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The potential of human induced pluripotent stem cells for modelling diabetic wound healing *in vitro*

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Abstract

Impaired wound healing and ulceration caused by diabetes mellitus, is a significant healthcare burden, markedly impairs quality of life for patients, and is the major cause of amputation worldwide. Current experimental approaches used to investigate the complex wound healing process often involve cultures of fibroblasts and/or keratinocytes *in vitro*, which can be limited in terms of complexity and capacity, or utilisation of rodent models in which the mechanisms of wound repair differ substantively from that in humans. However, advances in tissue engineering, and the discovery of strategies to reprogram adult somatic cells to pluripotency, has led to the possibility of developing models of human skin on a large scale. Generation of induced pluripotent stem cells (iPSC) from tissue donated by diabetic patients allows the (epi)genetic background of this disease to be studied, and the ability to differentiate iPSC to multiple cell types found within skin may facilitate the development of more complex skin models; these advances offer key opportunities for improving modelling of wound healing in diabetes, and the development of effective therapeutics for treatment of chronic wounds. (178 words)

Perspectives

- The cost of diabetic wounds to the National Health Service is estimated to be around £5 billion per year.
- Rodent models of wound healing fail to recapitulate human wound repair, and *in vitro* systems using human skin cells can be limited in terms of complexity and capacity
- Reprogramming of cells derived from human donor tissue into pluripotent stem cells may allow the development of fully translational skin models of diabetic wound healing, facilitating the drug discovery process.

Abbreviations Cardiomyocytes (CM); Cluster of differentiation (CD); Checkpoint kinase 1 (CHK1); Clustered regularly interspace short palindromic repeats (CRISPR); CRISPR associated protein 9 (Cas9); Diabetic cardiomyopathy (DCM) Hair follicle-associated-pluripotent (HAP); Human leukocyte antigens (HLA); Human skin equivalents (HSE); Human umbilical vein endothelial cells (HUVECs); Induced pluripotent stem cells (iPSCs); Krueppel-like factor-4 (Klf4); Laminin subunit beta 3 (LAMB) gene deficient Junctional Epidermolysis Bullosa (JEB); Maturity Onset Diabetes of the Young (MODY); Mesenchymal stem cells (MSC); Maternal inherited diabetes and deafness (MIDD); Myc proto-oncogene protein (c-Myc); Non-obese diabetic (NOD); Octamer-binding transcription factor 4 (Oct4) Platelet-derived growth factor (PDGF); Recessive Dystrophic Epidermolysis Bullosa (RDEB); Reduction, refinement and replacement (3Rs); Retinal pigment epithelial (RPE); Sex determining region Y-box 2 (Sox2); Transcription activator-like effector nuclease (TALEN) Transforming growth factor- β (TGF- β); Type 1/2 diabetes mellitus (T1/2DM); Zinc finger nucleases (ZFNs).

Introduction

Diabetes mellitus is a major global health concern, affecting around 425 million adults worldwide, a figure predicted to rise to 629 million people with diabetes by 2045 (International Diabetes Federation). Type 1 diabetes mellitus (T1DM) is an auto-immune disorder, caused by destruction of insulin-producing pancreatic β -cells [reviewed in 1], while type 2 diabetes mellitus (T2DM) is a polygenic condition characterised by loss of insulin secretion and sensitivity, strongly influenced by environmental factors such as obesity [2]. Single gene disorders, such as neonatal diabetes, Maturity Onset Diabetes of the Young (MODY), and mitochondrial mutations (m.3243A>G), can trigger diabetes in younger age groups [3]. Diabetes mellitus also presents in a number of rare genetic conditions (congenital lipodystrophies [4], Huntington's disease [5], Friedreich ataxia [6], and Turner syndrome [7]), genetic obesity (Prader-Willi syndrome [8], Alstrom syndrome [9] and premature aging conditions such as Werner [10] and Hutchinson-Gilford progeria [11] syndromes. It is a complex metabolic disorder, diagnosed clinically by hyperglycaemia [12] and associated with chronic inflammation, pro-coagulability, impaired fibrinolysis and macro- and microvascular defects, promoting cardiovascular disease (coronary heart disease and stroke) [13], renal dysfunction [14, 15], retinopathies [16], neuropathies [16, 17] and impairment of wound healing in the extremities [17-20]. Foot ulceration is major cause of morbidity in type 1 and type 2 diabetes, a significant healthcare burden, and results in markedly impaired quality of life for the patient. Globally, the prevalence of diabetic foot ulceration in 2017 was 6.3% (95% CI 5.4 to 7.3%) [20] and diabetes is estimated to cause loss of a lower limb (or part thereof) to amputation every 30 seconds worldwide (International Diabetes Federation).

