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Hope versus hype: what can additive manufacturing realistically offer trauma and orthopedic surgery?

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Additive manufacturing (AM) is a broad term encompassing 3D printing and several other varieties of material processing, which involve computer-directed layer-by-layer synthesis of materials. As the popularity of AM increases, so to do expectations of the medical therapies this process may offer. Clinical requirements and limitations of current treatment strategies in bone grafting, spinal arthrodesis, osteochondral injury and treatment of periprosthetic joint infection are discussed. The various approaches to AM are described, and the current state of clinical translation of AM across these orthopedic clinical scenarios is assessed. Finally, we attempt to distinguish between what AM may offer orthopedic surgery from the hype of what has been promised by AM.

Keywords: additive manufacturing • bone graft • orthopedic surgery • 3D printing

The hype of additive manufacturing

Additive manufacturing (AM) has been used to produce everything from guns to models of unborn babies. In excess of 1450 articles pertaining to AM are listed in PubMed, around a third of which were published in the last 2 years alone. Attempts are being made to use AM to form human organs; indeed, some authors have commented that for hard tissue applications, the barriers for clinical translation are now regulatory rather than scientific or technical [1]. By contrast, other researchers believe that there are still a number of problems that must be solved before we can see the availability of scaffolds for clinical purposes [2].

Unmet clinical needs in trauma & orthopedic surgery

To understand the potential of AM in orthopedics, it is necessary to first evaluate the clinical need and current limitations to treatment. Areas to be addressed include bone defects, spinal arthrodesis, chondral injury and periprosthetic joint infection (Figure 1).

Bone graft material is frequently required to fill voids to enable a mechanically stable reconstruction in trauma and orthopedic surgery. Currently, in excess of 2 million grafting procedures are performed annually [3], with the material used either isolated from the patient (autograft), a donor (allograft) or the application of synthetic materials. Harvesting of autograft results in patient morbidity [4] and the amount available is finite. Furthermore, the use of allograft may result in disease transmission or an immunological reaction at the graft site. To date, 59 synthetic bone substitutes are available for clinical practice in the UK [5], while their composition varies, in the mainstay, the materials are limited by poor control of porosity and rate of degradation. Ideally, bone graft material should be: osteogenic (contain bone-forming cells), osteoconductive (permit migration of bone cells) and osteoinductive (stimulate osteogenic differentiation). Autograft remains the only material to retain any significant osteogenicity. Furthermore, none of these three categories of graft are vascularized, nor can the materials, including autograft, be readily formed/moulded to match the defect site.

Spinal arthrodesis is a common surgical procedure [6] used to treat pain and restore

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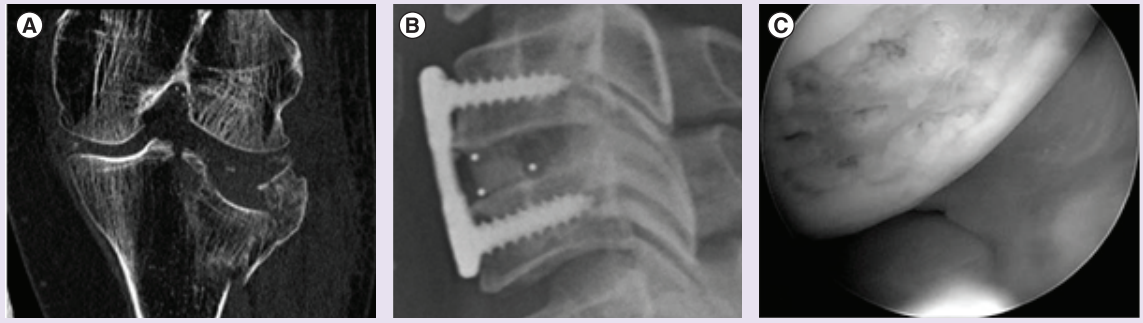

Figure 1. Clinical requirements in trauma and orthopedic surgery. (A) Tibial fracture requiring bone graft, **(B)** spinal cage device and **(C)** chondral defect post microfracture.

stability in a wide range of conditions including trauma, deformity and degenerative disease. Surgical techniques vary according to the disease, spinal location and patient and surgical factors [7]. In selected cases, cages may be used in combination with bone graft or an osteoinductive material to provide immediate mechanical support and to facilitate fusion. Devices are typically comprised of titanium or polyether ether ketone. However, disadvantages of using these permanent materials include subsidence and stress shielding [8].

Chondral injury, in other words damage to articular cartilage, is relatively common [9] and results in symptoms such as pain and loss of function for the patient. Current treatment strategies include microfracture and autologous chondrocyte implantation (ACI). Microfracture involves drilling through the lesion into the subchondral bone to stimulate the production of fibrocartilage from the underlying marrow. However, fibrocartilage remains biomechanically inferior to hyaline cartilage; this may explain the poorer long-term outcome in patients observed with this technique compared to ACI [10]. By contrast, ACI involves harvesting 'donor' chondrocytes from the patient's knee, *ex vivo* expansion, seeding of the expanded cells onto a scaffold and placement into the defect during a second surgical procedure. While ACI produces a superior long-term clinical outcome [10] compared with other treatment strategies, limitations include: requirement for 'donor' chondrocytes, subjecting the patient to two procedures, cell expansion and the formation of scaffold material to match the defect.

Periprosthetic joint infection is a devastating complication affecting between 1 and 3% of joint replacements. In addition to the human cost, given that in excess of 180,000 lower limb arthroplasties are performed annually in England and Wales [11], this represents a huge burden in terms of healthcare provision. The accepted gold standard treatment involves resection of implants and placement of an antibiotic-impregnated cement for a minimum of 6 weeks followed by reimplantation of the prosthesis [12]. While antibiotic-impregnated cement facilitates a prolonged release of antibiotics, cement may only be combined with antibiotics that are thermostable due to the exothermic nature of cement process and the high temperatures generated.

The focus of this article is on the current applications and limitations of AM for orthopedic translation. A number of AM reviews detailing the processes therein have recently been published [13,14,34]. Critically, the focus is on the identification of what the technology of AM may achieve, as opposed to the hype of what has, to date, been promised.

Additive manufacturing

AM technologies, known as rapid prototyping and solid freeform fabrication, are computer-directed layer-by-layer fabrication processes in which very thin layers of materials are stacked and adhered to shape a 3D physical model. AM technologies have been standardized and classified by the American Society for Testing and Materials International Committee F42 on AM Technologies into seven processes in accordance with the method of layers deposition and bonding, as described below [15].

Vat photopolymerization

Vat photopolymerization processes involve selective curing of predeposited photosensitive liquid polymer using light [15]. In stereolithography, the main vat photopolymerization technique, a laser beam or UV light source is used to project a cross-section of a single slice of the object onto a photopolymer resulting in the setting of the layer. This process is repeated until all the layers of the complete structure are created. Two-photon polymerization is a variation of the stereolithography process in which the photo initiator requires two photons to release a free radical that initiates polymerization; this approach results in significantly enhanced resolution.

Material extrusion

In this process, a continuous flow of materials in the form of paste or slurry is dispensed layer-by-layer using a 3D motion system incorporated with an extrusion nozzle. Material extrusion is diverse in concept but can be classified into two subgroups: processes based on material melting (comparable with fused deposition modeling, precision extrusion deposition [16], 3D fiber deposition [17] and multiphase jet solidification [18]) and processes without material melting (comparable with pressure-assisted microsyringe [19], 3D bioplotting [20], solvent-based extrusion freeforming [42], robocasting [21] and direct-write assembly [22]). Electrospraying describes the disruption of a liquid into a spray of charged particles when subjected to an intense electrical field, and was first described 25 years ago [23]. If the jet turns into very fine fibers instead of breaking into small droplets, the process is known as electrospinning. With the use of a coaxial needle comprising a central needle dispensing biological fluid containing cells and an outer needle dispensing biopolymer with low conductivity, electrospinning is capable of delivering viable cells [24] and active growth factors [25], as well as producing scaffolds with roughened surfaces to facilitate cellular migration [26] and control fiber orientation [27].

Material jetting

Material jetting is the use of inkjet printing or other similar techniques to deposit droplets of material that are selectively dispensed through a nozzle or an orifice to build the 3D structure. The material often turns into a solid subsequent to the deposition process via cooling (e.g., by crystallization or vitrification), chemical changes (e.g., through the cross-linking of a polymer) or solvent evaporation [20,33]. Commercial material jetting systems typically cure with a photopolymer ink using UV light in the inkjet printing process. In inkjet printing technology, two techniques are predominantly utilized for material droplet creation, namely, drop-on-demand and continuous inkjet.

Binder jetting

Binder-jetting techniques use nozzles to print material; however, instead of printing with the build material, the printed material is the 'glue,' which holds the powder together in the desired shape [15]. The 3D printing process is the main binder-jetting technique based on inkjet technology in which droplets of a binder material are deposited over the surface of a powder bed, adhering to the powder particles together where the part is to be shaped. The process is followed by lowering of the powder bed via a piston and a fresh layer of powder is then spread over the previous layer and, again, binder is deposited over the surface of the new layer. This procedure is repeated to build the whole structure.

Powder bed fusion

Powder-bed-fusion machines work in a manner similar to binder jetting; however, instead of printing glue onto a layer of powder, thermal energy is used to melt the powder into the desired pattern [15]. Most systems use laser power to melt the polymer, metal or ceramic material. Partial melting is termed selective laser sintering and full melting, selective laser melting. The application of an electron beam to melt the metal powder is known as electron beam melting. Finally, selective mask sintering offers a slightly different system that utilizes infrared light through a digitally printed optical mask to melt a thin layer of plastic powder.

