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ORIGINAL PAPER

Regenerative therapy for refractory vitiligo using cultured epithelial sheet grafting

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Abstract

Background Vitiligo, the most common refractory, acquired skin disease, has an estimated population incidence of 0.5 ~ 1.0%. The pathogenesis of vitiligo can be classified into two major types, segmental and non-segmental (generalised), both of which, depending on the site of onset, significantly reduce the quality of life of patients and affect their social life. Autologous cultured epithelial sheet grafting reduces surgical invasion of normal skin and is excellent for large-area skin defect treatment. We investigated the efficacy of cultured epithelial grafting in patients with refractory vitiligo who failed to respond to standard vitiligo therapy.

Methods In 16 patients (6 males; 10 females; average age, 33.06 [13–71] years), 21 vitiligo sites were treated, with a follow-up period of 3 months to 1 year. Vitiligo types were segmental type ($n=2$), non-segmental type ($n=11$ cases: generalised, acrofacial, and universal types, with nine, one, and one case, respectively), non-classified type ($n=2$), and Sutton's nevus ($n=1$). Spindle-shaped skin sample was obtained near the inguinal region for epithelial cell culture. The skin was digested enzymatically to obtain free cells. The epithelial cells and melanocytes contained in the obtained free cells were cultured separately. Thereafter, the cultured melanocytes were seeded onto the cultured epithelial cells. Under local anaesthesia, the area of vitiligo was abraded, and a cultured epithelial sheet containing melanocytes was grafted onto the abraded region. After grafting, the results were evaluated using the vitiligo area scoring index (VASI) score.

Results The preoperative and 3 months postoperative VASI were $4.62+/-0.74$ and $3.43+/-1.03$ (21 sites in 16 patients: $p=1.2\times10^{-4}$), respectively, with significant improvement. The VASI score was $2.56+/-1.20$ (18 sites in 13 patients, $p=8.1\times10^{-7}$) at 6 months and $2.25+/-1.61$ (16 sites in 11 patients, $p=2.5\times10^{-5}$) at 1 year. At 6 months and 1 year post grafting, the VASI significantly improved compared to that preoperatively.

Conclusions The melanocyte-containing cultured epithelial sheet grafting was effective for segmental vitiligo and showed some efficacy for non-segmental (generalised) vitiligo, but no effect in unclassified or some non-segmental vitiligo types. Therefore, further clarification of the mechanisms underlying vitiligo is necessary. Our cultured epithelium-containing melanocytes may be useful for treating skin deformities caused by pigmentation and/or depigmentation.
Level of Evidence: Level IV, therapeutic study.

Keywords Cultured epithelium · Cultured melanocytes · Vitiligo · Sheet career · Regenerative therapy

Introduction

Autologous cultured epithelial grafting, which reduces surgical invasion of normal skin, is excellent for the treatment of large-area skin defects. The use of cultured epithelium for large-area burns is a typical example [1]. On the other hand, it can also be used to treat giant nevi, vitiligo, and tattoos that require extensive excision [2–4]. However, when deep dermal excision is required, as in the case of giant naevi and tattoos, the cultured epithelium is not successfully grafted, and it is essential to reconstruct

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the dermis before grafting. In addition, scarring often persists postoperatively. Acquired vitiligo, caused by the loss or dysfunction of melanocytes, is pathologically limited to the superficial dermis, resulting in less invasion of the dermis, better engraftment of cultured epithelium, and scarless healing after grafting.

In particular, the epithelial cell culture technique established by Rheinwald and Green [5] can culture epitheliums in an environment that retains melanocytes, and the colour tone is often maintained after grafting [3]. Vitiligo is a common skin disorder that is often recognised as a cutaneous disfigurement. Depending on the site of onset, it may cause significant psychological distress to patients due to cosmetic problems, greatly reducing their quality of life [6]. Although many therapeutic methods have been devised, it is one of the most refractory skin diseases for which no standard therapy has been established.

In this study, we investigated the efficacy of cultured epithelial grafting in patients with refractory vitiligo who failed to respond to standard vitiligo therapy.

