Title page

Title:

Epidermal grafting for wound healing: A review on the harvesting systems, the ultrastructure of the graft and mechanism of wound healing

Authors:

Muholan Kanapathy[1,2] *MD MRCS MSc*

Nadine Hachach-Haram[2] *BSc MBBS MRCS*

Nicola Bystrzonowski[2] *MBBS MRCS MSc*

John T. Connelly[3] *PhD*

Edel A. O'Toole[3] *MB PhD RFCP*

David L. Becker[4, 5] *PhD*

Afshin Mosahebi[1, 2] *FRCS(Plast) PhD MBA*

Toby Richards[1,2] *FRCS MD*

Affiliations:

[1]Division of Surgery & Interventional Science, University College London, London, United Kingdom

[2]London Wound Healing Group, Department of Plastic and Reconstructive Surgery, Royal Free NHS Foundation Trust Hospital, London, United Kingdom

[3]Centre for Cell Biology and Cutaneous Research, The Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK

[4]Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore

[5]Institute of Medical Biology, A*Star, Immunos, Biomedical Grove, Singapore

Corresponding author:

Mr Muholan Kanapathy,

Clinical Research Fellow,

Division of Surgery and Interventional Science, University College London,

Fourth floor, 74 Huntley Street, London, United Kingdom, WC1E 6AU

Telephone number: +44207 6796454

Email: muholan.kanapathy.13@ucl.ac.uk

Author to request for reprints:

Professor Toby Richards,

Professor of Surgery and Consultant Vascular Surgeon,

Division of Surgery and Interventional Science, University College London,

Fourth floor, 74 Huntley Street, London, United Kingdom, WC1E 6AU

Telephone number: +44207 6796454

Email: toby.richards@ucl.ac.uk

Short running title

Epidermal grafting for wound healing

Key words

Epidermal graft, healing mechanism, skin graft, ultrastructure, wound healing

Key messages

- The superficial nature of epidermal graft enables it to be performed in a relatively pain free manner in an outpatient setting with minimal or no donor site morbidity, however little is known about the precise mechanism of healing by epidermal graft.
- This paper aims to explore the evolution of the harvesting systems, the ultrastructure of the epidermal graft and the current hypotheses on the mechanism of wound healing by epidermal graft.
- The healing by epidermal graft is influenced by the interplay of three main mechanisms: keratinocyte activation, growth factor secretion and re-epithelialisation from the wound edge.
- We explained how these processes work and integrate to promote wound healing based on the current in vivo and in vitro evidence and reviewed the ongoing clinical trials.

Abstract

Epidermal grafting for wound healing involves the transfer of the epidermis from a healthy location to cover a wound. The structural difference of the epidermal graft in comparison to the split thickness skin graft and full thickness skin graft contributes to the mechanism of effect. Whereas skin grafting is an epidermal transfer little is known about the precise mechanism of wound healing by epidermal graft. This paper aims to explore the evolution of the epidermal graft harvesting system over the last five decades, the structural advantages of epidermal graft for wound healing and the current hypotheses on the mechanism of wound healing by epidermal graft. Three mechanisms are proposed: keratinocyte activation, growth factor secretion and re-epithelialisation from the wound edge. We evaluate and explain how these processes work and integrate to promote wound healing based on the current in vivo and in vitro evidence. We also review the ongoing clinical trials evaluating the efficacy of epidermal graft for wound healing. Epidermal graft is a promising alternative to the more invasive conventional surgical techniques as it is simple, less expensive, and reduces the surgical burden for patients in need of wound coverage.

Introduction

Epidermal grafts (EG) for wound healing involves the transfer of the epidermal layer from an area of healthy skin to the wound bed. The EG are harvested by applying continuous negative pressure on the donor site to promote blister formation. The roof of the blister, which is the epidermis, is then excised and transferred to the wound. The superficial nature of the graft enables this autologous skin grafting to be performed in a relatively pain free manner in an outpatient setting with minimal or no donor site morbidity (1, 2).