The healing of diabetic wounds has traditionally been investigated using animal models, immortalised cell lines and primary cells. Rodents are often used to examine the complex healing process *in vivo*, but do not fully recapitulate the wound repair process in humans [21, 22]. Moreover, the requirement for reduction, refinement and replacement (3Rs) of animal models, and the severity of the procedures involved, mean that a valid replacement is ethically desirable. The use of human primary cells from dermal biopsies to study wound repair *in vitro*, particularly in 3-dimensional (3-D) organotypic models, has provided valuable insights into the wound healing process: for example, the incorporation of diabetic patient-derived fibroblasts into a 3-D model accurately replicated key features associated with chronic ulcers *in vitro* and *in vivo* [23]. This article will review these approaches, and

highlight recent advances in reprogramming of adult somatic cells to pluripotency, which offer exciting opportunities to develop improved models for diabetic wound healing.

Wound healing: acute and chronic wounds

Wound healing involves four distinct, but overlapping phases: haemostasis, inflammation, proliferation and remodelling (**Table 1**) [24-26]. Wound clotting is followed by re-epithelisation at the wound edge around 12-18h post-wounding; concomitantly, a granulation tissue is formed by dermal fibroblasts at the wound margin, some of which convert to contractile myofibroblasts. Bone-marrow derived mesenchymal stem cells contribute to granulation tissue which contains a dense network of capillaries, and contraction reduces the surface area requiring re-epithelisation [22-26]. Neutrophils are recruited to the site of wounding, to kill microorganisms, followed by macrophages which phagocytose cell and matrix debris; these inflammatory cells then either die or leave the site of injury, aiding final resolution of the wound.

Chronic (non-healing) wounds in diabetic patients are characterised by persistent, unresolved inflammation, mediated predominantly by infiltrating neutrophils, with a significant impairment in local bacterial invasion control. Diabetic conditions, such as inflammation, hyperglycaemia and hyperlipidaemia induce epigenetic changes, promoting an inflammatory macrophage phenotype [27] which fails to transition to a pro-healing phenotype within the local wound environment [28]. Keratinocytes at the epidermal edge are hyper-proliferative, adjacent to an ulcer base which contains exudate and necrotic debris; dermal fibroblasts appear senescent and recalcitrant to the migratory stimulant, transforming growth factor- β (TGF- β), and few myofibroblasts are present. Instead of granulation tissue, vessels are surrounded by fibrin cuffs and do not form an effective network, rendering the wound poorly vascularised [26, 29]. Advanced glycation end-products act in concert with inflammatory mediators, and commit fibroblasts and vascular cells to apoptosis, contributing to the demise of granulation tissue [30].

Primary cells, immortalised cell lines and skin explants

The simplest cell-based model of wound healing *in vitro* involves culturing a monolayer of primary fibroblasts, keratinocytes or immortalised cells (e.g. HaCaT keratinocytes) and introducing a 'scrape' across the surface using a sterile pipette tip: the 'scratch' wound assay [31, 32, 33]. Migration of cells across the wound can be measured using static or time-lapse microscopy, and the expression of proteins involved in inflammation, migration,

proliferation, differentiation and remodelling of the extracellular matrix investigated (**Table 1**). Variability can be introduced into the system through changes in oxygen tension, the addition of drugs, growth factors or cytokines, or the use of extracellular matrices and specialised culture surfaces. These models can also be used to examine differences between healing and non-healing wounds, using normal and diabetic cells and altered tissue culture environments [23, 34-38].

Since the 1970s there has been increasing interest in generating skin constructs which are physiologically relevant, capable of mimicking barrier formation, immune and sensory functions [39, 40]. Co-cultures of keratinocytes with other cell types, including immune cells and dermal fibroblasts, are often used for investigations of wound healing, but these cannot entirely replicate the cell-cell and cell-matrix interactions found in a 3-dimensional environment. The use of dermal fibroblasts encapsulated in gel/matrix, combined with differentiated keratinocytes forming a 3-D epidermis, often at the air-liquid interface, can address some of these limitations, and are useful for modelling changes in keratinocyte function, cytokine release after drug treatment, metabolism, irritation and sensitisation, and skin aging [23, 41-46]. Tissue-engineered skin products have also been used clinically (e.g. Apligraf, Dermagraft) to aid wound repair, particularly to provide barrier function [47, 48]. However, the complexity of skin tissue, including the roles of a number of specialised cell types such as melanocytes, immune cells (macrophages, Langerhans cells, T cells, dendritic cells) and stem cell niches, and of skin appendages (hair follicles), sweat and sebaceous glands, obviously cannot be wholly recapitulated in cultures of just keratinocytes and/or fibroblasts.