Patients

This study was reviewed and approved by the Technical Advisory Committee of the Extraordinarily Certified Committee for Regenerative Medicine of Yukeikai (no. NA8200002), recognised based on the Safety of Regenerative Medicine Act regulated by the Ministry of Health, Labour and Welfare (Approval No. PB3210086).

The selection criteria for patients with vitiligo indicated for cultured epithelial grafting were as follows: patients were diagnosed with vitiligo and did not respond to phototherapy, topical corticosteroid/immunosuppressive therapy, oral corticosteroid therapy, or surgical procedures such as mini-grafts according to the Vitiligo Treatment Guidelines 2012 (Japanese Dermatological Association) (Table 1). The exclusion criteria are also listed in Table 1.

Sixteen patients (6 males and 10 females) were included in the study, with a follow-up period of 3 months to 1 year between October 2019 and November 2021. Twenty-one vitiligo sites were treated in 16 patients. The average age distribution was 33.06 (13–71) years.

Methods

Epithelial cell culture

Spindle-shaped (1 × 2 cm) skin samples were obtained for epithelial cell culture near the inguinal region. The skin was minced to 1 × 2 mm and enzymatically digested with a 0.25% trypsin solution to obtain free cells. Free cells were cultured using Boyce and Ham's method [7].

Melanocyte culture

Melanocytes were cultured from enzyme-treated skin residues using migration culture. After confirming melanocyte migration from the tissue, the cells were cultured selectively for approximately 2 weeks according to the method described by Chen et al. [8]. These melanocytes were passaged using 0.05% trypsin solution and maintained using the same method.

Melanocyte-containing cultured epithelial sheet for grafting

Epithelial cells obtained using the method described in Epithelial cell culture were cultured to 95% confluence, and cultured melanocytes obtained using the method described in Melanocyte culture were seeded onto the cultured epithelium. After confirming melanocyte adhesion (approximately 24 h later), DME/F12 medium containing 10% FBS was used to induce differentiation, and melanocyte-containing cultured epithelial sheets were prepared.

The melanocyte-containing epithelial sheets were detached using a 600 U/mL Dispase solution and stored on a carrier for cell sheet grafting (ATTRAN, Nikkan Industrial Co. Tokyo, Japan) until the grafting.

Table 1 Therapeutic compliance criteria

Inclusion criteria
1. Being diagnosed with acquired vitiligo
2. Resistance to therapy recommended by the Vitiligo Treatment Guidelines 2012 (Japanese Dermatological Association)
Exclusion criteria
1. Untreated vitiligo
2. Congenital vitiligo and albinism
3. Malignant skin disease
4. When therapy of present illness takes precedence over treatment of vitiligo

Melanocyte-containing cultured epithelial sheets grafting

After local anaesthesia with 2% lidocaine was applied to the grafted region, the area of vitiligo was abraded to the superficial dermis, where the petechial haemorrhage occurred, using serrated wheels. Melanocyte-containing cultured epithelial sheet was then grafted onto the abraded region using ATTRAN as the carrier sheet. After the ATTRAN was removed from the grafted MCE sheets, the grafted wound region was protected using a non-adherent gauze (Adaptic: Johnson & Johnson Medical Inc., Arlington, TX, USA) coated with white Vaseline. It was kept in place for 1 week after grafting, after which the old Adaptic was removed. The grafted wound was subsequently redressed using Adaptic with a steroid-containing ointment or antibiotic ointment.

Post-grafted management

For up to 2 weeks after grafting, the grafted surface was exposed to open air for several hours per day for complete epithelialisation of the grafted skin. Thereafter, a steroid ointment was used if there were any symptoms such as itching in the wound region. Two weeks after the grafting, the wound was exposed to sunlight. However, patients were prohibited from potent sunlight exposure for at least one year to prevent blistering of the graft site at least 1 year. Occasionally, phototherapy was administered according to the guidelines as needed.

Statistical processing

The vitiligo area before and after transplantation was evaluated using the vitiligo area scoring index (VASI) described by Hamzavi et al. [9]. Statistical significance was determined using the Student's *t*-test, and $p < 0.0167$ was considered significant after compensation using Bonferroni's multiple comparison procedure.