Directed energy deposition

Directed energy deposition uses a laser beam to melt and fuse particles of the powder material delivered from the material deposition head. The X-Y table is moved to shape the cross-section of each desired layer. This process is repeated until all the desired cross-sectional layers of the structure are created. Other types of this technology are known as laser engineering net shape and direct metal deposition.

Sheet lamination processes

Sheet lamination techniques work by selective cutting and bonding sheets of material to form an object. The original system used glue or binder to bond paper or plastic sheets and is called Laminated Object Manufacturing (LOM), whereas ultrasonic welding of metal sheets is named Ultrasonic Consolidation (UC).

Table 1 summarizes specifications and applications of various AM processes.

Advances in the use of AM in trauma & orthopedic surgery

Clinical implants

The use of AM to produce permanent implants is outside the scope of this article. However, implants do demonstrate properties unique to AM, and in the case of cranio-maxillofacial implants, an area where the most clinical translation has occurred. These developments will be briefly reviewed. AM technologies can extract digital information from cross-sectional imaging routinely used in clinical practice such as computed tomography (CT)/MRI scans and apply this to build custom implants. The main advantages of AM technologies are manufacturing flexibility and the capability to fabricate implants of complex external shape and internal structures including the capacity to

Table 1. Classification and applications of different additive manufacturing techniques.

Process	Typical AM techniques	Advantages	Disadvantages	Applications	Living cells and growth factors	Experimental or commercial in medical field
Photopolymer vat	SL, 2PP	High-dimensional accuracy, offering transparent materials	Only photopolymers, single composition, cytotoxic photoinitiator, incomplete conversion thus postcuring required, limited cells for incorporation, nonhomogeneous cell distributions	Printing clinical implants and surgical guides, tissue engineering scaffolds, 3D micro-vasculature networks, biological chips, cell-incorporated 3D biological constructs	Yes	Experimental
Material extrusion	Melting extrusion: FDM, PED, MJS, 3D fiber deposition	Rapid, no toxic materials, good material properties	Low-dimensional accuracy, delamination, weak bonding between dissimilar polymers	Printing clinical implants, tissue-engineering scaffolds	No	Experimental
	Extrusion without melting: PAM, 3D bioplotting, solvent-based extrusion freeforming, robocasting, direct-write assembly, electrospinning	Simple and cheap mechanism, no trapped materials, low material waste, fairly high fabrication speed, cell-friendly environment	Relatively low-dimensional accuracy and mechanical strength, solvent is sometimes used, precise control of ink rheology is crucial	Printing tissue-engineering scaffolds, cell-incorporated 3D biological constructs, organ bioprinting	Yes	Commercial
Powder bed fusion	SLS, SLM, EBM, SMS	Wide range of materials, great material properties, high material strength	Thermal stress, degradation, accuracy limited by the particle size of materials, requirement for atmosphere control for metals	Printing surgical implants with complex internal and external structures, tissue-engineered scaffold, medical devices	No	Commercial
Directed energy deposition	LENS, DMD, LC	Wide range of materials, good material properties	Low-dimensional accuracy, thermal stress, requirement for atmosphere control, requirement for machining process for finishing the part	Printing orthopedic implants	No	Experimental

2PP: Two-photon polymerization; 3DP: 3D printing; AM: Additive manufacturing; DMD: Directed metal deposition; DoD: Drop-on-demand; EBM: Electron beam melting; FDM: Fused deposition modeling; LC: Laser cladding; LENS: Laser engineering net shape; LOM: Laminated object manufacturing; MJS: Multiphase jet solidification; PAM: Pressure-assisted microsyringe; PED: Precision extrusion deposition; PJT: PolyJet technology; SL: Stereolithography; SLM: Selective laser melting; SLS: Selective laser sintering; SMS: Selective mask sintering; UC: Ultrasonic consolidation.

Table 1. Classification and applications of different additive manufacturing techniques (cont.).

Process	Typical AM techniques	Advantages	Disadvantages	Applications	Living cells and growth factors	Experimental or commercial in medical field
Sheet lamination	LOM, UC	Low-temperature effects	Shrinkage, significant amount of waste, delamination	Printing orthopedic implants	No	Experimental
Material jetting	DoD inkjet printing, PJT	Rapid process, wide range of biomaterials, yet jettable materials, use of existing inexpensive technology, multiple compositions, multicell printing	Nozzle blockage an issue, low viscosity prevents build-up in 3D, low strength	Printing clinical implants and surgical guides, printing tissue-engineering scaffolds, printing cell incorporated biological constructs, organ bioprinting	Yes	Experimental
Binder jetting	3DP	Low-temperature process, rapid process, multiple compositions	Requirement of powder, high porosity, low surface quality, accuracy limited by the particle size of materials, powder entrapment, cell-challenging environment	Printing clinical implants and tissue-engineering scaffolds	No	Experimental

2PP: Two-photon polymerization; 3DP: 3D printing; AM: Additive manufacturing; DMD: Directed metal deposition; DoD: Drop-on-demand; EBM: Electron beam melting; FDM: Fused deposition modeling; LC: Laser cladding; LENS: Laser engineering net shape; LOM: Laminated object manufacturing; MJS: Multiphase jet solidification; PAM: Pressure-assisted microsyringe; PED: Precision extrusion deposition; PJT: PolyJet technology; SL: Stereolithography; SLM: Selective laser melting; SLS: Selective laser sintering; SMS: Selective mask sintering; UC: Ultrasonic consolidation.

create porous structures for weight reduction, tailoring stiffness and improving osteointegration. Critically, AM-manufactured implants accurately fit the defect site as the implant is developed from patient-specific digital data. In addition, an implant with a carefully designed lattice incorporating specific geometries confers the central advantage reducing stress shielding as the implant can closely match the stiffness of the bone. Moreover, material properties of AM-derived implants are normally better than investment-casted implants as the microstructure of the metal is finer resulting in higher tensile and flexural strengths [28].

Figure 2 depicts different 3D-printed clinical implants for use in reconstructive surgery.

Bone graft

The optimal properties of bone graft material include biological properties discussed previously, which encompasses control of porosity and the ability to match the graft to the defect site. As previous authors have stated the ideal bone graft substitute for all situations does not exist. Thus, with different clinical problems, different substitutes or combinations are required [30].

Allograft and autograft may be modified intraoperatively, but limitations on size, topography available and the ability to remodel fragments using an osteotome render accurate contouring of the graft to the defect site challenging. Traditional methods for the manufacture of synthetic bone graft, such as solvent casting/salt leaching, phase separation and foaming also have a number of limitations including shape restrictions, inconsistency and inflexibility, rendering these approaches poorly suited to produce graft material that accurately matched to the defect site. Klammert *et al.* were able to produce calcium phosphate implants precisely matching cranial defects mapped with the use of CT in an *ex vivo* model using AM [31]. The authors employed a binder-jetting process with phosphoric acid deposited onto tricalcium phosphate (TCP) powder that was subsequently hardened with additional phosphoric acid and subsequent autoclaving. Resulting material could be drilled, and held with plate and screw fixation as required. However, while demonstrating the ability of AM to delicately control graft topography, the pores remained smaller than the optimal size required and biodegradation was prolonged.

A key limitation of synthetic bone substitutes is the lack of control on porosity and pore interconnectivity, which are known to be of crucial importance in bone regeneration [32]. By contrast, AM offers delicate and exquisite control of these parameters, as well as other critical variables including filament size and alignment [33]. Extrusion-based AM is a technique that is particularly well suited to the formation of bone graft material. The additive nature of extrusion freeforming ensures minimal waste of biomaterial and makes this process suitable for mass production of tissue-engineering

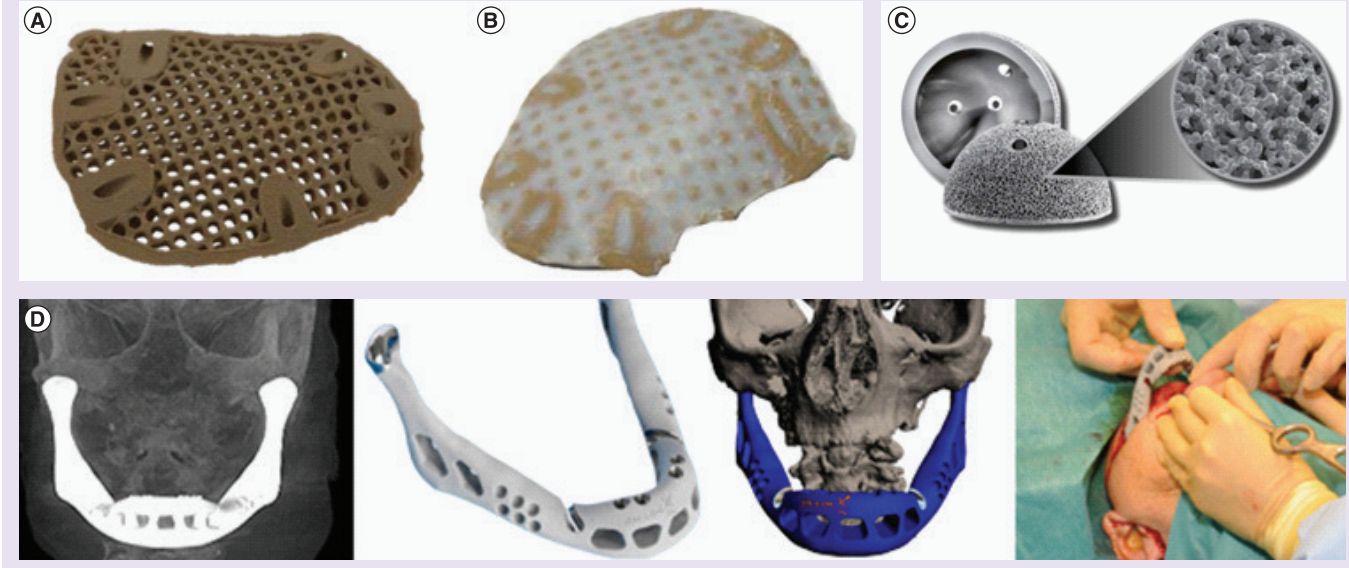

Figure 2. Examples of clinical implants produced using various additive manufacturing technologies for use in reconstructive surgery. **(A)** Cranial implant printed using selective laser sintering process from polyether ether ketone (PEEK) HP3 (PEEK material developed for use in selective laser melting EOS GmbH [Electro Optical Systems, Germany] sintering machine). **(B)** Infiltrated PEEK implant with a bioabsorbable polymer/hydroxyapatite hybrid material [29]. **(C)** An acetabular cup with porous surface printed using electron beam melting technology in a single process. **(D)** Total lower jaw implant in titanium printed by LayerWise using SLM process. **(C)** Figure courtesy of Arcam AB (Mölndal, Sweden); **(D)** Reproduced with permission from Layerwise NV (Leuven, Belgium).

scaffolds, multiple material porous bioactive structures [34] and microscale structures [13]. Solvent-based extrusion freeforming processes [35–37] have been successfully used for making high-resolution (<60-µm filament diameter) bioceramic scaffolds. Unique nozzle selection, paste formulation and paste rheological properties of this technique have enabled the finest ceramic scaffold fabricated using powder-based ceramic materials to be processed [38].

