBMX were measured by Western blot. The expression of β-actin was used as control

Discussion

Pigment barrier skin disease is thought to be caused by a variety of external environmental factors and physiological factors including ultraviolet radiation, the influence of inflammation and ageing; skin cells produce autocrine and paracrine hormones and cytokines, formed in the local autocrine and paracrine network and playing an important regulating role in skin pigmentation [11]. The aetiology of pigmented disease is complex, with studies showing that at least 125 genes are directly or indirectly involved in this process [12].

The main purpose of clinical treatment for pigmented diseases is to inhibit the activity and proliferation of melanocytes, to inhibit the formation and transport of melanin particles or to damage pigment particles to accelerate their metabolic degradation [13]. The curative effect of drug treatment was relatively ineffective with many adverse

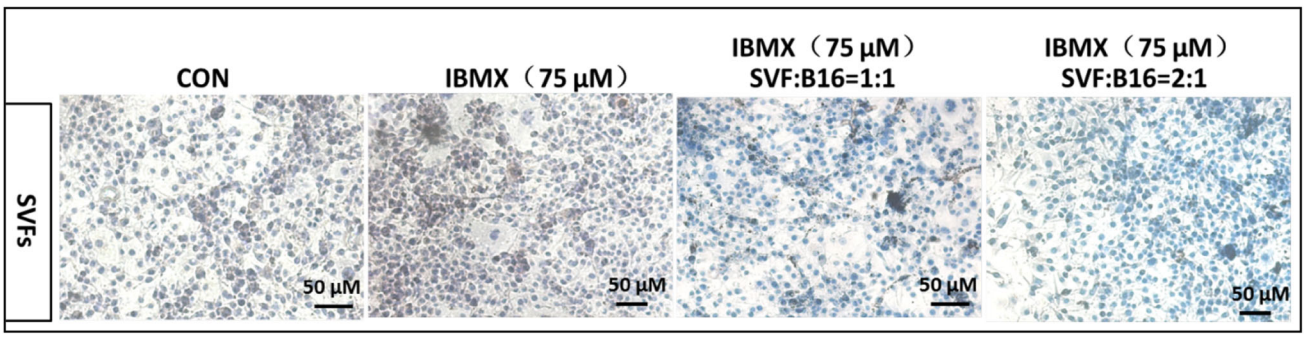

Fig. 1 Immunohistochemical analysis of HMB-45 in B16 cells (con), B16 cells induced by IBMX, co-culture of SVFs and B16 cells induced by IBMX. The expression of HMB-45 decreased with the increase in SVFs

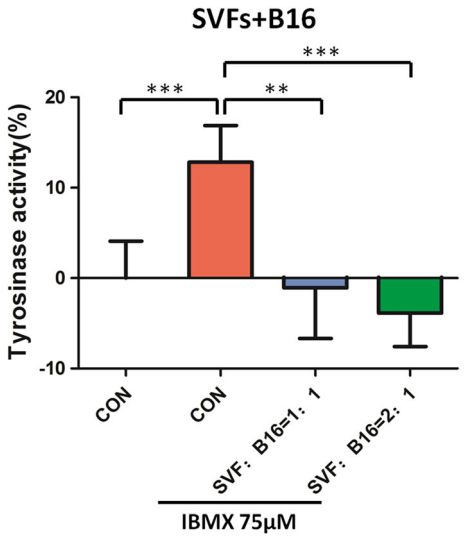

Fig. 4 The activity of tyrosinase of B16 cells (con), B16 cells induced by IBMX, co-culture of SVFs and B16 cells induced by IBMX. SVFs inhibited the activity of tyrosinase in B16 cells. The data represent the mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Table 1 SVFs effect on the activity of B16 melanoma tyrosinase ($\bar{X}$ ± s) ($n = 3$)

Groups	Ratio	TYR relative activity
Control group	–	100 ± 0.00
SVFs, B16	1:1	80.15 ± 1.45
SVFs, B16	2:1	70.6 ± 2.45

reactions. The q-switched laser technology of "selective photothermal effect" reduced the thermal damage to normal skin [14]. However, for Asian people with the most common Fitzpatrick III–IV [15], simultaneous laser treatment of pigment often causes pigmentation. The mechanism of skin pigmentation is the basis of skin beauty research. The search for a safe and effective whitening agent is a current hot research topic.