Results

Vitiligo classification (Table 2)

The classification breakdown of vitiligo was as follows: segmental type, 2 cases; non-segmental type, 11 cases (generalised type, 9 cases; acrofacial type, 1 case; and universal type, 1 case); non-classified type, 2 cases; and Sutton's naevus, 1 case.

*Follow-up (*Table 2*)*

All patients were observed up to 3 months postoperatively, 14 patients were observed for 6 months, and 11 patients were observed for 1 year postoperatively.

In the physician's visual evaluation, the degree of improvement compared to that before grafting was 6/16 patients rated as a score of 4 (good) or 5 (excellent) at 3 months, 6/14 at 6 months, and 5/11 at 1 year. Similarly, the

Table 2 Therapeutic compliance criteria

Patient	Gender	Age	Type	Subtype	Donor site	Grafted site	Result		
							3 months	6 months	1 year
1	F	39	UC		Inguinal	Shoulder	3	4	5
2	M	13	SN		Inguinal	Nose	5	3	
3	F	33	S		Inguinal	Lip	4	4	
4	M	29	NS	G	Inguinal	Neck	3	3	4
5	F	37	NS	G	Inguinal	Nuchal	4	5	
6	F	26	S		Inguinal	Face and neck	3	3.7	3
7	M	22	NS	G	Inguinal	Neck	3	4	5
8	M	16	NS	G	Inguinal	Leg	3	4	4
9	F	36	NS	G	Inguinal	Lip	2	1	1.5
10	F	34	NS	U	Inguinal	Abdomen	4	4	5
11	F	48	NS	G	Inguinal	Nuchal and lip	4	1.5	2.5
12	F	70	NS	G	Inguinal	Back	0.5	3	4.5
13	M	37	UC		Inguinal	Submaxillary	2	1	1
14	F	63	NS	A	Inguinal	Hand	2		
15	M	14	NS	G	Inguinal	Face	3	3	3
16	F	12	NS	G	Inguinal	Abdomen	4		

Gender: *F*, female; *M*, male. Type: *UC*, unclassified; *SN*, Sutton nevus; *S*, segmental; *NS*, non-segmental. Subtype: *G*, generalized; *A*, acrofacial; *U*, universal. Result: 0; no-change, *1*, poor; *2*, slightly improved; *3*, improved; *4*, good; *5*, excellent; blank, not evaluated

scores of 2 (slightly improved) and 3 (improved) were 9/16 at 3 months, 5/14 at 6 months, and 3/11 at 1 year. Similarly scores of 0 (no change) and 1 (poor), which indicated no effect, were 1/16, 3/14, and 3/11, respectively.

Evaluation of pigment regeneration using the vitiligo area scoring index (VASI) method (Figs. 1 and 2)

Melanocyte-containing cultured epithelial sheets were grafted onto 21 sites in 16 patients. Four sites in four patients showed no pigment regeneration 3 months after grafting. Pigment regeneration was observed at 17 sites in 12 patients (Fig. 1).

The preoperative VASI was $4.62 +/- 0.74$ (21 sites in 16 patients). At 3 months postoperatively, the VASI was $3.43 +/- 1.03$ (21 sites in 16 patients; $p = 1.2 \times 10^{-4}$) and improved significantly.

The VASI was $2.56 +/- 1.20$ (18 sites in 13 patients; $p = 8.1 \times 10^{-7}$) at 6 months and $2.25 +/- 1.61$ (16 sites in 11 patients: $p = 2.5 \times 10^{-5}$) at 1 year. At 6 months and 1 year post-grafting, the VASI significantly improved compared to that preoperatively (Fig. 2).