To circumvent the problems associated with the incorporation of growth factors or cells in AM, attempts have been made to enhance bone formation with the incorporation of silicate and metallic particles. Fielding and Bose investigated the osteoinductive potential of silicate and zinc oxide [39]. SiO_2 and ZnO particles were incorporated into β-TCP scaffolds produced using a binder-jetting technique and laser sintering. β-TCP scaffolds with and without SiO_2/ZnO particles were placed in rat femoral defects and subjected to a variety of tests including push-out testing, histology and micro-CT. Addition of SiO_2/ZnO particles was demonstrated to promote osteogenesis. This technique could represent a useful therapeutic strategy; however, given the significant problems resulting from metallic particulate matter stimulating immunological reactions following metallic hip implants [40], further investigation will be required.

Meseguer-Olmo *et al.* used silicate hydroxyapatite particles to enhance the osteoconductive ability of polycaprolactone (PCL) [41]. Hydroxyapatite and PCL were dispersed and dissolved using a solvent, and following printing, the scaffold was heated to 50°C overnight to ensure full evaporation of the solvent. Resulting scaffolds, alone or in combination with demineralized bone matrix, were implanted ectopically and orthotopically in rabbits. At 4 months, bone regeneration was seen in peripheral areas of all scaffolds using histology, while demineralized bone matrix appeared to promote bone regeneration centrally. While these results suggest the ability of this scaffold material to regenerate bone, the data would be further enhanced through comparison to control groups, as well as analysis using techniques such as CT.

Reichert *et al*. successfully used AM to produce bone graft material capable of mediating reconstruction of large bone defects [42]. Fused deposition molding of medical-grade PCL-TCP was used to produce cylindrical scaffolds. These scaffolds alone and in combination with BMP-7 or bone marrow cells were evaluated in a critical-sized defect in an ovine tibial model. Comparison was made with empty defects, and treatment representing the current clinical gold-standard, autologous bone grafting. The authors reported scaffolds combined with BMP-7 produced bone regeneration equivalent to autologous bone graft, while addition of bone marrow cells to scaffold material was not seen to augment bone formation. This ovine study including histological, biomechanical and micro-CT analysis at 3 and 12 months represents perhaps the closest to clinical translation attained, thus far, in the application of AM to treat large bone defects.

A current limitation with AM techniques to produce bone graft materials described above [31,39,41,42] remains the need to enhance cell and growth factor compatibility – currently limited by the application of thermal or chemical treatment. It is noteworthy that while Reichert *et al.* have demonstrated significant success in their technique, growth factor (BMP-7) and cells were applied as a separate process following production and sterilization of the scaffold [42].

Inkjet and extrusion-based AM systems such as 3D bioplotting can be used for simultaneous scaffold formation, cell and growth factor delivery when used under sterile conditions. Inkjet printing systems have previously been limited by loss of cell viability and cell/debris obstruction. Moon *et al*. were able to overcome these problems with the use of mechanical valves permitting printing of high-viscosity hydrogel precursors containing cells [43]. This bioprinting platform enabled synthesis of multilayered 3D hydrogel structures seeded with muscle cells at high densities, albeit with a dramatic reduction in resolution, with droplets >0.5 mm. While smooth muscle cells were used in this study, this technology could be applied to other cell types including bone marrow stromal cells. Other studies have also demonstrated the capability of AM to print complex 3D constructs containing multiple living cell types. Marga *et al.* reported bioprinting of multiple cell types (bone marrow and Schwann cells) and agarose cylinders to build a three-lumen tube using an extrusion-based bioprinter [44]. Xu *et al*. mixed human amniotic fluid-derived stem cells, canine smooth muscle cells and bovine aortic endothelial cells separately with ionic cross-linker calcium chloride [45]. Each cell type was dispensed from separate ink cartridges using a modified thermal inkjet printer (Figure 3B & C). The biological functions of the 3D-printed constructs were evaluated *in vitro* and *in vivo*. Critically, printed cell types maintained their viability, normal proliferation rates, phenotypic expression and physiological functions within the heterogeneous constructs.

Spinal fusion

Spinal fusion can be facilitated with the use of autologous bone graft, allograft or synthetic bone, alone or supplemented with bone marrow cells or osteoinductive factors. Fischer *et al.* performed a systematic analysis of the outcomes of these modalities and concluded that

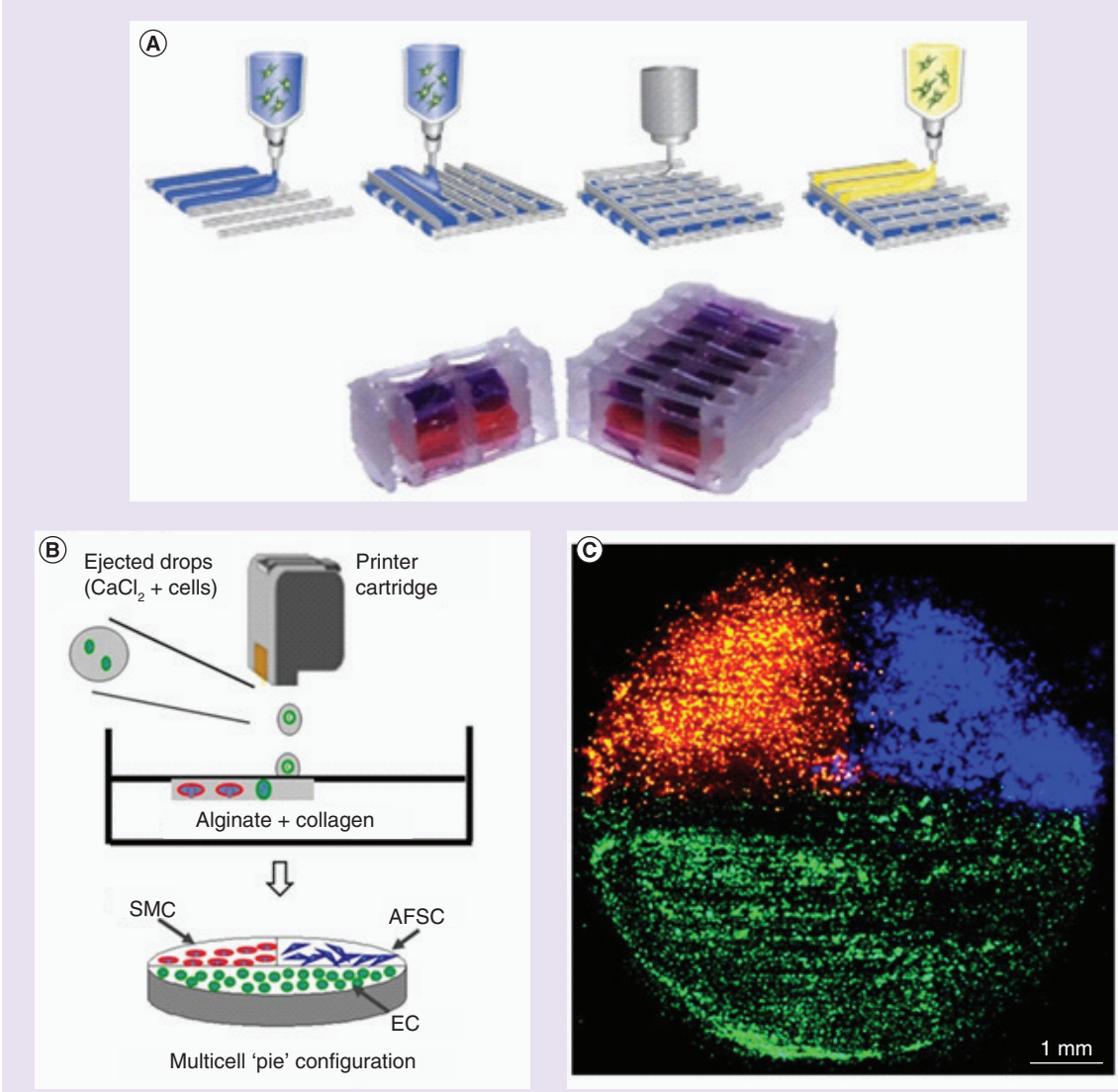

Figure 3. Biofabrication strategies adopted for direct 3D tissue printing. (A) Fabrication of solid biodegradable materials with cell-laden hydrogels: schematic illustration of a hybrid bioprinting process including alternating steps of printing biodegradable polymer and cell-laden hydrogels, and layering of the dye-containing alginate results in specific confinement of the printed hydrogels [46]. **(B)** 3D tissue constructs using simultaneous ink-jetting of multiple cell types. Human AFSCs, canine SMCs and bovine aortic ECs are separately mixed with ionic cross-linker $CaCl_2$, loaded into separate ink cartridges and printed using a modified thermal inkjet printer. The three cell types were delivered layer-by-layer to predetermined locations in a sodium alginate–collagen biocomposite located in a chamber under the printer. The reaction between $CaCl_2$ and sodium alginate results in a rapid formation of a solid composite gel and the printed cells are anchored in designated areas within the gel. The printing process is repeated for several cycles leading to a complex 3D multicell hybrid construct [45]. **(C)** Microscopic top views of a complete 3D multicell 'pie' construct before implantation. The cells that appear in green are bovine aortic ECs labeled with PKH 26 dyes; the cells that appear in blue are humanAFSCs tagged with CMHC dyes; the cells that appear in red are canine SMCs labeled with PKH 67 dyes [45].
AFSC: Amniotic fluid-derived stem cell; EC: Endothelial cell; SMC: Smooth muscle cell.
(A) Reproduced with permission from [46]. **(B & C)** Reproduced with permission from [45].
For color images please see www.futuremedicine.com/doi/full/10.2217/rme.14.20