Vascularisation can be achieved by the addition of endothelial cells, stimulated to form capillaries within the dermal environment, and to form a vascular network when supported by a suitable scaffold and perfusion system [49]. Other approaches have focused on the introduction of melanocytes, to study melanin transfer to keratinocytes, allowing studies of photoprotection and acquired (drug-induced) hyper-pigmentation [50], and the inclusion of hair follicles, sweat and sebaceous glands, to facilitate study of cosmetics, treatments for alopecia and sebo-regulating drugs [40]. The addition of immune cells, innervation of skin models, and introduction of the hypodermis (adipose tissue) are desirable for effective drug and allergen testing.

At present, however, it can be difficult to achieve some of these outcomes with primary cells derived from human skin biopsies, due to their limited availability and/or growth potential

[40, 50]. Tissue biopsies are small, making it possible to isolate and expand in culture only the most numerous cell types, i.e. dermal fibroblasts and epidermal keratinocytes [50], and a negative relationship exists between the extent of expansion of keratinocytes in culture, and their longevity in a tissue-engineered epidermis [51, 52]. Minority skin cell populations, such as Merkel cells, cannot be greatly expanded in culture [50] and many cell types found in skin, such as macrophages, are transient, entering the skin only in response to a pathological challenge. While the use of multiple donors is possible, this approach ignores individual responses, and can greatly complicate data analysis [50]. Immortalisation of primary cell lines can solve some of these problems [32, 33, 53], but the constitutive expression of oncogenes can markedly influence cellular phenotype and proliferation rate, which may limit their value for wound healing studies and negate their usefulness clinically.

Animal models in wound healing studies

Animal models have been used to replicate the complexity of wound healing *in vivo*. Mice are the most widely used species for *in vivo* studies, although other larger species, such as pig, more closely mirror human wound healing and are often used in pre-clinical trials [29, 54]. The epithelial architecture, extracellular matrix, vascular networks and innervation in porcine skin are similar to those in human skin, but swine are difficult to house in most vivariums, do not lend themselves easily to *in vivo* imaging experiments and are not fully characterized at cellular and physiological levels. Moreover, the availability of swine-specific reagents, such as antibodies and growth factors, may be restricted. Acute wound healing in rodents is commonly monitored following excisional (biopsy punch, surgical scissors or laser) or full thickness incisional (scalpel) wounds, made to the foot, thigh or back, although considerable variance exists as to the size and number of wounds per animal, the tools employed, the presence of occlusive dressings, splints, or non-occlusive bandages of varying types, and the use of sutures to close the wound margins [29, 54].

Chronic wound healing models involve introducing an acute wound, usually in a murine model, within the clinical context involved, such as diabetes or ischaemic injury [22, 29]. Type 1 diabetes mellitus can be induced by transgenic breeding (e.g. non-obese diabetic (NOD) mice), spontaneous autoimmunity, chemical ablation of pancreatic β -cells (streptozotocin) or viral infection [55, 56]. High fat feeding or genetic deletion is often used to induce a condition resembling T2DM in mice [56]: the most widely used models of T2DM are leptin (*ob/ob*) or leptin receptor (*db/db*) deficient rodents, which become obese around six weeks of age, and subsequently develop T2DM with marked delays in wound healing.

Polygenic diabetic strains, such as NONcNZO10/LtJ mice which model human metabolic syndrome and obesity-induced diabetes, have also been developed which exhibit defects in wound repair [56-58].

Despite the common use of animal models to study acute and chronic wound healing, physiological differences between species need to be considered [22, 59, 60]. Mouse and human skin have markedly different architecture, responsiveness and functionality, due to differences in thickness, hair density and appendages; murine skin is also largely devoid of sweat glands. Murine subcutaneous tissue has a thin layer of muscular tissue, the panniculus carnosus, which has substantive contractive potential; up to 90% of excisional wounds in mice close by contraction. By contrast, re-epithelization and formation of granulation tissue is the predominant mechanism of cutaneous wound healing in human dermis [22, 59, 60]. Attempts have been made to address this issue, by splinting excision wounds to more closely replicate wound healing in human skin; topical administration of platelet-derived growth factor (PDGF), for example, fails to promote closure of a splinted wound by re-epithelisation in db/db mice [61], despite its efficacy in other murine models of wound healing. While wound splinting does allow histologic monitoring of wound bed granulation, the healing process still does not fully mimic human wound healing [22, 59, 60]. Outcomes from wound healing studies in rodents may therefore not be fully translatable to humans, possibly explaining the dearth of effective treatments currently available.