Typical cases by pathological classification (Figs. 3, 4, 5, 6, and 7)

The following are pre- and post-grafting photographs of a patient classified as unclassified type (case 1). Three months after grafting, vitiligo on the shoulder improved, and one year later, it disappeared (Fig. 3)

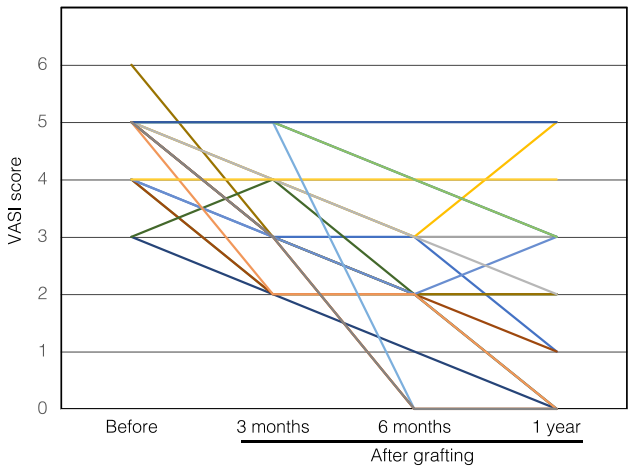

Fig. 1 Change of vitiligo area scoring index (VASI) score after grafting of cultured epithelial sheet containing melanocytes. The VASI score was evaluated as described by Hamzavi et al. [9]. Each line shows the change at 3 and 6 months and 1 year after grafting compared with that before therapy. The changes in the 16 patients (21 sites) are shown in figure

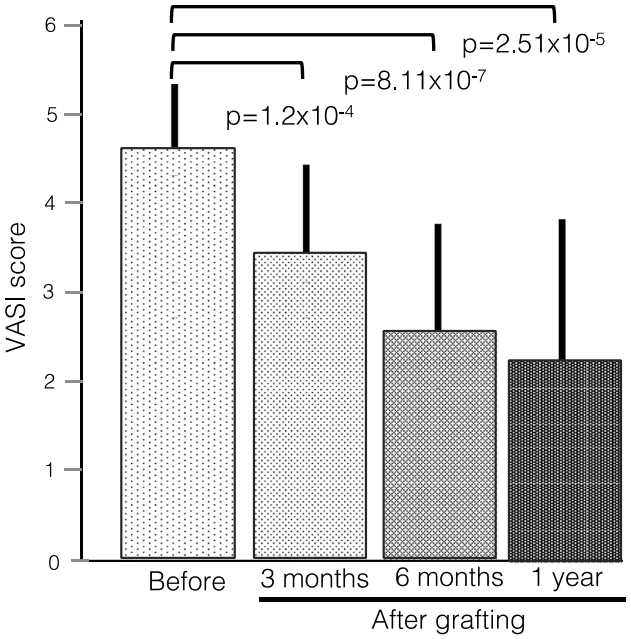

Fig. 2 Effects of cultured epithelial sheet containing melanocytes on vitiligo. Data are shown as mean +/− SD of the VASI score. Student's *t*-test with Bonferroni's multiple comparison procedure was used for statistical analysis. Vitiligo symptoms improved significantly after grafting

The following are pre- and post-grafting photographs of a patient classified as segmental type (case 3). Pigment regeneration was observed 3 months after grafting, with more extensive improvement at 6 months; no observations were made more improvement at 1 year (Fig. 4)

A patient classified as non-segmental (generalised type) (case 7) is shown pre- and post-grafting. Good pigment regeneration was observed and the number of vitiligo sites was reduced. After one year, the patient wished to have the remaining sites of vitiligo from the neck to the mandible and lower lip treated (Fig. 5)

The following are pre- and post-implantation photographs of a patient classified as a non-segmental type (universal type; case 10). Three months after grafting, the vitiligo had almost disappeared, and six months after grafting, hyperpigmentation was evident (Fig. 6)

A patient classified as unclassified (case 13) is shown in the grafting photos. Pigmentation did not regenerate for one year after grafting (Fig. 7)

Discussion

Vitiligo is well known as the most common refractory acquired skin disease. The incidence of vitiligo is estimated to be 0.5 ~ 1.0% in the population [10]. The pathogenesis of