the use of ceramics in combination with bone marrow aspirate showed a significant promise [7]. While limitations of current therapies include pseudoarthrosis and migration of cage devices, it should be acknowledged that in general terms levels of arthrodesis are high [7] and the correlation between arthrodesis and clinical outcome remains uncertain [47].

Abbah and colleagues sought to overcome the limitations of stress shielding and pseudoarthrosis by using fused deposition modeling to produce a

poly-ε-caprolactone scaffold with an internal fiber architecture, and compressive modulus specifically designed to match those of the cancellous bone [48]. Pore geometry was tailored to allow bone ingrowth. medical-grade PCL-TCP scaffold combined with BMP-2 demonstrated favorable outcomes compared with autologous bone grafting in a porcine model of lumbar interbody fusion. Furthermore, these results were supported by a recent study of medical-grade PCL with BMP-2 in a sheep thoracic spine fusion model [49].

Cage designs can lead to higher interference stresses and result in graft subsidence. In order to overcome this problem, Murphy *et al.* patented the concept of using AM to produce a biodegradable cage device in which the fixation plate could be integrated with the scaffold [50]. Devices based on this concept were designed for cervical interbody fusion and were produced using PCL. Additional PCL scaffolds were further modified with the inclusion of a calcium phosphate coating or collagen sponge containing BMP-7. These three scaffold types were then assessed in a porcine cervical fusion model [51]. In contrast to the other biodegradable scaffolds [52], these materials demonstrated sufficient mechanical strength for the 18-month duration of the experiment. Interestingly, the calcium phosphate coating and BMP-7 scaffolds demonstrated similar degrees of bone formation, both of which were superior to the unmodified PCL scaffold. Based on these findings, it may be hypothesized that coating of osteoinductive particles may circumvent the need for expensive and thermosensitive growth factors. Comparison of these scaffolds with a cage device incorporating autologous bone grafting would permit further evaluation of the effectiveness of this appealing strategy.

Osteochondral reconstruction

Tissue engineering approaches for the reconstruction of osteochondral defects can be described as a 'top-down' approach in which the scaffold is the key. The focus remains provision of a microenvironment to facilitate cell migration and differentiation. A polar opposite of this approach is the 'bottom-up' approach whereby scaffolds are eliminated, the rationale being that appropriate cells at high densities can produce the desired matrix [53]. Such scaffold-only [54] and cell-only techniques [55] represent two ends of a continuous spectrum with many authors employing a combination of cells, growth factors and scaffolds to facilitate tissue regeneration. In studies using cell delivery, controversy continues as to the cell type of choice, bone marrow stromal cells or chondrocytes [56,57], and in the case of chondrocytes if zonal isolation is important. Such a debate is beyond the scope of this article and has been detailed by others [57,58].

It is generally accepted that the zonal organization of type II collagen, chondrocytes and proteoglycans is of crucial importance to the function of articular cartilage [59]. It is also widely accepted that mechanical stimulation affects chondrocytes and synthesis of cartilage [60]. Scaffold pore size, pore geometry [61] fiber size [62] and pore interconnectivity [63] have all been shown to affect cartilage regeneration. Traditionally fiber size and porosity result from the choice of chemical or manufacturing process, whereas AM enables the specific selection of these parameters. Thus, AM enables production of scaffolds with specific variations porosity, enabling optimal properties to be elicited for specific regenerative requirements. Functionally graded nanocomposite structures [64] that are more suited to reconstruction at tissue interfaces such as osteochondral region can also be fabricated using AM.

Fedorovich *et al.* were able to dispense cells, control fiber spacing and the angle of deposition in 3D constructs produced using printing [65]. Heterogeneous scaffolds containing chondrocytes in alginate and bone marrow cells in alginate supplemented with biphasic calcium phosphate and hydroxyapatite were created. These constructs underwent *in vitro* culture or subcutaneous implantation in mice. The authors demonstrated heterogeneous tissue formation and the contribution of transplanted cells to extracellular matrix formation. Although this approach indicated a significant advance in the use of AM to produce tissue, the alginate material used is unlikely to confer sufficient mechanical strength to offer clinical translation without further modification.

Shim *et al.* used a six-nozzle extrusion system to form osteochondral tissue [66]. Two nozzles were heated and dispensed molten PCL to provide mechanical strength, while four nozzles dispensed a liquid alginate hydrogel at 20°C containing encapsulated human osteoblast-derived cells or chondrocytes derived from human nasal septum. The authors were able to control the porosity and left pores vacant in an attempt to enhance diffusion of oxygen to central areas of the construct. The study demonstrates the possibility of using AM to produce a porous scaffold seeded with two cell types with an assessed end point cell viability (live–dead stain) at 1 week. Successful tissue engineering will ultimately require that any incorporated cells are not only viable in the long term, but also remain in the chosen state of differentiation. The ability to maintain the cells in the desired state of differentiation is a recognized challenge in therapies targeting osteochondral injury [67], although timing of postoperative biopsy may also have an effect on tissue formation [68].

Cohen *et al.* have presented a novel concept for the *in situ* repair of osteochondral defects [69]. A modified

Fab@Home AM system was used to extrude alginate cross-linked with $CaSO_4$ prior to loading into a syringe and demineralized bone paste in a Gelatin carrier (BioSet™). The authors produced an alginate hydrogel and alginate-demineralized bone matrix plugs that matched the size and shape of defects formed on an *ex vivo* bovine femoral condyle. The requirement that the materials undergo cross-linking in a process compatible with the *in vivo* environment understandably places enormous limitations on the choice of materials, recognized by the authors. Materials that are dependent on laser, UV or chemical cross-linking to achieve phase deposition post printing are not suitable for *in situ* printing. Furthermore, in this specific case the paste must be of sufficient viscosity to enable retention of strength post extrusion, while sufficiently fluid to enable the material to pass through a relatively small needle. For ultimate clinical application, resolution of issues around porosity and mechanical composition (strength) will, thus, need to be addressed.

Periprosthetic infection

As discussed, standard therapy for deep infection in joint replacements involves removal of prosthesis, debridement, implantation of an antibiotic-impregnated cement spacer and, finally, revision to a definitive prosthesis following eradication of infection. In this treatment strategy only heat-stable antimicrobials can be used. Cement spacers are typically produced intraoperatively [77]; this takes time and may also result in a spacer of suboptimal dimensions. These temporary spacers are required to remain *in situ* for at least 2 months, and must be correctly formed to facilitate patient mobility and protect adjacent soft tissue. AM is capable of forming implants that provide a sustained release of heat-sensitive antibiotics [78]. As the amount of tissue debridement required in periprosthetic infection cannot be accurately determined by imaging, it is not likely that antibiotic spacers could be fabricated preoperatively for individual patients. However, AM could be used to produce an 'off-the-shelf' selection of spacers varying in size and topography, loaded with a selection of antibiotics. This could represent a clinically superior solution at reduced cost compared with current standard practice.

Other applications of AM

Several authors have reported on the use of additive manufacture to provide a 3D model to aide surgical planning [70] or form graft material to the specific clinical requirements [71,72]. Indeed, Lethaus *et al.* presented a study of 20 patients in which AM model mandibles were used to precontour plates to facilitate reconstruction [73].

Ongoing challenges in the use of AM in trauma & orthopedic surgery

Notwithstanding the tremendous progress in this field, key challenges are: vascularization of grafts, integration of the graft into surrounding tissue, sourcing of cells and growth factors, demonstration of long-term cell function, sterility, and ability to upscale production in an economically viable manner.

Vascularization

Construction of any tissue in excess of 100–200 μm in thickness requires a form of perfusion, preferably via a functioning vascular network to provide sufficient nutrient and gaseous exchange for the tissue [74]. Attempts have been made to incorporate pores within printed scaffolds to allow diffusion of adequate nutrients [66]. Miller *et al.* described the formation of a patterned vascular network in tissue material formed by AM [75]. A sugar-based template coated with a material to protect cells from osmotic damage was used to template the vascular network. This sugar-based material was subsequently removed revealing a tubular network, which was then seeded with human umbilical vein endothelial cells. While these strategies are innovative, the key will be the demonstration of a functional vascular network, comprised of endothelial cells that modulate permeability and direct cellular activity [76], surrounded by a muscle layer able to regulate flow. To date, a functional vascular network produced by any method remains to be fully demonstrated *in vivo*.