The EG has been reported to behave more like a tissue engineered skin graft or a cultured keratinocyte sheet, which stimulates the wound to regenerate by itself rather than to provide instant wound coverage as seen with FTSG and SSG (3, 4). Cultured keratinocytes have been used for resurfacing burn wounds and in the treatment of skin ulcers since the 1970s (5). However, the clinical application of the cultured keratinocytes has been limited by the short-term and long-term results: variable graft take rate, limited mechanical resistance, hyperkeratosis, scar contracture, ulceration and blister formation due to reaction towards foreign fibroblasts in feeder media (6-8). These results, accompanied by the long culture time (typically requiring three to four weeks), the fragility of the sheets, and the high cost, has limited the use of this technique to only specialised facilities (9).

Newer methods developed to overcome these drawbacks, include pre-confluent keratinocytes combined with various delivery systems such as dermal substitute (9), polymer matrix (10, 11), fibrin glue suspension (12), and aerosol spray (13) as well as co-culture with melanocytes (9) require advanced logistics and handling capacity which involves clean room facilities and the use of clinical-grade reagents that are compliant with the Advanced Therapy

Medicinal Products (ATMPs) guideline (9). Similar challenges are faced by tissue engineered skin grafts, which are often not easy to handle, lack durability, are expensive and not available off-the-shelf (14).

EGs are advantageous as they do not require a carrier system, additional culture time, or a specialised facility. We have previously reported good clinical results with the use of EGs for wound healing in the outpatient setting with over two thirds of patients achieving successful wound healing within six weeks (1). Comparable clinical outcomes were reported by several other groups (3, 4, 15). However, little is known about its mechanism of healing.

The goal of this review is to explore the mechanism of healing by EG. We will first highlight the evolution of the harvesting system over the last five decades and the structural advantages of EG, before exploring the current hypothesis on its healing mechanism. We end with proposing the possible models to study the mechanism of healing by EG along with an overview on the ongoing clinical trials aimed at evaluating the efficacy of EG for wound healing.

EG harvesting systems

Various EG harvesting devices were used over the last fifty years with clear refinement in technology over the years (1, 16-19). The three harvesting systems which were most commonly used to harvest EG were the Dermovac system (Oy Instrumentarium, Helsinki, Findland), the syringe system, and the CelluTome Epidermal Harvesting System (Acelity, San Antonio, Texas) (Figure 1). These devices rely on the same principle of applying continuous

negative pressure onto healthy skin to promote blister formation, although they vary in the amount of the negative pressure generated and the size of graft harvested.

The earliest device used was the Dermovac, first developed by Kiistala in 1968, which enabled separation of the epidermis from the dermis using purely mechanical forces without causing any chemical or thermal damage(19). This device consisted of a transparent plexiglass suction cup and a hand pump that generated negative pressure of 250-300 mmHg with a blistering time of about 1-2 hours (Figure 1a). The suction cup was equipped with an adapter plate, which allowed the user to determine the number and size of blisters to be harvested. The suction blisters were then excised separately by the surgeon and transferred to the site of interest. Smaller grafts were more convenient as larger grafts tended to curl at the edges, making the transfer challenging (20). The long harvest time and the size of the equipment meant the techniques did not gain popularity (21).

The EG harvesting system more commonly associated with EG employs syringes (21-23). The syringe system was simple, comprising a syringe with the piston removed, placed onto the skin then suction applied through the nozzle. This could be simply achieved by a three-way connector linked to a larger syringe, which had two to three times the suction capacity of the smaller one (Figure 1b). The syringe system had a blistering time of one hour and raised blister measuring 1.5 cm in diameter which required surgical excision for transfer (21-23). Variations on the system include use of a smaller syringe or subepidermal local anaesthesia infiltration (23). However, the reliability of the syringe system is dependent on numerous patient and environmental factors(15). Furthermore, its clinical applicability has been limited by the long harvest time, the requirement for repeated grafting due to the small graft size as well as being tedious with inconsistent blister formation (15).