An alternative *in vivo* approach involves the generation of humanised mouse models. Shunmugamet *al* (2015) grafted human skin biopsies from elective abdominoplasty surgery onto athymic (*nu/nu*) mice for three months, before inducing an excision wound into the graft, and monitoring wound healing using near infra-red fluorescent imaging [62]. Human skin constructs have also been transplanted onto immunodeficient mice: a 3-D matrix, enriched with fibrin from human blood plasma was seeded with human fibroblasts to form a dermis, before adding an epidermal layer using human keratinocytes [63]; the same group have also described a humanised model of delayed wound healing in streptozotocin-induced diabetic mice [64]. It is clear, however, that all of these approaches to the study of wound healing in animals are classified as 'severe', likely to cause pain and distress: an ethical imperative exists to develop an *in vitro* translational model of human wound healing.

Induced pluripotent stem cells

The concept of cell plasticity originates from the groundbreaking work of John Gurdon in the 1960s [65-67], culminating in the award of the Nobel Prize in Physiology or Medicine (2012) jointly with Shinya Yamanaka, more than forty years later; Yamanaka, together with Kazutoshi Takahashi, discovered how mature somatic cells can be reprogrammed to become pluripotent stem cells [68-70]. Induced pluripotent stem cells (iPSCs) can be derived from individuals with differing genetics, disease conditions or ethnic origins, and differentiated to cell types of the three germ layers, revolutionising research and drug discovery, and increasing the prospect of personalised regenerative medicine [68-74]. While early reprogramming strategies used retroviral and lentiviral vectors to deliver the transcription factors (e.g. Oct4, Sox2, Klf4, c-Myc) required to reprogram adult somatic cells to the pluripotent state [68-71], current reprogramming approaches avoid genomic integration [75], by utilising integration-free strategies such as Sendai virus, mRNA, microRNA, episomal vectors and non-nucleotide based methods [76-79] (**Figure 1**).

Induced pluripotent stem cells are characterised by the ability to self-renew indefinitely, stable karyotype, and the potential to differentiate into cell types of the ectoderm, mesoderm and endoderm [80-83]. Robust protocols are then needed to differentiate iPSCs into cells and tissues of the desired type, characterised by function and/or expression of key biomarkers, and the presence of the disease phenotype; disease mechanisms can then be investigated, or potential therapeutics tested [80-83]. Age and/or sex matched control donor material can be subjected to equivalent reprogramming and differentiation procedures; alternatively, isogenic controls can be generated by gene correction of the pluripotent cells, using clustered regularly interspace short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9), zinc finger nucleases (ZFNs) or transcription activator-like effector nuclease (TALEN) systems [84, 85].

The direct reprogramming and trans-differentiation of somatic cells to specialised cell types, including those involved in diabetes and wound healing, has also been described; this approach bypasses the pluripotent stage and may therefore prove safer for cell therapy [86-88]. Primitive stem cells, such as hair follicle-associated-pluripotent (HAP) stem cells resident in the skin, also have potential to be converted to keratinocytes and melanocytes for therapeutic epidermal regeneration without the risk of tumour formation [89]. These approaches do not, however, offer the same opportunities for expansion and banking as conversion to pluripotency.

Pluripotent stem cells and generation of human skin constructs *in vitro*

The ability to model the complex interactions of whole tissues and organs using entirely human biology is a major goal for tissue engineering and stem cell research, and may eventually lead to the production of whole organs for transplantation [90]. Indeed, dermatology may be the ideal context for application of iPSC-based therapies, as it is readily accessible and easy to monitor, and excision is possible if adverse side effects occur [91]. Many of these challenges are also relevant to the development of accurate representations of tissue for disease modelling, including skin for wound healing assays.

The generation of iPSCs from dermal fibroblasts, keratinocytes, melanocytes and dermal papilla cells is established, with reprogramming occurring at higher efficiency in keratinocytes and melanocytes than in fibroblasts [39, 40, 50]. Moreover, iPSCs have been derived from patients with genetic skin disorders, including Type VII collagen-deficient Recessive Dystrophic Epidermolysis Bullosa (RDEB), laminin subunit beta 3 (LAMB) gene deficient Junctional Epidermolysis Bullosa (JEB), Epidermolysis Bullosa simplex with a dominant R125C keratin 14 mutation, and from gene corrected RDEB fibroblasts [92-95]. Regeneration of human epidermis *in vivo* using iPSCs is yet to be achieved, but the potential of this approach was recently demonstrated by Hirsch *et al* (2017) who utilised autologous transgenic keratinocyte cultures to renew the epidermis in a seven year old child suffering from severe JEB [96]. In this case, the epidermis was sustained by a limited number of long-lived stem cells or holoclones, self-renewal of which provided progenitor cells capable of replacing terminally differentiating keratinocytes [96].