Cell source

Multiple studies have reported on the ability to use AM to distribute viable cells. These studies have typically explored viability at 1 week [66], although the long-term cellular viability remains undetermined. Studies of longer duration often do not include phenotypic or genotype analysis. In such cases, any damage to genetic material or changes in cell phenotype would not be manifest. This is of concern as studies have identified that even piezoelectric printing techniques can result in cell lysis [77]. In addition to the challenge of incorporating cell delivery into printing techniques, cells also need to be sourced. In the case of chondrocytes, this typically involves removal of 'donor' chondrocytes from a patient and *ex vivo* expansion. An alternative strategy may be use of bone marrow stromal cells obtained via intraoperative marrow aspiration. This approach has been successful in facilitating bone regeneration [78], spinal fusion [7] and osteochondral repair [79] in clinical studies.

Factors

Growth factors used, such as BMP-7, would also need to be sourced; typically the factors are expensive and

subject to a rapid decline in activity *in situ*. Following cell and growth factor preparation any printing process would have to be compatible with this biological material, performed in a sterile manner and on a timescale that maintains cell and or growth factor viability.

Barriers to commercialization of AM devices

While AM has been used to produce a range of products from implants to artificial organs, most of these applications have been developed through experimental systems; availability of commercial AM systems for biomedical applications is not that remarkable. Commercial AM systems developed primarily for industrial manufacturing purposes have been adapted or modified to perform specific applications in the medical sector. In the mainstay, these modifications are undertaken by biomedical research groups and institutes. Possibly the greatest obstacle to the commercial development of 3D printing applications is the lack of information and confidence of commercial AM systems suppliers in the biological performance of 3D-printed parts. This may, in part, be explained by the difficulty in demonstrating and validating predictable and reproducible biological viability of printed materials prior to and after biofabrication processes. To make current commercial AM systems suitable for use in orthopedic or advanced biomedical applications such as formation of tissue or artificial organs, a critical challenge is the modification of the process/device to enable processing of a wide range of biomaterials and to ensure a cell-friendly process environment. Current biofabrication processes such as extrusion-based systems in production of cell-seeded scaffolds may not offer satisfactory reproducibility. Similarly, production of precise tissue constructs with features less than 100 μm remains challenging, while integration of biomanufacturing systems with micro/nanosystems is limited due to the requirements for a clean environment. Furthermore, resulting specialized AM-based applications must meet stringent regulatory requirements, let alone be economically viable. In essence, evidence for biological performance, technical and regulatory challenges are the main barriers that need to be addressed in order to commercialize AM applications.

Future perspective

Bone graft

AM enables production of bone graft with biological and biomechanical features tailored for the particular clinical task. AM-fabricated bone graft is likely to achieve clinical superiority to allograft, and when combined with growth factors may indeed achieve efficacy equal to that of autograft in the near future.

Currently, the incorporation of growth factors and cells during printing severely limits the materials and processes that can be used and imposes significant logistical and time constraints (resulting material would require surgical placement prior to loss of biological activity). One potential economically and clinically viable solution remains the seeding of the bone marrow aspirate taken and delivered intraoperatively, and the delivery of growth factors frozen in a paste, which may be thawed and also delivered at time of surgery. Thus, a selection of 'off-the-self' sizes of graft material with tailor-printed graft used only in complex cases could benefit from this approach. An economically viable model may be the delivery, ultimately, of AM devices at level 1 trauma centers, or solely at a single national location. Electronic transmission of imaging files would permit remote fabrication of desired product and dispatch via a courier. Complex reconstructions requiring such graft material are generally planned in advance, and as such this system could provide the tailor-made graft in the requisite time.

Spinal arthrodesis

AM has been used to graft material of optimal biomechanical and biological properties for spinal arthrodesis, and has shown significant success in large animal models. When growth factors have been used, these factors have been applied intraoperatively following the production of the scaffold and this approach is likely to continue. While preoperative imaging offers the potential to produce patient-specific cage/fixation devices, such imaging cannot predict the amount of tissue tension or the amount of tissue that will be resected. For these reasons, and for cost control, it would seem likely that except for exceptional cases, a range of off-the-shelf products would be used rather than products produced for a specific patient.

Osteochondral reconstruction

In this clinical scenario, we envisage the predominant use of off-the-shelf products. While imaging may permit mapping of the defect size, it is unlikely to determine the health of surrounding tissue which may require debridement, leaving imaging unable to predict/map with accuracy the dimensions of the subsequently required graft. A role for *in situ* repair of defects is unlikely as the limitations on which materials can used are likely to outweigh potential advantages.

Models of orthopedic disease

While vascularization of tissue produced using AM is likely to remain a challenge for some time, we anticipate that AM may be harnessed to produce models of structural bone disease such as osteogenesis imperfecta, thus facilitating development of treatments. Printed tissue, such as bone, is also likely to partially replace animal testing in the pharmaceutical industry [80].

Financial & competing interests disclosure

The work of the authors is supported by a strategic longer and larger grant (sLOLA) from the Biotechnology and Biological Sciences Research Council, UK (BB/G010579/1) to R Oreffo, as well as partial funding from EU Biodesign (EU FP7) and Rosetrees Trust. M Vaezi has received funding from the Institute for Life Sciences, University of Southampton, and Invibio, UK. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Additive manufacturing

- Additive manufacturing (AM) is an umbrella term encompassing a variety of techniques that involve computer-directed material fabrication in 3D.
- A variety of AM techniques have been successfully used to manufacture biocompatible scaffolds and to seed the scaffolds with viable cells.
- Cross-sectional imaging has been used to produce scaffolds that accurately match defect sites.

Advances in the use of AM in trauma & orthopedic surgery

- Surgeons have used AM to provide printed models to guide complex reconstruction and to produce graft material to match specific patient needs.
- Materials used in patients thus far remain far from optimal for application in long bone defects; however, evidence is emerging of the efficacy of such an approach including the demonstration of medical-grade polycaprolactone–tricalcium phosphate scaffolds combined with BMP-7 in a large animal model.
- AM-derived spinal fusion cages have been used with success in animal models; however, these cages are yet to translate into clinical practice.
- In general terms, AM will produce superior materials for a variety of clinical scenarios, but essentially, until new developments are forthcoming, growth factors and cells are likely to be applied intraoperatively rather than as part of the printing process.

Ongoing challenges in the use of AM in trauma & orthopedic surgery

- While presenting new opportunities, AM will need to address long-standing hurdles such as graft vascularization, cell and growth factor availability.

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Review

Regenerative Medicine

Progress towards cell-based burn wound treatments

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Cell therapy as part of the concept of regenerative medicine represents an upcoming platform technology. Although cultured epidermal cells have been used in burn treatment for decades, new developments have renewed the interest in this type of treatment. Whereas early results were hampered by long culture times in order to produce confluent sheets of keratinocytes, undifferentiated proliferating cells can nowadays be applied on burns with different application techniques. The application of cells on carriers has improved early as well as long-term results in experimental settings. The results of several commercially available epidermal substitutes for burn wound treatment are reviewed in this article. These data clearly demonstrate a lack of randomized comparative trials and application of measurable outcome parameters. Experimental research in culture systems and animal models has demonstrated new developments and proof of concepts of further improvements in epidermal coverage. These include combinations of epidermal cells and mesenchymal stem cells, and the guidance of both material and cell interactions towards regeneration of skin appendages as well as vascular and nerve structures.

Keywords: burns • cell therapy • keratinocyte transplantation • mesenchymal stem cell • scar • skin substitutes • tissue engineering • wound healing

Burns are the fourth most common type of trauma worldwide [1]. A high total burned surface area (TBSA) is in particular an increased risk, because open wounds present a high risk of infection and sepsis, and a poor prognosis. The prognosis of the patient is dependent on timely wound excision and fast wound closure in order to limit inflammation, fluid loss, catabolism and microbial invasion as well as to provide durable and long-term cover [2]. Consequently, one of the main clinical priorities in treatment of patients with extensive burns is rapid wound closure.

Due to the developments in primary support and treatment such as fluid resuscitation [3], prophylaxis for deep venous thrombosis and venous thromboembolism [4,5], and glucose control [6], more severely burned patients can nowadays survive the initial phase of the burn trauma. This results in more patients with a large percentage of open wounds, which continues to be a challenge for the multidisciplinary treatment team.

Partial-thickness defects potentially heal without a surgical intervention due to spontaneous re-epithelialization. Conservatively treated deep dermal and full-thickness defects inevitably heal with scar formation and always require surgical treatment [7]. A correct diagnosis of the depth of the burn wounds is therefore important in determining the appropriate initial treatment. This can nowadays be performed reliably by using laser Doppler imaging [8,9]. Currently, transplantation of autologous split-thickness skin grafts harvested from healthy donor sites is used as the standard of care (Figure 1) [10,11]. With this technique the entire epidermis and part of the dermis is removed, creating a new partial-thickness wound area on the patient at the donor site. In severely burned patients, lim-