The most recent harvesting system, which has been commercially developed, is the CelluTome Epidermal Harvesting System (3, 4, 15). This system consists of an automated harvester, a vacuum head and a control unit (Figure 1c). It combines negative pressure of 400-500 mmHg and temperature of 40°C, allowing 128 micro-blisters (each of 2mm diameter, 2mm apart) to be raised within 30 minutes(1). The harvester is equipped with an in-built blade to excise the roof of the blister and the EG is then transferred by use of an adhesive dressing to the designated wound site. Being an automated device, it ensures consistency in the graft size and number. In contrast to the previous devices, the shorter harvest time of the CelluTome Epidermal Harvesting System comes from the high negative pressure, which is applied concurrently with the thermal energy of 40°C and its design which harvests an array of micro-blisters (15). It also offers painless graft harvest without anaesthesia, which is easily performed in the outpatient and community setting due to the straightforward nature of the procedure (3, 4). Serena et al highlighted several advantages of this technique in resource-poor setting, including simplicity, affordability, reproducibility, efficiency and the capacity of non-surgically trained clinician to perform the procedure (4).

Histology of EG

The epidermis is the upper most layer of the skin. The EG harvesting systems separate the epidermis from the dermis at the dermal-epidermal junction (DEJ) while preserving the histological architecture of the epidermis(19). Ultrastructurally, the DEJ consists of four zones (Figure 2): first, the membrane of the basal keratinocytes which contains hemidesmosomes; second, the lamina lucida, an electron-lucent region as seen by electron microscopy, which

anchoring filaments traverse; third, the lamina densa, an electron-dense area as seen by electron microscopy; and fourth, the sub-basal lamina which contains anchoring fibrils(24). The anchoring filament links the basal keratinocytes to the lamina lucida while the anchoring fibrils link the lamina densa to the underlying dermal matrix (24, 25). Histological study of the EG harvested from seven healthy volunteers showed that the separation is sub-epidermal, at the level of the sub-basal lamina, with a well-defined basement membrane lining the blister (26). Immunohistochemical staining for collagen type IV, the primary component of lamina densa, further confirmed that the basement membrane components were contained within the micrografts (27, 28).

Electron microscopic analysis of the EG harvested from healthy volunteers using Dermovac at -200 mmHg within 90 to 120 minutes revealed that the ultrastructure of the epidermis is preserved, although vacuoles were seen within the keratinocyte cytoplasm (29). Similar finding of vacuoles within the cytoplasm was observed in another study analysing EGs harvested using the syringe system (30). Despite the presence of vacuoles, the nuclear membrane remained intact (30). Furthermore, the epidermal cells were found to be viable in a study that analysed EG harvested from healthy volunteers using the CelluTome Epidermal Harvesting System, which demonstrated the presence of Ki67 stained proliferative cells at the basal layer of the grafts (27). The presence of the nuclear protein Ki67, which is expressed in cycling cells (G1, S, G2, and M phases) and absent in resting G0 cells, indicates that the proliferative potential of the EG is preserved upon separation (31).

The separation at the DEJ can be accelerated by heat, with the temperature ranging between $40^{o}C$ to $45^{o}C$ being reported as the optimal temperature for rapid suction blister formation (32). In a systematic review on the suction blistering time, skin temperature was identified as

the strongest predictor for the blistering time, indicating that the DEJ loses its strength with the increasing temperature due to temperature related detachment of the hemi-desmosomes and/or the inflow of blister fluids (33). The ability of the CelluTome Epidermal Harvesting System to raise blisters in a short period of time is due to negative pressure coupled with a temperature of about 40°C.

Mechanism of wound healing by EG

The separation at the DEJ maintains the entire ultrastructure of the epidermis, constituents of which contributes to its unique wound healing mechanism. The healing by EG is influenced by the interplay of three main mechanisms: keratinocyte activation, growth factor secretion and re-epithelialisation from the wound edge (Figure 3). Each of these mechanisms will be explored in detail in this section.

Keratinocyte activation and migration onto the wound bed

The first of these mechanisms is the activation of the basal keratinocytes within the EG. Whilst, keratinocyte activation in response to epidermal injury has been well reviewed (please see references (34, 35)) keratinocyte activation within EG, has not. Keratinocyte activation within EG was proposed to occur in addition to the well understood phases of skin graft healing: plasmatic imbibition, inosculation, and revascularization (36). The direct interaction between the basal keratinocytes within the EG and the wound bed contributes to this additional phase that is not seen in FTSG and SSG, which instead have a layer of dermis that

interacts with the wound bed (36). This phase was proposed based on the pronounced expression of Ki67 (marker of cell proliferation) and β_1 integrin subunit (a putative keratinocyte stem cell marker) in the basal keratinocyte layer and on the wound bed after epidermal grafting (36). Both the Ki67 and β_1 integrin was seen in the first week post grafting and disappeared at the fourth week, suggesting that the keratinocyte activation phase begins as part of the inosculatory phase and persists into the early stages of the revascularisation phase. The activated phenotype is also marked by changes in the cytoskeleton and increased expression of the cytoskeletal keratins involved in re-epithelialisation, namely KRT6, KRT16 and KRT17 (34, 35).