Notably, it seems that reprogrammed cells retain epigenetic features of the cell type of origin (although these disappear on continued passaging) and it has been suggested that this residual epigenetic memory may facilitate differentiation back to the corresponding original cell type [91, 97-99]. Intriguingly, the process of reprogramming down-regulates senescent pathways, elongates telomeres and restores mitochondrial function [100-102] effectively 'rejuvenating' the cellular phenotype; indeed, 'rejuvenated' fibroblasts can also be generated from iPS cells derived from very old patients, offering new strategies for treatment of chronic wounds in the elderly [91, 102].

Keratinocytes, fibroblasts and melanocytes have been successfully derived from human iPSCs, and iPSCs can be differentiated into immune cells (T-lymphocytes, macrophages and dendritic cells), endothelial and smooth muscle cells, Schwann cells and peripheral

neurons [39, 40, 50, 103-107] and iPSC-derived ectodermal precursor cells can contribute to hair follicle morphogenesis *in vivo* [108]. Thus, iPSC-derived skin cells have huge potential for skin tissue engineering, and for the development of *in vitro* skin models for healthy and diseased skin, and acute and chronic wound healing studies.

Human skin equivalents (HSE) have been generated using iPSC-derived fibroblasts and/or keratinocytes [106-109]. The transcriptome of iPSC-derived keratinocytes proved very similar to primary healthy human keratinocytes, while incubation of iPSC-derived keratinocytes at an air/liquid interface resulted in epidermal stratification and the development of a functional permeability barrier [109]. Itoh *et al* (2011) generated an *in vitro* 3-D skin equivalent, using a type I collagen matrix to support iPSC-derived fibroblasts in the dermis, and iPSC-derived keratinocytes to form an epidermis, and demonstrated normal morphology, stratification and terminal differentiation at the air-liquid interface [107]. Enriched complexity was achieved by Gledhill *et al* (2015), by introduction of iPSC-derived melanocytes, responsible for both skin colour and protection against ultraviolet radiation, into a 3-D HSE [50]. A collagen I matrix was seeded with iPSC-derived fibroblasts, to support iPSC-derived keratinocytes and melanocytes. The iPSC-derived melanocytes localised to the basal layer, extending dendrites into the suprabasal layer of the epidermis, and produced melanin which was taken up by iPSC-derived keratinocytes [50].

Endothelial and smooth muscle cells, differentiated from human iPSC, cooperate to enhance formation of tubular networks *in vitro*, and in athymic nude mice; co-transplantation of these cells markedly increased neovascularisation and wound healing in a murine dermal wound model [110] compared to primary somatic cells or implantation of differentiated endothelial cells alone. Vascularisation of HSE, comprised of primary neonatal dermal fibroblasts and keratinocytes, has also been achieved using iPSC-derived endothelial cells [39, 110]. The former study focused on developing an *in vitro* platform capable of recapitulating the cutaneous microcirculation, allowing perfusion and evaluation of endothelial barrier function [39]. A 3-D printing technology was used to create the desired patterns of vasculature, including inlet-outlet tubes for perfusion, from sacrificial microchannels of cross-linked alginate embedded in a dermis consisting of uncrosslinked collagen I gel and fibroblasts. Within this context, the diffusion barrier function provided by iPSC-derived endothelial cells proved similar to that provided by human umbilical vein endothelial cells (HUVECs) [39].

Finally, using a rather different approach Zhang *et al* (2015) developed patient-specific mesenchymal stem cells (MSC) from human iPSC, and isolated the exosomes released into the extracellular milieu [111]. Exosomes, positive for cluster of differentiation (CD) 9, CD63, CD8a, are nano-sized vesicles (30-100nm in diameter) that contain proteins, mRNA and micro RNA, and are thought to facilitate wound healing in a paracrine manner. In this study, exosomes derived from iPSC-MSCs enhanced the proliferation and migration of fibroblasts and human umbilical vein endothelial cells (HUVECs), increased the secretion of collagen and elastin and promoted the formation of tubular networks of endothelial cells *in vitro*; in a rat wound model, the introduction of iPSC-MSC derived exosomes enhanced wound repair, collagen synthesis and angiogenesis [111].