Future Medicine part of fsg

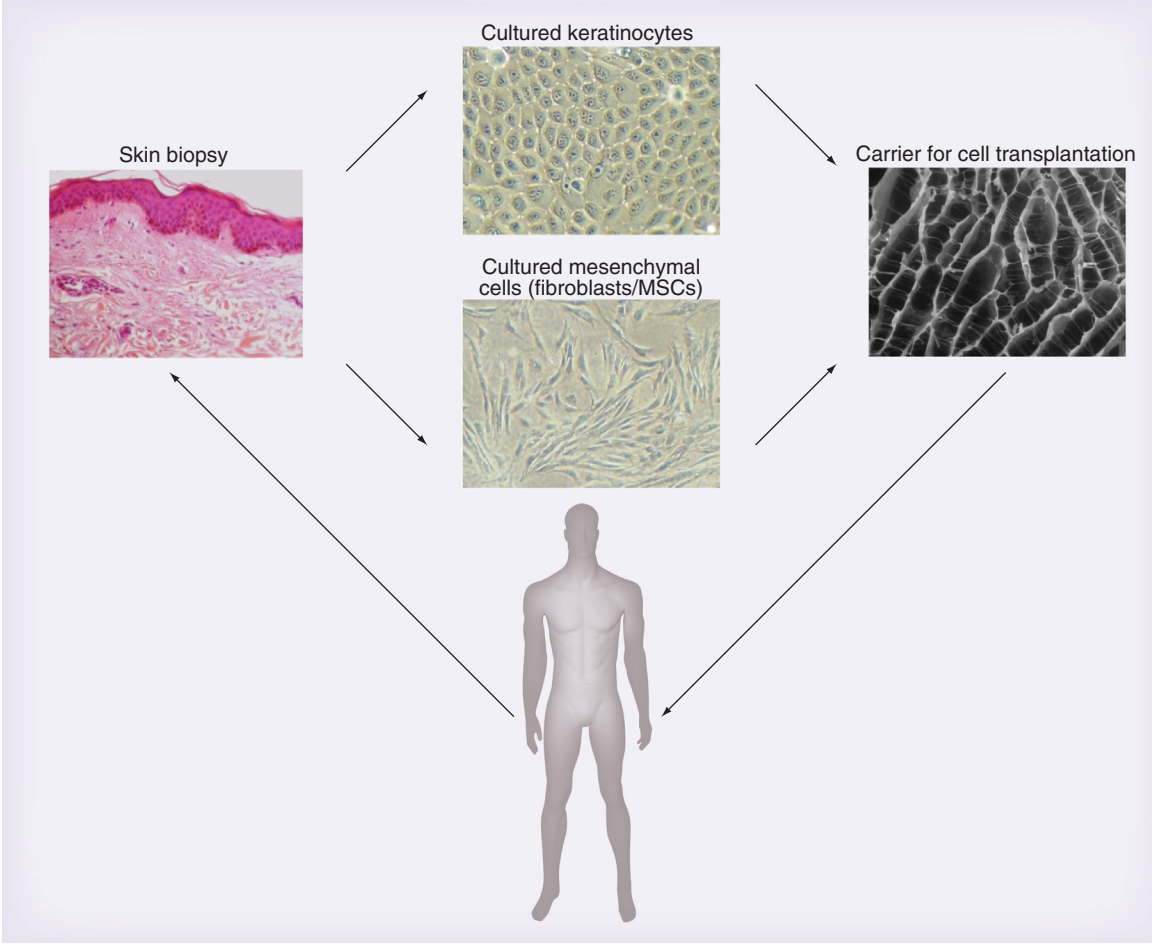

Figure 1. Schematic representation of the generation of an autologous skin substitute. To generate autologous skin substitutes, a biopsy is obtained from the patient and transferred to the laboratory. Keratinocytes and fibroblasts or MSCs are isolated and cultured. The cultured cells are transplanted back to the patient on a carrier. Please see color figure online at www.futuremedicine.com/doi/pdf/10.2217/rme.13.97
MSC: Mesenchymal stem cell.

ited healthy donor sites are available for transplantation. Consequently, the surgical treatment of this group of patients is the most technically demanding burn care. Expansion techniques such as Meek-Wall and mesh are employed to cover a larger wound surface area [12–14]. However, this often results in disappointing scar qualities with hypertrophy and contracture [14]. The importance of the scar quality should not be underestimated and has become increasingly important for the patient [15,16]. The different techniques of primary surgery to cover the wounds as soon as possible, and of reconstructive surgery in a later stage, are related to the resulting scars. New developments for the coverage of burn wounds in the severely burned patient are necessary first to overcome the limited availability of healthy donor sites and second to improve the resulting scar quality.

The need to provide skin cover in a situation of insufficient donor sites leads to interest in laboratory-based tissue expansion through the development of cultured skin substitutes [17–22].

In the last four decades, advanced cell biological techniques in tissue engineering methods were developed for the treatment of patients with extensive burns, aimed at support the body and closing the wounds as soon as possible, using limited donor sites and potentially leading to improved outcomes with less scar formation and better scar quality.

This article provides an overview of cell therapy in burn wound healing and revies the history of tissue engineering with regard to the different cell types, their cell biology, the performed clinical trials and the future directions.

Historical overview

The human skin is structured in multiple layers of several cell types. The keratinocytes and melanocytes are the predominant cell types of the epidermal barrier layer. The fibroblasts are the prominent cells in the dermal layer. Since the 1960s, the first steps of *in vitro* culture of human skin epithelial cells were taken [23].

Initially, the skin layers were separated with trypsin, thus retaining the cell viability, followed by reassembly of the skin layers in culture [24]. In 1975, Rheinwald and Green were the first to succeeded in the culture of keratinocytes from single-cell suspensions of human epidermal cells [25]. Subsequently, it was shown that keratinocytes were able to survive and proliferate in tissue culture; the production of small sheets of epidermis consisting of two or three layers of cells were a fact [26]. The culture method made use of animal-derived feeder layers. This feeder layer technique contained 3T3 murine fibroblasts that were lethally irradiated to prevent overgrowth of the fibroblasts. Murine fibroblast feeder layers supported the growth of human keratinocytes, but this included a risk that remnants of animal components (e.g., murine DNA) would be introduced into the epithelial transplants [27]. The elimination of risks of transmission of animal-derived disease components such as viruses or prions to the patient nowadays represents serious regulatory and safety issues, since from a regulatory point of view these undefined xenobiotic materials and cells should not be used for clinical treatment of patients.

The first clinical applications of cultured epithelial autograft (CEA) were in the 1980s [28–30]. The innovation to culture a 'football field' full of keratinocytes, from a small healthy skin biopsy, had become reality.

This was the start for many of the leading clinical burn centers to use this novel development in the treatment for severely burned patients [17,19–22]. In 2006 Wood *et al.* described and summarized the clinical studies available on the use of CEA [31].

In patients with an extensive burned surface area and limited healthy donor sites, this was a new treatment strategy. However, the promise of the perfect solution quickly scattered. Problems occurred, such as unpredictable take rates, the fragility of the cell sheets (Figure 2) [32] and long culture times.

This time lag to treatment caused an increased risk for the patients such as the risk of colonization or infection of the open wound, sometimes even leading to the inability to transplant the cultured keratinocytes back to the patient [33].

The unpredictable culture time and hence the planning of the operation in combination with the clinical status of the patient represented serious constrictions. This still is almost an unmanageable challenge for the clinicians and laboratory technicians and a tremendous limitation for the practical clinical use of these CEAs in burns. In addition, disappointing long-term results like scar formation and contractures [34] became apparent as a result of the lack of dermal tissue. Analysis at the electron microscope level revealed the lack of anchoring elements between epidermis and dermis, resulting in fragile skin with blister formation [35,36]. This led to more research for new and more sophisticated solutions.

Cell-based treatments of burns

Keratinocytes

Although much progress has been made with the culture of autologous epidermal cells and the use in the clinical setting has improved substantially, there are still some drawbacks: the procedure still requires a minimal culture time of 10–14 days, which might interfere with early wound excision, and the method is expensive. In addition, due to more stringent safety instructions enforced by the different authorities (e.g., the EU), elaborate quality and safety checks have to be performed for all individual products. Therefore, allogeneic keratinocyte transplantation is still considered as an alternative. Allogeneic cell products allow the construction of fully characterized and screened cell banks [27,37]. However, there are risks associated with allogeneic transplantation. Due to the rejection reaction these cells are only temporarily present and their effects on the wound healing process are probably due to the delivery of cytokines or growth factors that activate the autologous cells to accelerate the healing process.

Eisinger *et al.* showed that extracts of cultured keratinocytes and even conditioned media of these cells induce keratinocyte proliferation [38]. In addition they reduced fibroblast proliferation and inhibit contraction of fibroblast-populated collagen sponges. *In vivo* application on surgical wounds showed increased re-epithelialization through increased migration and proliferation of keratinocytes from remnants of glands, hair follicles and wound edges.

Duinslaeger *et al.* have taken this even a step further; they prepared a lyophilized keratinocyte extract [39].

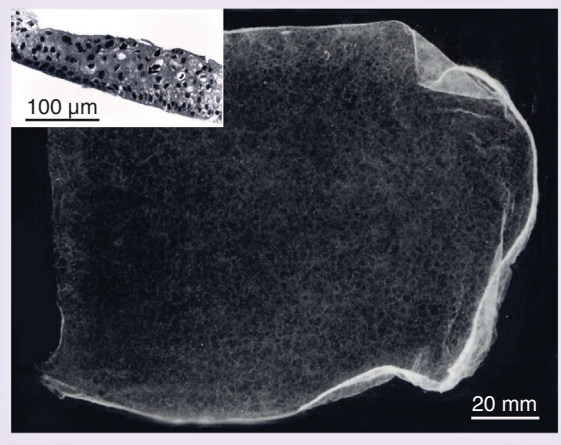

Figure 2. Cultured epidermal autograft. Insert demonstrates the microscopic view of the cultured epithelial autograft.

They showed that application of this concentrated keratinocyte lyzate in combination with a meshed split skin autograft increases the rate of re-epithelialization.

Culture media & feeder layers

The present culture methods for keratinocytes preferably avoid the use of xenobiotic cells or materials in culture media for clinical application. A desirable protocol would be to culture cells without using irradiated 3T3 murine fibroblasts as a feeder layer and media that do not contain bovine fetal calf serum (FCS) or bovine or porcine pituitary extracts. Research has shown that collagen type IV can replace the irradiated 3T3 mice fibroblast feeder layer. Coolen *et al.* showed that they could effectively culture keratinocytes to a fully differentiated epidermis, without the need for fibroblast feeder layers and FCS [40]. Another example is the work of MacNeil *et al.*, who developed a feeder layer including irradiated human dermal fibroblasts to culture keratinocytes under serum-free conditions [41,42]. Initially, fibroblast were seeded in a medium containing FCS and in a later phase transferred to co-cultures with keratinocytes in serum-free media. Notably, other products used in the 'serum-free' culture media may still contain low levels of animal-derived proteins. The use of only human recombinant materials could theoretically solve this issue; however, at present these materials are considered too expensive for routine clinical application in an already expensive culture system.