Arguably, keratinocyte activation could potentially be initiated upon separation of the EG from the DEJ during the graft harvest. As seen in epidermal injury, the exposure of the keratinocytes to their surrounding initiate the keratinocytes activation cycle(34). This activation process is achieved by the expression of several cytokines, with interleukin-1 (IL-1) being the most common initiator (34, 35). This cytokine, which is present in the cytoplasm of the keratinocytes in an unprocessed form, is converted by cellular injury to a processed form and released extracellularly, enabling the surrounding cells to perceive the injury (37). The IL-1 serves as an autocrine signal to activate the surrounding keratinocytes and as a paracrine signal to the dermal fibroblasts, enhancing their migration, proliferation, and production of dermal extracellular components (34, 38, 39). The other common initiator of keratinocyte activation is the pro-inflammatory cytokine, tumor necrosis factor-α (TNFα) (40). Similar to IL-1, TNFα acts in an autocrine fashion to stimulate keratinocyte migration, and in a paracrine fashion activating fibroblasts(40).

The activated keratinocytes are mitotically active and are capable of outgrowth from the multiple small epidermal islands onto the wound bed (27). The proliferative capacity of these small islands is immense, as exemplified by the ability of Cultured Epidermal Autografts (CEA), harvested from a small area, to rescue patients with burn wounds over 30% of their total body surface area (41). As the keratinocytes migrate away from the EG, these hyper-proliferative, migratory keratinocytes secrete components of basement membrane into the microenvironment of the wound bed(34). EG from healthy donors cultured in vitro synthesized and secreted components of basement membrane, whereby fibronectin, laminin 332 and type IV collagen were prominently stained at the expanding peripheries of the epidermal islands compared to the terminally differentiated upper layers of the epidermis (42, 43). This suggests that keratinocytes deposit basement membrane components on the wound which assist in the anchorage and migration of the keratinocytes(44). This ability of keratinocytes to secrete products of basement membrane and extracellular matrix is being exploited in efforts at producing tissue engineered skin grafts(45). These cell-derived matrices are advantageous for bioengineering as they are entirely cell-type specific and are processed and deposited onto the surface containing a full portfolio of ligands such as growth factors and proteoglycans(45). The synergy between the extra-cellular matrix and cytokines plays a pivotal role in the regulation of keratinocyte proliferation during re-epithelialisation.

Expression of cytokines to activate wound bed

Activated keratinocytes are the principle source of cytokines in the epidermis (46). The cytokines secreted can be broadly divided by their biological activities into three categories: growth factors, interleukins, and colony stimulating factors (46-48). The production of these

cytokines are mediated by the change in cell cycle, cell-differentiation state, a wide range of biological and physiological agents, and even the cytokines themselves (46).

A number of growth factors, including epidermal growth factor (EGF), transforming growth factor alpha (TGFα), heparin-binding EGF, and keratinocyte growth factor, are known to stimulate keratinocyte motility and proliferation in a wounded epidermis (34, 48). EG harvested from three healthy donors and cultured in vitro for seven days have been shown to secrete vascular endothelial growth factor (VEGF), transforming growth factor-α (TGF-α), platelet-derived growth factors AA (PDGF AA), platelet-derived growth factors AB/BB (PDGF AB/BB), hepatocyte growth factor (HGF), and granulocyte colony-stimulating factor (G-CSF)(27). These growth factors are known to modulate wound healing response and are able to stimulate endogenous process of wound healing (3). Such benefit is seen even with allogenic cell therapy that has shown impressive therapeutic value in wound healing (49). The allogenic cells, despite not attaching and covering the wound permanently, release growth factors, dermal extracellular matrix and basement membrane components to accelerate epithelialisation from the wound edge and promote granulation formation from the wound bed (49).