Thus, the technology exists for use of pluripotent stem cell-based systems in creating physiologically relevant and translatable 3-D models of human wound healing *in vitro*, to aid the development of novel therapeutics, identifying effective treatments and reducing attrition in the later and more costly stages of drug development. Some barriers exist, however, to widespread adoption of these models. Financial constraints may ultimately be addressed by optimisation of cell culture, reprogramming and differentiation protocols; the labour intensive nature of the work may also be resolved by high throughput automated approaches [112]. Other limitations include variability among iPSC cell lines [113] and genomic instability [114, 115]. The establishment of selection criteria for iPSC and iPSC-derived cells, such as cell-specific markers, assessment of proliferation rate and lifespan, and investigation of the transcriptome [112, 113], can help to minimise variability, while limiting replication stress during reprogramming, either genetically by targeting checkpoint kinase 1 (CHK1) or by using nucleoside supplementation, can help to reduce genomic instability [115]. The quality of 3-D skin constructs should also meet key criteria such as histological morphology, cell viability and barrier function [106-109].

Pluripotent stem cells derived from diabetic individuals: disease modelling

Induced pluripotent stem cells have been derived from individuals with monogenic forms of diabetes, and from patients with type 1 and type 2 diabetes mellitus, facilitating research into multiple aspects of the complex pathogenesis of these disorders (**Table 2**), although it is clear that the potential for utilisation of these cells in studies of diabetic wound healing is yet to be fully realised. The autoimmunity that arises in T1DM results from a complex interaction between genetic and immunologic factors [1]. Risk of T1DM progression is polygenic, with a large number of genes conferring small risk effects, and a small number of

genes having large effects, most particularly the human leukocyte antigens (HLA) DR/DQ alleles (e.g. DRB1*03-DQB1*0201 (DR3) or DRB1*04-DQB1*0302 (DR4) [1]. To date, genomic wide association studies have identified at least 75 independent genetic loci for T2DM although whether all of these variants are causal is not known, and their mechanism of action requires further clarification [2, 136, 137]. Further, most of the common variants identified confer a relatively low risk of T2DM (odds ratio 1.0 to 1.4) and explain only 10-15% of the heritability of this disease [136, 137]. These complex inheritance patterns highlight the importance of generating iPSCs from diabetic individuals to develop skin wound healing models *in vitro*, although they may effectively negate the possibility of generating isogenic controls.

Epigenetic changes, including those due to an early metabolic insult, are also critical in the development of T2D: changes in DNA methylation markers have been identified in blood samples and pancreatic islets from T2D patients [137]. At present, it is not clear whether these epigenetic changes would be retained during reprogramming to pluripotency, a process which induces metabolic changes, including a shift from oxidative to glycolytic metabolism of glucose. However, Harvey *et al* (2018) recently demonstrated retention of metabolic memory in human iPSC: in response to challenge with reduced oxygen concentration, iPSC cell lines did not respond appropriately, indicating that metabolism had not been functionally reprogrammed, failing to recapitulate the metabolic responsiveness of embryonic stem cells [138].

This may pose a significant problem in cell replacement therapy designed to correct defects in diabetic patients; for example, the generation of functional insulin-secreting pancreatic β -cells from iPSCs, for potential cell replacement therapy represents a key goal for treatment of type 1 and type 2 diabetes [139]. Induced pluripotent stem cells have been generated from fibroblasts from ulcerated skin of diabetic foot ulcer patients, and compared with those from non-ulcerated diabetic skin and from healthy individuals: all of the skin fibroblasts were reprogrammed to iPSC with similar efficiencies, indicating that even repair-deficient fibroblasts may be useful therapeutics for wound healing [134]. Human iPSC cells from Type 1 diabetic patients have also been differentiated into early vascular cells and mature endothelial cells which can assemble into 3-D networks when embedded in engineered matrices, incorporate into developing zebrafish vasculature, and may prove to be a useful in vascular repair for diabetic patients [133]. The challenges currently facing this type of

regenerative cell therapy, which are outwith the scope of this review, have been recently and comprehensively reviewed [73, 112-115, 140].