De Corte *et al.* introduced neonatal foreskin keratinocytes as source of cultured epithelial allografts [27]. As described in their paper, an important drawback of delivering subconfluent allogeneic cells is the fact that ultimately these allogeneic cells will be rejected [27]. The potential benefit for burn patients relies on an accelerated healing by potent mitogenic stimulation by the allogeneic keratinocytes on remnant cells in burn wounds or donor sites. Consequently, this allograft method can only be used in partial-thickness wounds. However, one of the biggest advantage of cultured epithelial allogeneic cells is the optional off-the-shelf use.

Lamb *et al.* showed that two serum-free, feeder cell-free growth media, sufficient for propagation of primary epidermal keratinocytes, did not support epidermis development in an *in vitro* skin equivalent model [43]. They did show that a stratified epidermis was formed using serum-free media supplemented with 10% fetal bovine serum [43]. Also, a stratified epidermis was formed after addition of heat-inactivated but not boiled serum, indicating that biologically activated serum factors are required for *in vitro* epidermis development [43].

The ongoing developments to minimize the use of xenobiotic materials and cells are already an important step towards fulfilling regulatory requirements for clinical application. Next to the elimination of xenobiotic cells and materials, the removal of antibiotics in the culture media is another goal to achieve in optimizing the production requirements for tissue-engineered skin [44].

New developments in keratinocyte culture techniques are mainly characterized by the use of non-confluent proliferating cells in and on a carrier system (Figure 3) or as a spray technique. These new treatment modalities will be discussed under 'Clinical data'.

Mesenchymal stem cells

Scar formation in burns, at least in part, is thought to be due to the lack of sufficient dermal material in the transplanted split skin autograft. To improve the outcome of healing of deep skin defects several dermal substitutes have been developed [45,46].

In contrast to the epidermis, which mainly consists of cells, the dermis consists of a tight network of extracellular matrix in which different cell types reside. The main cell type in the dermis is the fibroblast, which is responsible for the production and maintenance of the extracellular matrix. Several studies were performed to evaluate the use of autologous or allogeneic fibroblasts in combination with a scaffold that mimics the dermal extracellular matrix.

The limited clinical use worldwide is probably due to the high costs for production of these constructs and the possible immunological reaction to the allogeneic products. The use of mesenchymal stem cells (MSCs) in tissue engineering has met great interest since the discovery of these cells in many tissues. MSCs are considered to be very promising for tissue engineering purposes due to their multilineage differentiation capacity and the immune-modulating effects.

MSCs are thought to play an important role in tissue homeostasis and to facilitate repair of damaged tissue to restore the function of the injured organs. The MSCs migrate towards the damaged tissue [45] under the influence of chemoattractants such as chemokines and growth factors, which are mainly produced by inflammatory cells.

In 2006 the International Society for Cellular Therapy (ISCT) postulated minimal criteria for defining multipotent mesenchymal stromal/stem cells [47]. These criteria, among others, describe that these cells have to express a specific cluster of differentiation (CD) marker pattern. They have to be positive (≥95%) for CD73, CD90 and CD105, and negative (≤2%) for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR. This suggests that the cell iso-

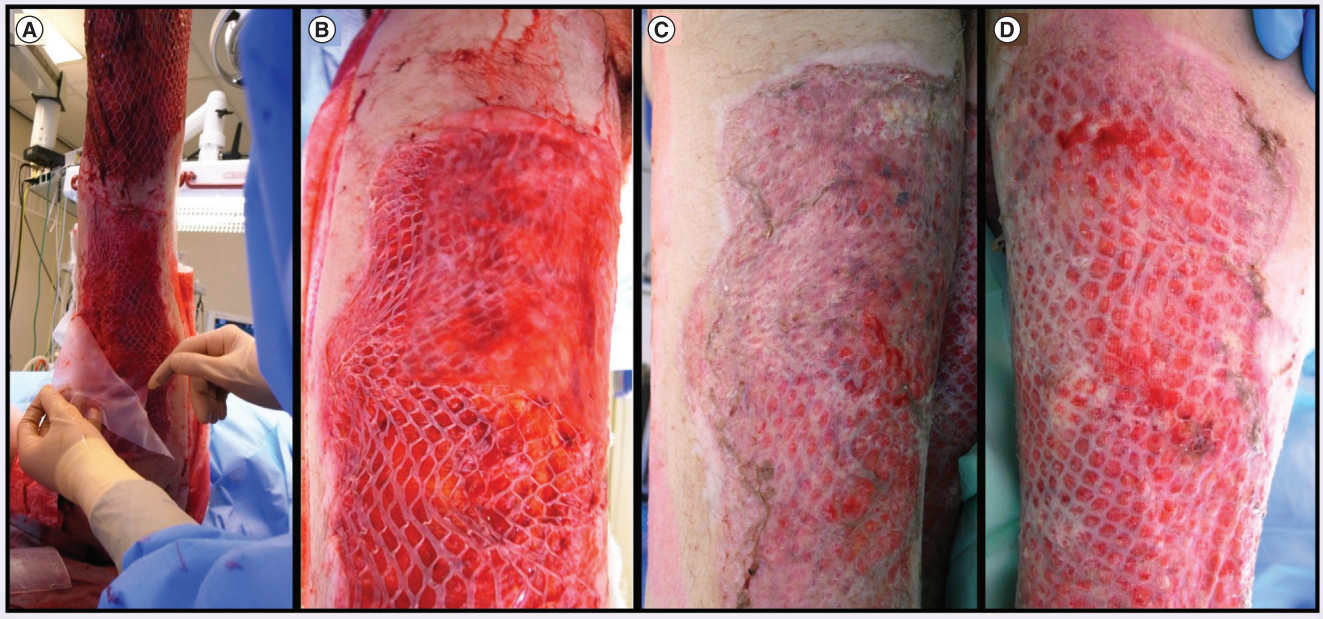

Figure 3. Treatment of a patient with proliferating keratinocytes seeded on a collagen matrix as a carrier. The autologous keratinocyte-containing carrier was applied on top of a widely meshed split-thickness skin autograft. **(A & B)** Application of the keratinocyte-containing carrier onto the wound. **(C)** Keratinocyte-treated wound 6 days after application. **(D)** Control wound without cultured keratinocytes.

lates represent a homogeneous cell population. However, the MSC populations from various tissues are a very heterogeneous population of cells of which only a small percentage possess multilineage differentiation potential [48]. Until proper discriminating markers have been found to identify MSCs and other cell types it is unknown how the different cell types in these populations contribute to the healing process.

Application of MSCs have been shown to improve the healing in different wound models [49,50]. The exact mechanism by which this is accomplished is not known. Some studies show the incorporation of MSCs into the newly formed tissue [51], while others believe that they are active in a paracrine way [52].

MSCs & their immune-modulatory effects

It was shown that MSCs are capable of reducing the immune response by suppression of the activation of T cells, B cells and natural killer cells, and reduction of the maturation of dendritic cells [53].

In addition, Adutler-Lieber *et al.* showed that MSCs derived from cardiac adipose tissue were able to skew macrophages towards the anti-inflammatory M2 phenotype [54]. It was shown that IL-6 secreted by the MSCs induced secretion of the anti-inflammatory cytokine IL-13 by the macrophages and induced the M2 phenotype. Whether this latter mode of action is beneficial for burn wound healing is debatable since the M2 phenotype is profibrotic. Kobayashi *et al.* showed that predominantly monocytes carrying a M2 phenotype could be isolate from the blood of burn patients [55]. The same group demonstrated in a mouse model that different M2 subtypes were already detectable at day 1 after the infliction of the burn wound [56]. The lack of M1 macrophages in the burn wound could explain the susceptibility for wound colonization because this M1 subtype is the major effector cell against microorganisms and M2 is thought to have diminished phagocytic capacity [54]. However, the phagocytic capacity of M2 cells is controversial as other papers have described an increased phagocytic capacity in this phenotype [57].

MSCs have reduced expression levels of MHC class I and II and they do not express the costimulatory molecules CD80, CD86 and CD40 [58]. Because of this the cells are immune privileged, meaning that they don't elicit an immune response and hence they can be used in an allogeneic setting. This is a major benefit because this would allow an off-the-shelf product.

However, there are also concerns with the use of these cells. For example, they might lose their immune-suppressive and immune-privileged status during differentiation or during the culture procedure [59,60]. In addition, they might differentiate into a wrong/unwanted phenotype or even become tumor cells due to their high capacity for self-renewal. Currently, these uncertainties limit the progress towards clinical application.

Site-specific differentiation of MSCs

MSCs have been shown to possess the ability to differentiate into different lineages, *in vitro* as well as *in vivo*. For *in vitro* differentiation, specific media conditions have been described. *In vivo* differentiation is thought to be driven by site-specific signals. Liechty *et al.* showed that intraperitoneal injection of human bone marrow-derived MSCs into fetal sheep home to different tissues [61]. The human cells could be detected up to 13 months after birth. In addition, they showed that these human cells displayed tissue-specific characteristics. During the cell transplantation procedure wounds were inflicted and after birth the human cells were detected in the dermis and dermal appendages and displayed fibroblastic features, indicating that the MSCs were involved in the healing process. The specific signals needed to guide the MSC to the defined cells are largely unknown.