It is known that growth factors in combination are more stimulatory for wound healing in vivo than topical application of isolated growth factor therapy (47). However, the combination of growth factors has to be tailored to the needs of the wound at any given time. This points to the benefit of EGs, which have the potential to deliver a cocktail of growth factors continuously in keeping with the stage of healing.

Stimulation of wound edge keratinocytes

Given the potent mitogenic and motogenic effects of the many growth factors, it is likely that the EG enhances wound edge keratinocytes to proliferate and migrate into the wound, stimulating re-epithelialisation from the wound edge (48, 50). Several authors have reported that the EG do not exhibit graft take on the underlying wound bed, however observed re-epithelialisation occurs from the wound edge, dubbed the 'edge effect' (3, 51). Gabriel et al. and Serena et al., on the other hand, reported visible graft take and subsequent re-epithelialisation from the wound edge as well as from within the wound bed when the EG exhibited graft take (4, 15). Costanzo et al similarly reported graft take in 8 out of 29 cases but highlighted that the major effect appears to be the stimulation of re-epithelialisation from the wound edge (51).

For re-epithelialisation to occur from the wound edge, keratinocytes must first disassemble their cell-cell and the cell-substratum adhesion. Numerous regulators modulate the proliferation and migration of keratinocytes during epithelialisation (35). A key event in breaking the polarity between the tightly organised epithelial cells is the loss of epithelial junctions, mediated by the downregulation of the tight and adherens junction proteins, zonula occludens 1 (ZO-1) and E-cadherin, respectively. These molecules are the transmembrane proteins, which mediate cell-cell interaction and communication (52). These transmembrane proteins are known to be co-localized and co-assembled in a multiprotein complex with the gap junctional protein, Connexins, especially Connexin 43, the most ubiquitous Connexin in the epidermis (Figure 4) (52). Connexins play a vital role in the migratory property of keratinocytes in addition to other physiological processes, which includes cell differentiation, proliferation, electrical transmission and inflammation(53, 54).

Furthermore, Connexins form the centre of a protein complex or “nexus” acting as a master gene that can influence the expression of over 300 other genes at the transcriptional level (55). The cytoplasmic tail of Connexin 43 is associated with actin cytoskeletal proteins via E-cadherins, ZO-1, α- and β-catenin, either directly or via adaptors (52, 54). These interactions affect both the cell adhesion and cytoskeletal dynamics and therefore the cell migration and wound healing. In acute wounding, Connexins are downregulated about 6 hours after injury which correlates with the keratinocyte adopting a migratory phenotype as they start to crawl across the wound bed to re-epithelialise the wound (53). The upregulation of Connexin 43, Connexin 30 and Connexin 26 at the wound edge, as seen in chronic wounds, is known to reduce the migratory activity of keratinocytes and fibroblasts due to the substantially increased adhesion between cells (56, 57).

The modulation of the gap junctional proteins by growth factors and cytokines has been reviewed extensively by Schalper et al (58). The growth factors expressed by the EG are likely to downregulate Connexins at the wound edge, initiating keratinocyte migration. Although the exact type and concentration of growth factors expressed by the EG in vivo is yet to be outlined, the concentration of growth factors expressed by the grafts in vitro suggests that it is likely sufficient to modulate the gap junctional proteins at the wound edge (27, 58).

Models to study wound healing mechanism of EG

There is currently a paucity of data on the precise in vivo wound healing mechanism by the EGs. As EGs stimulates both the wound edge and wound bed to regenerate, analysis should

involve tissues taken from these two locations. This could be performed by taking tissue biopsies prior to treatment and repeated again at week 1 post treatment or done repeatedly at several fixed intervals throughout the treatment. The skin biopsies taken at the wound edge can confirm the activation and proliferation of the keratinocytes upon grafting. This can be done by observing the morphologic changes of the keratinocytes by a simple haemotoxylin and eosin (H&E) staining as well as by immunostaining for proliferative markers and gap junctional proteins. The morphological changes and the downregulation of the gap junctional proteins can confirm the change of the keratinocytes into a migratory state (56, 59). Tissue biopsy from the wound bed, on the other hand, will be able to confirm the activation of the wound bed and the presence of components of the basement membrane. Furthermore, staining for keratinocyte markers, such as KRT5, KRT6 and KRT14 can confirm the presence of the graft on the wound bed (60), as several studies have reported that graft take was not clinically visible in most cases (15, 51). Besides tissue biopsy, non-invasive investigation such as the analysis of wound fluid collected throughout the treatment will be able to provide invaluable information on the expression of cytokines and growth factors (61). As well as confirming the type and concentration of growth factors expressed, this will provide insight into the changes in expression with treatment.