Tyrosinase is a rate-limiting enzyme in the biosynthesis of melanin. Tyrosinase activity is proportional to the amount of melanin produced. Because of the key role of tyrosinase in the synthesis of melanin and its unique physiological function in biology, tyrosinase has been studied in the fields of medicine, agriculture, cosmetics, pharmacy and chemistry [16–18].

Some investigators cultured melanin B16 cells with ADSC-CM (adipose-derived stem cell-conditioned medium) and found that TGF-β1 in ADSC-CM reduced the activity of tyrosinase, reduced the synthesis of tyrosinase, and reduced the production of melanin [19]. In addition, some studies suggest that TGF-β1 can interfere with the maturation and transport of melanocytes and have the effect of reducing pigment. After the ADSC suspensions were injected into the skin of C57BL/6 mice, the mice were exposed to UVB irradiation for 2 days, and melanin synthesis in the experimental group was reduced compared to that of the control group. Therefore, ADSC may inhibit the synthesis of melanin and the activity of tyrosinase by secreting antioxidant substances and other cytokines and play a role in skin whitening [20]. Nevertheless, the mechanism remains unclear and requires further study.

Clinical use of autologous culture of ADSC has limitations. In vitro culture cannot meet clinical needs. In vitro amplification takes a long time, at least 2 to 3 weeks. Furthermore, in vitro cultivation is tedious, often becomes contaminated, tends to be unsafe, and imposes strict requirements on facilities and equipment, with high costs. Adipogenic stromal vascular fractions (SVFs) are composite cell components rich in adipogenic stem cells, with the ability of self-proliferation, long-term survival and multi-directional differentiation. SVFs can differentiate into bone, cartilage, fat, blood vessel and nerve [21]. Compared with adipogenic stem cells, the procedure can be completed in one stage without in vitro amplification and with a high degree of safety. Compared with human epidermal melanocytes, mouse melanoma B16 cells have greater similarity and are easier to acquire, cultivate, and grow rapidly. After several generations of repeated cultivation, the ability of melanin production remains strong. In the previous experiment, SVFs were co-cultured with B16 cells. SVFs reduce the activity of B16 cells and their proliferation. However, the contents of melanin and the expression of tyrosinase were not significantly changed, and melanin production of B16 cells was limited when the culture was considered.

Studies have shown that IBMX promoted the activity of tyrosinase [2, 3] and activated the generation of melanin. In this study, after the treatment of B16 cells by IBMX, the generation of melanin was induced and the production of melanin increased significantly in B16 cells. HMB-45 is a marker of melanocyte activation and an important melanogenesis-related protein. We measured expression levels of HMB-45 by immunohistochemistry to reflect the content of melanin indirectly. After co-culture of SVFs and B16 cells, the expression of HMB-45 decreased significantly and positively correlated with the increase in SVFs. Moreover, we also found that SVFs had a significant inhibitory effect on the activity of tyrosinase in B16 cells, and the inhibitory effect was more substantial with the increase in dose; however, the content of tyrosinase was not significantly changed.