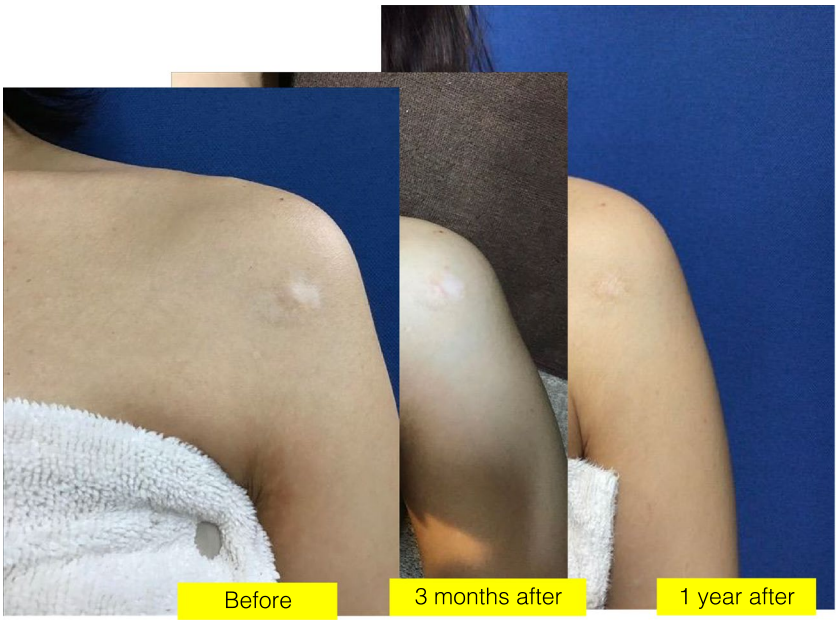

Fig. 3 Typical patient photograph before and after grafting of cultured epithelial sheet containing melanocytes. Patient 1 (shown in Table 2): unclassified vitiligo. The physician outcome was assessed as score 5 (excellent). The VASI score improved from 4 before therapy to score 1 at 1 year after grafting

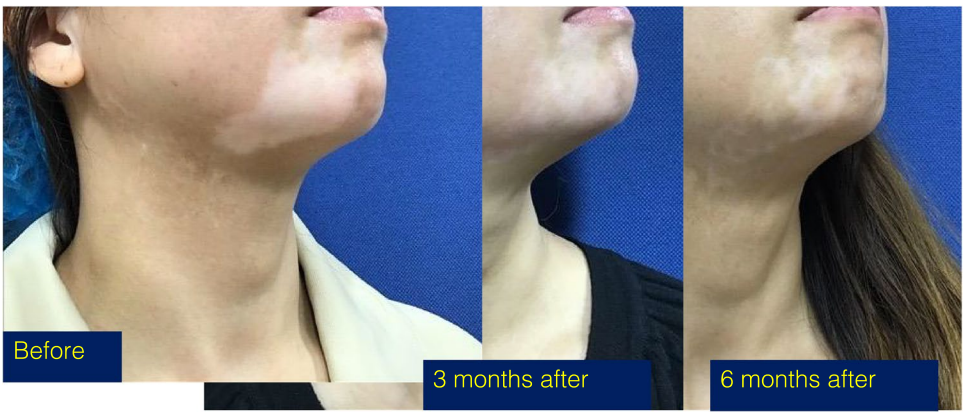

Fig. 4 Typical patient photograph before and after grafting of cultured epithelial sheet containing melanocytes. Patient 3 (shown in Table 2): segmental vitiligo. The physician outcome was assessed as score 4 (good). The VASI score improved from 5 before therapy to score 2 at 6 months after grafting

vitiligo can be classified into two major types: segmental and non-segmental (generalised), both of which, depending on the site of onset, significantly reduce the quality of life of patients and affect their social life [11].