Several clinical trials are currently underway to investigate the efficacy of EG in the clinical setting using the Cellutome Epidermal Harvesting System. We are currently undertaking a randomised controlled trial to evaluate the efficacy of EG against SSG (EPIGRAAFT Trial) (62, 63). This trial will also include mechanistic analysis to further understand the difference in the mechanism of wound healing between the two techniques. Another large randomised multicentre controlled trial is comparing the safety and effectiveness of EG combined with multi-layered compression therapy for the healing of venous leg ulcers (64). Similarly, the

effectiveness of EG for chronic wounds in the outpatient setting is being investigated by a non-randomised study which compares EG against SSG from historical controls (65). Besides chronic wounds, the efficacy of EG for wounds secondary to inherited connective tissue disease, epidermolysis bullosa, is also being evaluated (66). The findings from these high quality trials will define the efficacy of this technique and further improve our understanding of the mechanism of healing by EG.

Conclusion

EG for wound healing holds promise as a potential alternative to the more invasive conventional surgical techniques as it is simple, inexpensive, and reduces the surgical burden for patients in need of wound coverage. The increased number of publications in the last couple of years testifies the growing clinical popularity of this technique as a form of autologous skin grafting in the outpatient setting. In this review, we have highlighted the possible mechanisms of wound healing by EG based on the current in vitro and in vivo evidence. However, more work needs to be done to better understand the mechanism of healing at the cellular level in order to propose an evidence based clinical pathway.

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Competing interests

The author declares that they have no competing interests or financial disclosures.

Authors' contributions

MK, NH, NB, JTC, EAO, DLB, AM and TR jointly contributed to the conception and design of the study. MK performed the literature review and drafted the manuscript. NH, NB, JTC, EAO, DLB, AM and TR revised this manuscript. All authors read and approved the final manuscript and are accountable for all aspects of the work.

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Figure 1

Epidermal graft harvesting systems. (A) The Dermovac system which consists of a pair of transparent plexiglass suction cups and a handheld pump. (B) The syringe system which consists of a small syringe with the piston removed and connected to a larger syringe via a three-way connector. The three-way connector is locked to maintain the negative pressure throughout the procedure. (C) The CelluTome Epidermal Harvesting System which consists of a control unit connected to a vacuum head.

Figure 2

Ultrastructure of DEJ and blister cavity. The DEJ consists of four zones: membrane of the basal keratinocytes, lamina lucida, lamina densa, and sub-basal lamina. Hemidesmosomes, present at the dermal pole of the basal keratinocytes, link to anchoring filaments that connect the basal keratinocytes to the lamina lucida. Achoring fibrils link the lamina densa and the dermal matrix. Continuous negative pressure forms a blister at the level of sub-basal lamina.

Figure 3

Mechanism of healing by epidermal graft (EG). (A, B, C) The aerial view of four EGs on a healthy wound bed. (D, E) The cross-sectional view of an EG on a wound bed. Upon grafting (B), the keratinocytes within the EGs are activated and migrate onto the wound bed (yellow arrows resembles keratinocyte migration). The activated keratinocytes concurrently secrete growth factors to the wound bed to stimulate endogenous process of wound healing (E) (green arrows resembles growth factor expression). The activated keratinocytes and the growth factors stimulate the wound edge keratinocytes to migrate into the wound, accelerating re-epithelialisation from the wound edge (C) (blue arrows resembles the migration of the wound edge keratinocyte into the wound).

Figure 4

The structural organisation of the gap junctional protein, the Connexin. Each Connexin is made of a paired hemi-channel known as a Connexon, which consists of six Connexin protein sub-units. Each Connexin protein subunit has four alpha-helical transmembrane proteins, two extracellular loops, a cytoplasmic loop, and a N- and C-terminus located within the cytoplasm(52). The C-terminus binds to cytoskeletal elements within the cells to regulate cellular migratory properties(52).