There are several limitations to the current study. First, we detected the effect of the stromal vascular fraction on changes in melanin formation in mouse melanoma B16 cells instead of human epidermal melanocytes. Whether SVFs inhibited melanin formation in human epidermal

melanocytes remains to be seen in further experiments. Furthermore, it was an animal experiment, which was still some way from clinical application. Second, we found that co-culture of SVFs and B16 cells decreased the production of melanin significantly and SVFs had a significant inhibitory effect on the activity of tyrosinase in B16 cells in the study. But the underlining mechanisms were still unclear and needed to be further explored. Third, there were two main ways for SVF isolation, mechanical dissociation or enzymatic dissociation [22]. We isolated the SVF from adipose tissue using collagenase in the study. The main disadvantage of this mean of enzymatic dissociation is not only that it is time-consuming and expensive, but also that enzymatic treatment disrupts all communicative connections that exist between the cells as well as between the cells and extracellular matrix. What is more, clinically applied cell-based products that are derived with collagenase are not allowed in the legislation of several countries [23].

There are various treatment modalities for hyperpigmentation currently, including external used drugs, oral agents and procedural techniques [24]. Combination products containing hydroquinone, retinoic acid, and corticosteroids are the most effective external used drugs for hyperpigmentation. Oral agents contain tranexamic acid, polypodium leucotomos extract, rucinol, emblica, pycnogenol, mulberry, coffeeberry, green tea, silymarin, grape-seed extract, orchids and belides. Procedural treatment for hyperpigmentation includes chemical peeling, dermabrasion, laser and intense pulsed light therapy. SVF has been used clinically for soft tissue repair such as the reconstruction of breast and face, and in pathologic disorders like lipodystrophy. SVF populations have also been used for the cure of inflammatory diseases such as muscular sclerosis, and for immune suppression in graft-versus-host disease and Crohn's disease-induced fistula [25]. Furthermore, the use of SVF transplantation has been evaluated to treat patients with radiation-induced severe burns [26]. There are fewer studies about the clinical application of SVF in hyperpigmentation so far. Our study may provide theoretical basis for the new treatment method for hyperpigmentation.

Conclusion

SVFs have a significant inhibitory effect on melanogenesis of B16 cells induced by external factors. By inhibiting the activity of tyrosinase, they reduce the production of melanin and play a role in reducing pigment, while the content of tyrosinase is not significantly changed. This experiment aimed to study the treatment basis and method of pigmentation so as to facilitate the establishment of a systematic and effective treatment for pigmentation. Due to the complex mechanism of pigmentation, further experimental studies are needed.

Acknowledgements This work was supported by the Guangdong medical science and technology research fund project (No. B2018073).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest to disclose.

Human and Animal Rights This article does not contain any studies with human participants performed by any of the authors. The animal care and experimental protocols were approved by the Animal Research Committee of Southern Medical University.

Informed Consent For this type of study, informed consent is not required.

References

1. Son YO, Lee SA, Kim SS et al (2011) Acteoside inhibits melanogenesis in B16F10 cells through ERK activation and tyrosinase down-regulation. J Pharm Pharmacol 63(10):1309–1319
2. Lee HD, Lee WH, Roh E et al (2011) Manassantin A inhibits cAMP-induced melanin production by down-regulating the gene expressions of MITF and tyrosinase in melanocytes. Exp Dermatol 20(9):761–763
3. Shibahara S, Takeda K, Yasumoto K et al (2001) Microphthalmia-associated transcription factor (MITF): multiplicity in structure, function, and regulation. J Investig Dermatol Symp Proc 6(1):99–104
4. Gimble JM, Bunnell BA, Chiu ES et al (2011) Concise review: Adipose-derived stromal vascular fraction cells and stem cells: let's not get lost in translation. Stem Cells 29(5):749–754
5. Zuk PA, Zhu M, Ashjian P et al (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13(12):4279–4295
6. Hunt G, Todd C, Cresswell JE et al (1994) Alpha-melanocyte stimulating hormone and its analogue Nle4DPhe7 alpha-MSH affect morphology, tyrosinase activity and melanogenesis in cultured human melanocytes. J Cell Sci 107(1):205–211
7. Lu S, Slominski A, Yang SE, Sheehan C, Ross J, Carlson JA (2010) The correlation of TRPM1 (Melastatin) mRNA expression with microphthalmia-associated transcription factor (MITF) and other melanogenesis-related proteins in normal and pathological skin, hair follicles and melanocytic nevi. J Cutan Pathol 37:26–40
8. Slominski A (2002) Coming of age of melanogenesis-related proteins. Arch Pathol Lab Med 126(7):775–777
9. Carlson JA, Ross JS, Slominski AJ (2009) New techniques in dermatopathology that help to diagnose and prognosticate melanoma. Clin Dermatol 27(1):75–102
10. Busam KJ (2004) The use and application of special techniques in assessing melanocytic tumours. Pathology 36(5):462–469
11. Imokawa G (2004) Autocrine and paracrine regulation of melanocytes in human skin and in pigmentary disorders. Pigment Cell Res 17(2):96–110
12. Bennett DC, Lamoreux ML (2003) The color loci of mice–a genetic century. Pigment Cell Res 16(4):333–344