Many patients with vitiligo have anti-melanocyte antibodies that inhibit a series of enzymes (tyrosinase, TRP1, and TRP2) involved in melanin synthesis. Impairment of melanin synthesis leads to depigmentation; however, melanocytes remain viable [11]. Owing to these pathologies, topical corticosteroids and immunosuppressive drugs are the first choice of therapy. Recently, the efficacy of phototherapy has been reported [12]. In Japan, narrowband type B ultraviolet (UVB) therapy is recommended as first-line therapy according to the treatment guidelines of the Japanese Dermatological Association [13]. Surgical treatment of vitiligo, on the other hand, is used for vitiligo that is refractory to a series of conservative treatments [14]. In general, segmental skin grafting and epithelial skin grafting are known [15]; however, mini-grafts and other techniques have recently been designed [16]. Skin grafting can leave scars at the site of skin excision; in the case of mini-grafts, the grafted site may have a paving stone or polka dot appearance [17], and scarring may also be present [18].

Kumagai et al. [19] initially used cultured epithelial grafts for extensive severe burns. In cultured epithelium, melanocytes are also maintained; thus, vitiligo therapy has been attempted. However, Rheinwald and Green's method of culturing the epithelium maintains melanocytes but does not proliferate them. Chen et al. [8] selectively cultured melanocytes from skin tissue and transplanted a suspension of cultured melanocytes into the superficial areas of vitiligo. In this case, the grafting efficiency was low because there was no carrier for melanocyte grafting. Therefore, we cultured

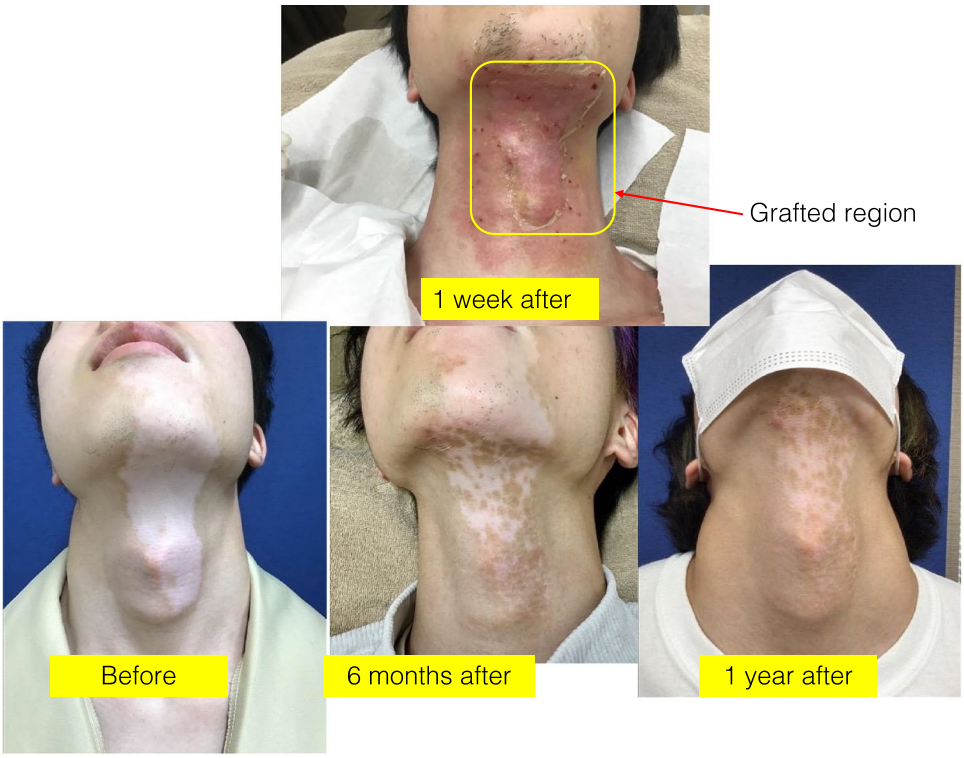

Fig. 5 Typical patient photograph before and after grafting of cultured epithelial sheet containing melanocytes. Patient 7 (Table 2): non-segmental (generalised) vitiligo. The physician outcome was assessed as score 5 (excellent). The VASI score improved from 5 before the therapy to score 2 at 1 year after grafting