Arguably, however, retention of the diabetic epigenome, together with the presence of genetic variations which increase the risk of T2D, may facilitate the development of authentic models, including wound healing, of this disease *in vitro*. To date, dermal fibroblasts, from patients with the mitochondrial A3243G mutation, associated with maternal inherited diabetes and deafness (MIDD) have been used to generate heteroplasmic iPSC clones for studies of mitochondrial function [117]. Isogenic iPSC clones with either high levels of this mutation or undetectable levels of the mutation, from the same individuals; complex I activity, mitochondrial respiration and ATP production were compromised in some of the mutation-high clones, while those from the mutation-undetectable clones were similar to those of iPSC cells from healthy subjects [117]. Induced pluripotent stem cell clones derived from patients with the same mitochondrial mutation were differentiated into retinal pigment epithelial (RPE) cells [141]. The RPE cells contained morphologically abnormal mitochondria and melanosomes, and marked functional defects in phagocytosis of photoreceptor outer segments, facilitating dissection of the complex tissue-specific pathology associated with this mitochondrial mutation [141]. Insulin-resistant iPSC cells, derived from patients with genetic defects in the insulin receptor, have also been shown to exhibit altered mitochondrial size and function, and changes in cellular metabolism [127].

The power of disease modelling using human iPSC is clearly illustrated by Drawnel *et al* (2014), who utilised this approach to develop a patient-specific iPSC model and drug-screening platform for diabetic cardiomyopathy (DCM) [135]. Firstly, the authors developed a surrogate DCM phenotype; iPSC-derived cardiomyocytes (CM), which more closely resemble neonatal CMs, were induced to an adult pattern of metabolic activity in two differing experimental conditions. A maturation media, supplemented with insulin and fatty acids, which requires the cells to maintain ATP by fatty acid β -oxidation, was used to mimic the metabolic substrate of adult ventricular CMs, while the re-introduction of glucose, and hormonal mediators of diabetes (endothelin-1, cortisol) generated a pattern of gene expression associated with hypertrophic stress, and recapitulated the DCM phenotype *in vitro*. Secondly, iPSC cells were derived from patients with two extreme DCM phenotypes: fast progression to cardiovascular disease (within 5y of diagnosis of diabetes) and slow progression (no cardiovascular disease despite 15y of T2D). Dermal fibroblasts were reprogrammed to patient-specific iPSC with normal karyotype and differentiation potential;

the patient-specific iPSC-derived cardiomyocytes, cultured in the presence of maturation media, displayed a basal cardiomyocyte phenotype which corresponded to the clinical status of the original donor, in the absence of diabetic stimulus, suggesting retention of epigenetic factors. Suppression of the diabetic phenotype in the environmental model of DCM was then successfully employed as a screenable endpoint for small molecules which could rescue the phenotype of patient-specific cardiomyocytes [135]. An equivalent approach to modelling diabetic wound healing could prove equally insightful and valuable therapeutically.

Concluding remarks: challenges for the future

The development of robust protocols for reproducible derivation of iPSC from diabetic patients, and for differentiation of iPSC into the multiple cell types found within human skin, is increasingly being performed using chemically defined and animal-origin free cell culture conditions; this is key in limiting batch variability, reducing the use of animal models and animal-derived materials, and in improving models of human diabetic wound healing. These goals rely on the altruistic donation of tissue from patients, a process facilitated by creation of large Biobanks (or Biorepositories) associated with universities, research organisations and the National Health Service in the UK. Biobanks provide ethically donated human tissue (normal and diseased), and associated anonymised clinical data, for individual studies or to smaller research tissue banks (e.g. GCU Skin Research Tissue Bank: www.gcu.ac.uk/hls/research/researchgroups/gcuskinresearchtissuebank/), dedicated to the study of human diseases involving complex genetic backgrounds. The derivation of diabetic pluripotent cells, capable of indefinite self-renewal and expansion, and differentiation into multiple lineages, may provide new insight into disease mechanisms and hold considerable promise for the development of effective therapeutics for treatment of chronic wounds in diabetic individuals

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Table 1: Summary of the stages of wound repair

Stage	Main processes	Key molecules	REF
Haemostasis	Platelet-dependent vasoconstriction and fibrin clot formation	ADP, ATP, GDP, 5-HT P2Y ₁₂ receptor axis Integrin α IIb β 3 Von Willebrand Factor Fibrinogen	[24]
Inflammation	Release of cytokines Invasion of inflammatory cells (neutrophils, monocytes)	Transforming growth factor- β (TGF- β 1, β 2, β 3)/TGF β receptor I/II Platelet-derived growth factor (PDGF)	[22, 25,26]
Proliferation	Dermis Macrophages and fibroblasts release growth factors Migration of fibroblasts and proliferation Biosynthesis and deposition of matrix proteins Angiogenesis Epidermis Migration, proliferation and differentiation of keratinocytes Contributions from hair follicle stem cells/interfollicular epidermal stem cells	TGF- β Matrix metalloproteinases (MMPs) Fibronectin, type (III) collagen, type (I) collagen Vascular Endothelial growth Factor (VEGF)	[22, 25, 26]
Remodelling	Wound fibroblasts adopt a contractile myofibroblast phenotype Degradation and reorganisation of the extracellular matrix Apoptosis of a variety of cell types Wound contraction	TGF- β MMPs Type (I) collagen	[22, 25, 26]