13. Quevedo WC Jr, Holstein TJ, Dyckman J, McDonald CJ, Isaacson EL (2000) Inhibition of UVR-Induced Tanning and Immunosuppression by Topical Applications of Vitamins C and E to the Skin of Hairless (hr/hr) Mice 1. Pigment Cell Res 13(2):89–98
14. Arora P, Sarkar R, Garg VK et al (2012) Lasers for treatment of melasma and post-inflammatory hyperpigmentation. J Cutan Aesthet Surg 5(2):93–103
15. Alexis AF (2013) Lasers and light-based therapies in ethnic skin: treatment options and recommendations for Fitzpatrick skin types V and VI. Br J Dermatol 169:91–97
16. Denning MF (2012) Specifying protein kinase C functions in melanoma. Pigment Cell Melanoma Res 25(4):466–476
17. Park HY, Wu C, Yonemoto L et al (2006) MITF mediates cAMP-induced protein kinase C-beta expression in human melanocytes. Biochem J 395(3):571–578
18. Bae-Harboe YS, Park HY (2012) Tyrosinase: a central regulatory protein for cutaneous pigmentation. J Invest Dermatol 132(12):2678–2680
19. Kim WS, Park SH, Ahn SJ et al (2008) Whitening effect of adipose-derived stem cells: a critical role of TGF-beta 1. Biol Pharm Bull 31(4):606–610
20. Chang H, Park JH, Min KH et al (2014) Whitening effects of adipose-derived stem cells: a preliminary in vivo study. Aesthetic Plast Surg 38(1):230–233
21. Bunnell BA, Flaat M, Gagliardi C et al (2008) Adipose-derived stem cells: isolation, expansion and differentiation. Methods 45(2):115–120
22. Nürnberger S, Lindner C, Maier J, Strohmeier K, Wurzer C, Slezak P, Suessner S et al (2019) Adipose-tissue-derived therapeutic cells in their natural environment as an autologous cell therapy strategy: the microtissue-stromal vascular fraction. Eur Cells Mater 37:113–133
23. van Dongen JA, Stevens HP, Parvizi M, van der Lei B, Harmsen MC (2016) The fractionation of adipose tissue procedure to obtain stromal vascular fractions for regenerative purposes. Wound Repair Regen 24(6):994–1003
24. Chaowattanapanit S, Silpa-Archa N, Kohli I et al (2017) Postinflammatory hyperpigmentation: A comprehensive overview: Treatment options and prevention. J Am Acad Dermatol 77(4):607–621
25. Kokai LE, Marra K, Rubin JP (2014) Adipose stem cells: biology and clinical applications for tissue repair and regeneration. Trans Res J Lab Clin Med 163(4):399–408
26. Rigotti G, Marchi A, Galie M, Baroni G, Benati D, Krampera M, Pasini A, Sbarbati A (2007) Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing process mediated by adipose-derived adult stem cells. Plast Reconstr Surg 119(5):1409–1422 **discussion 23–24**