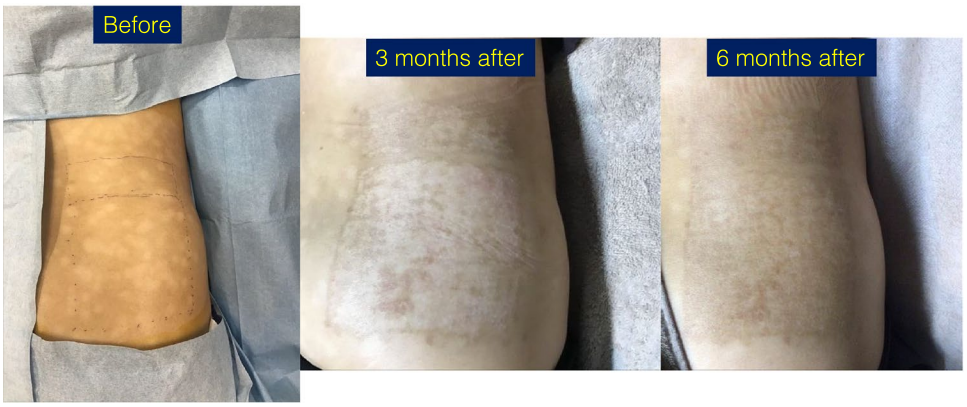

Fig. 6 Typical patient photograph before and after grafting of cultured epithelial sheet containing melanocytes. Patient 10 (Table 2): non-segmental (universal) vitiligo. The physician outcome was assessed as score 5 (excellent). The VASI score improved from 3 before therapy to 0 1 year after grafting

the epithelium and melanocytes separately from the obtained skin and seeded the cultured epithelium with the separately cultured melanocytes and co-cultured them. This new technique facilitates the efficient implantation of melanocytes and wound management because it can cover the entire surface of the recipient site by grafting the cultured epithelial sheets.

The present graft of melanocytes-containing cultured epithelial sheets for vitiligo was effective for segmental vitiligo, but also showed some efficacy for non-segmental (generalised) vitiligo. Matsuzaki et al. [20] reported that it was effective for segmental vitiligo but had little or no effect on non-segmental (generalised) vitiligo.

Furthermore, they reported a statistically significant improvement in segmental vitiligo compared with non-segmental vitiligo. In the present study, most cases were classified as the non-segmental type. This is probably due to the fact that we treated patients with vitiligo who had failed to respond to standard therapy or mini-grafts. Therefore, statistical comparisons between the segmental and non-segmental types could not be made. However, the authors observed efficacy in 9 out of 11 (81%) grafted patients with the non-segmental type. This result may be due to the difference between our cultured epithelium containing melanocytes and that of Rheinwald and Green, since melanocytes do not proliferate in their