Table 2: Establishment and utilization of iPSC cells from diabetic patients

Mutation	Patient characteristics	Uses	REF
Mitochondrial tRNA (A3243G)	Maternal Inhibited Diabetes and Deafness (MIDD)	Analysis of mitochondrial (dys)function associated with diabetes	[116, 117]
Glucokinase (GCK)	Maturity Onset Diabetes of the Young (MODY) 2	Defects in insulin production and gene correction.	[118]
Hepatocyte nuclear factor (HNF) 1A	MODY 3	Defects in insulin production	[118, 119]
Pancreatic and duodenal Homeobox 1 (PDX1)	MODY4	Generation of glucose-responsive insulin secreting cells	[120]
Heterozygous PDX1	MODY4	Pathogenesis of MODY4 and T2DM	[121]
HNF1B	MODY5	Pancreatic hypoplasia Development of pancreatic β -cells	[122] [123]
Heterozygous activating mutation in potassium inwardly-rectifying channel, subfamily J, member 11 (KCNJ11)	MODY13	Pathogenesis of MODY13	[124]
Arginine vasopressin (AVP) gene carrying an adFND1 causing variant in exon 1	Autosomal dominant familial neurohypophyseal diabetes insipidus (adFND1)	Pathogenesis of adFND1	[125]
Activating germline mutation in signal transducer and activator of transcription (STAT3)	Neonatal diabetes	Pancreatic endodermal development	[126]
Insulin receptor mutations	Genetic insulin resistance	Mitochondrial (dys)function	[127]
Loss of paternal gene expression in an imprinted (epigenetic) interval on 15q11.2-q13	Obesity (Prader-Willi syndrome)	Pathogenesis of Prader-Willi syndrome and associated morbidities	[128]
Lamin A/C (LMNA)	Hutchinson Gilford Progeria syndrome (HGPS) (accelerated aging,	Pathology of HGPS	[129]

	lipodystrophic insulin resistance)		
Berardinelli-Seip congenital lipodystrophy 2 (BSCL2/SEIPIN) mutations	Congenital generalized lipodystrophy	Defects in adipogenesis and pathology of human lipodystrophy	[130]
-	Fulminant type 1 diabetes	Cytokine-induced apoptotic reactions of β -like insulin producing cells	[131]
-	Type 1 diabetes	Expression of pancreas-specific microRNAs	[132]
-	Type 1 diabetes	Autologous vascular therapy	[133]
-	Diabetic foot ulcer Type 2 diabetes	Future regenerative therapies for diabetic foot ulcer	[134]
-	Type 2 diabetes	Disease modelling and drug screening for diabetic cardiomyopathy	[135]

Figure 1: Modelling of diabetic wound repair using induced pluripotent stem cells

Patient skin biopsies (diabetic and non-diabetic) can be used to supply frozen tissue or wax embedded sections for interrogation by Q-PCR, immunohistochemistry or immunofluorescence; alternatively, primary fibroblasts and keratinocytes isolated from the dermis and epidermis can be utilised directly in 2- and 3-dimensional studies of wound healing, or reprogrammed to pluripotency by delivery (viral, episomal plasmids, mRNA, microRNA) of combinations of transcription factors such as octamer binding transcription factor 4 (Oct4), Krueppel-like factor-4 (Klf4), sex determining region Y-box 2 (Sox2), Myc proto-oncogene protein (c-Myc), lin-28 homolog A (Lin28) or Nanoghomeobox (Nanog). Retention of epigenetic 'memory' can be assessed from gene expression profiles, persistence of donor-cell gene expression and ease of differentiation and both 'memory' and 'non-memory' iPSC which are capable of indefinite self-renewal and expansion can be banked; iPSC cell lines can also undergo targeted gene correction by CRISPR/Cas9, TALEN or ZFN systems. Established and developing protocols can be used to differentiate iPSC into a range of cell types found within human skin (fibroblasts, keratinocytes, melanocytes), and immune cells (T-lymphocytes, macrophages, dendritic cells and neutrophils), endothelial and smooth muscle cells, Schwann cells and peripheral neurons. When combined with tissue engineering methodologies, utilisation of skin models derived

from diabetic iPSC may provide new insight into disease mechanisms, and facilitate the development of effective therapeutics for treatment of chronic wounds in diabetic individuals.

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