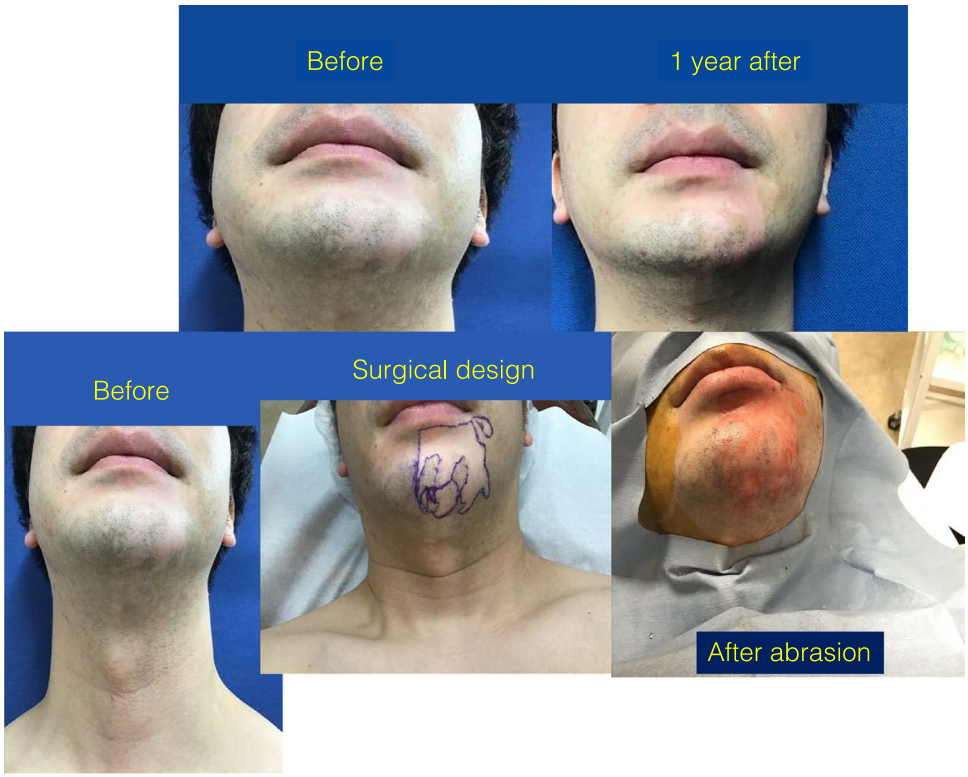

Fig. 7 Typical patient photograph before and after grafting of cultured epithelial sheet containing melanocytes. Patient 13 (Table 2): unclassified vitiligo. The physician outcome was assessed as score 1 (poor). The VASI score improved from 4 before therapy to 4 1 year after grafting

culture method. Therefore, the cultured epithelium created from patients with non-segmental (generalised) vitiligo may have a lower density of melanocytes. Functional melanocytes may not be retained in autoimmune reactions. Therefore, insufficient melanocytes remain, to cause visual changes.

On the other hand, UV therapy and steroid application, which were performed on some patients after this grafting, may promote pigmentation for postoperative vitiligo and may influence the judgement of the effectiveness of grafted the cultured epithelium containing melanocytes. However, these therapies had already been performed before cultured epithelium grafting and showed no effect. Therefore, even if these therapies were to show efficacy, it is likely to be derived from the grafted cultured melanocyte function.

In our method, melanocytes that are confirmed to produce melanin are cultured separately and mixed with the epithelium; therefore, the melanocyte content was higher. This difference is apparent in our results. However, no effect was observed in unclassified or non-segmental vitiligo types. Therefore, further clarification of the mechanisms underlying vitiligo is necessary.

In addition, mini-graft technique in the vitiligo region that was a standard surgical approach was found remaining scar or scar contraction in many cases (shown in Fig. 3). In these results, the melanocytes-containing cultured epithelial sheets that we developed may be useful for the treatment of skin deformities caused by pigmentation and/or depigmentation. However, the pigmentation of pre- and postoperative should be compared to using an internal color standard. Because the current investigation attempted on the feasibility of this therapy, such considerations were not made. This culture technique will be able to freely regulate the skin color after grafting by adjusting the number of melanocytes contained in the cultured epithelium or by preparing an epithelial sheet containing melanin-induced cultured melanocytes [21]. In the next stage, it may be necessary to conduct clinical research according to a research protocol that enables objective evaluation.

Author contribution All authors contributed to the study conception and design. The surgical operation was performed by Hirose Yoshie and Kamikawa Mayuko. Epithelial sheet preparation for grafting and data collection was performed by Fuijta Chiharu and Inoue Hajime. The data analysis was performed by Inoue Hajime. The first draught of the manuscript was written by Inoue Hajime, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethical approval All procedures involving human participants performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and the 1964

Helsinki Declaration and its later amendments or comparable ethical standards. This study was reviewed and approved by the Technical Advisory Group of the Extraordinarily Certified Committee for Regenerative Medicine of Yukeikai (ref. No. NA8200002) recognised by the Safety of Regenerative Medicine Act regulated by the Ministry of Health, Labour, and Welfare (Approval No. PB3210086).

Consent to participate Informed consent was obtained from all the patients for the inclusion of their data (clinical and patient-reported outcomes) in the publication.

Consent for publication The authors affirm that the human research participants provided informed consent for the publication of the images in Figs. 3 and 7.

Competing interests Hirose Yoshie, Fujita Chiharu, Kamikawa Mayuko, and Inoue Hajime declare no competing interests.

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