Nowadays most papers describing MSCs confine their characterization of the cell population to the different markers that were defined by the ISCT. Only a few studies have shown that the cell population containing stem cells also contains α-SMA-positive cells [62,63]. It is unclear whether the MSCs themselves express α-SMA or whether these cells are differentiated myofibroblasts; the cell type involved in scar formation and fibrosis.

Recently we have shown that during the first 10–14 days post burn MSCs migrate into the wound [64]. We hypothesize that these cells contribute to scar formation. The microenvironment created and experienced by these myofibroblast-like cells is distinctly different from normal dermal tissue and because cell function and tissue performance are largely dependent on the cellular microenvironment the healing process can become trapped in a vicious circle. Ideally, tissue engineering could play an important role in this by the development of scaffolds that are able to guide the stem cells into the proper phenotype.

Stem cells in burn wound healing

In the past decade several animal studies were performed to demonstrate the efficacy of MSCs in burn wound healing [49,65–68].

In 2003 Shumakov *et al.* published one of the first studies on MSC application for burn wounds in rats [67]. In this study the wound healing effects of application of autologous and allogeneic bone marrow-derived MSCs and fetal (lung) fibroblasts were evaluated. Application of either cell type resulted in faster wound closure in comparison with the control group (no cells). However, the best results were obtained with the bone marrow-derived MSCs, with a slight better (not statistically significant) performance for the autologous MSCs. This study shows that allogeneic MSC transplantation indeed could be used to enhance burn wound healing. In another study they showed that application of either fetal fibroblasts or allogeneic MSCs reduced the inflammatory response [49].

It was suggested that MSCs not only accelerate wound healing but may potentially also restore the appendages in the skin [68]. Labeled bone marrow-derived MSCs were co-cultured with sweat gland cells prior to transplantation into full-thickness excision wounds in rats. It was shown that these cells were incorporated in the appendages in the wound area. The appendages in the nonwounded skin did not contain labeled cells, suggesting that these cells were only integrated in newly formed appendages.

Clinical data

Clinical studies with cell-based therapy

All this innovative research provided a framework for new standard treatment options in patients with skin diseases. A considerable number of clinical trials on dermal substitutes has been performed and nowadays many dermal substitutes are commercially available. However, most of these were developed for chronic wounds, such as diabetic neuropatic or venous ulcers.

Materials specifically developed for burn wound treatment are unlikely to be as economically profitable compared with materials that were developed for chronic wounds, due to the higher prevalence of the latter [69]. Consequently, a whole body of research on dermal substitutes was performed in patients with chronic wounds. The currently available dermal substitutes have been described in detail elsewhere [46,63,70–74]. In 2002, Jones *et al.* produced a schematic representation of the components of the main commercial artificial skin substitutes [69].

Notably, despite the extensive clinical problems in burn patients, only a handful of clinical studies were published on the effects of cultured autologous epidermal substitutes in these patients, and most of them were case reports or retrospective cohort studies [75,76]. The clinical use of CEA in burn patients has been discussed extensively previously [31,77].

Table 1 provides an overview of clinical trials with epidermal substitutes in burn patients. The literature search was carried out in the PubMed database. The additional search filters in PubMed were activated for the section 'Article types' ('Clinical Trial') and for the section 'Species' ('Human'). Subsequently, the following keywords were included in the literature search: ("skin, artificial"[mesh] AND "burns"[mesh]) AND "transplantation, autologous"[mesh]). Only six English-language papers were found, of which four ful-

Table 1. Overview of clinical trials including commercially available epidermal substitutes in burn patients.

Product name (manufacturer)	Website	Source	Study design	Application area	Patients/wounds treated (n)†	Intervention	Results	Ref.
Bilayered (dermoepidermal) substitutes								
Apligraf®‡ (Organogenesis Inc., MA, USA; product formerly known as Graftskin or Living Skin Equivalent)	www.organogenesis.com	Al	Ra, P, Pi	PT skin donor sites	10	Apligraf vs Apligraf without epidermis vs polyurethane film	No difference between the three treatment modalities in establishing basement membrane in donor site wounds, cosmetic outcome and time to wound healing	[82]
			Ra, P	Deep dermal wound (removal tattoo)	43 (wounds)	Apligraf + Mepitel® vs SSG vs Mepitel only	Apligraf does not persist long-term and merely serves to act as a biological dressing	[83]
			Ra, P	Mmsw	12	Apligraf vs heal by secondary intention	Apligraf appears to be a safe, well-tolerated biological dressing	[84]
			Ra, P	FT or PT bw	38	Apligraf + SSG vs SSG alone	Suitable and clinically effective treatment (cosmetic and functional advantages) for burn wounds with Apligraf when applied over SSG	[85]
OrCel™‡ (Forticell Bioscience Inc., NY, USA; product formerly known as Composite Cultured Skin or Ortec)	www.forticellbioscience.com	Al	Ra, P	PT skin donor sites	82	OrCel vs Biobrane®-L	OrCel is well tolerated, promotes more rapid healing and results in reduced scarring compared with convential therapy Biobrane-L	[86]
Epidermal substitutes								
Epicel®‡ (Genzyme Tissue Repair Corp., MA, USA)	www.epicel.com	Au	C, P	FT bw	28	Noncomparative	Extensive, permanent burn coverage achieving a high survival rate and high beneficial value in the management of burns >60% TBSA	[87]

†Data represent numbers of patients treated unless indicated otherwise.
‡Product also investigated in clinical trials on other skin diseases (e.g., ulcers and/or epidermolysis bullosa and/or congenital melanocytic nevus).
Al: Allogeneic; Au: Autologous; bw: Burn wound; C: Cohort study; Ca: Case report; FT: Full thickness; Mmsw: Mohs micrographic surgery wounds; NS: Not specified; P: Prospective study; Pi: Pilot study; PT: Partial thickness; Ra: Randomized study; Retro: Retrospective study; SSG: Split skin graft; TBSA: Total burned surface area.

Table 1. Overview of clinical trials including commercially available epidermal substitutes in burn patients (cont.).

Product name (manufacturer)	Website	Source	Study design	Application area	Patients/wounds treated (n)†	Intervention	Results	Ref.
Laserskin®‡ (Fidia Advanced Biopolymers, Abano Terme, Italy; product formerly known as Vivoderm)	www.fidiapharma.it	Au	Ra, P	Deep dermal wound (removal tattoo)	20 (wounds)	Partial or total esterified hyaluronic acid matrices	Less wound contraction and stimulation of neoangiogenesis using a tissue-engineered dermal substitute after 2 weeks dermal regeneration of the wound bed using hyaluronic acids matrices. Recommend the total esterified material	[88]
			Ca	Reconstructive surgery	3	Noncomparative	Eliminates skin harvesting, creating a painful donor site. Initial take rate of 50–100% in three patients (time undefined)	[89]
Spray								
HP802-247‡ (Healthpoint Ltd, TX, USA)	www.smith-nephew.com	Al	Ra, Pi	Mmsw	8	HP802-247 vs bacitracin ointment	HP802-247 was found to provide a modest, incremental benefit in the healing of Mmsw. Scores for signs and symptoms and scar were similar for both groups but, in general, were numerically better for HP802-247	[90]
Keraheal™ (MCTT, Seoul, South Korea)	www.mctt.co.kr	Au	Retro	FT bw	12	NS	Good potential to save lives by providing epidermal cover	[91]
			C	FT bw	29	Noncomparative	Enhanced the take rate of a wide meshed autograft in massive burns	[92]
ReCell®‡ (Avita Medical, MA, USA [formerly Clinical Cell Culture Europe Ltd, Cambridge, UK])	www.avitamedical.com	Au	Ra,P, Pi	PT bw	13	ReCell + Biobrane vs Biobrane only vs standard treatment	Early intervention (ReCell + Biobrane and Biobrane only) was associated with a decreased time to healing with fewer dressing changes, less pain and better scar outcomes	[93]

†Data represent numbers of patients treated unless indicated otherwise.
‡Product also investigated in clinical trials on other skin diseases (e.g., ulcers and/or epidermolysis bullosa and/or congenital melanocytic nevus).
Al: Allogeneic; Au: Autologous; bw: Burn wound; C: Cohort study; Ca: Case report; FT: Full thickness; Mmsw: Mohs micrographic surgery wounds; NS: Not specified; P: Prospective study; Pi: Pilot study; PT: Partial thickness; Ra: Randomized study; Retro: Retrospective study; SSG: Split skin graft; TBSA: Total burned surface area.

Table 1. Overview of clinical trials including commercially available epidermal substitutes in burn patients (cont.).

Product name (manufacturer)	Website	Source	Study design	Application area	Patients/wounds treated (n)†	Intervention	Results	Ref.
			NS	Vitiligo	15	ReCell vs adjacent normally pigmented area	ReCell is a feasible, simple and safe technique. Repigmentation >75% was recorded in 12 (80%) and 25–50% repigmentation in three (20%) of 15 patients treated	[94]
			Pi	Vitiligo	5	ReCell vs DMEM	Repigmentation was comparable for both the techniques. Studies on larger series of patients are required to confirm the effect of ReCell to treat vitiligo	[95]
			Ra, P	Deep PT bw.	82	ReCell vs SSG	Gives similar results to SSG but harvesting minor areas, resulting in less pain on the biopsy site, can open possible future applications in the management of large-burns patients	[96]

†Data represent numbers of patients treated unless indicated otherwise.
‡Product also investigated in clinical trials on other skin diseases (e.g., ulcers and/or epidermolysis bullosa and/or congenital melanocytic nevus).
AI: Allogeneic; Au: Autologous; bw: Burn wound; C: Cohort study; Ca: Case report; FT: Full thickness; Mmsw: Mohs micrographic surgery wounds; NS: Not specified; P: Prospective study; Pi: Pilot study; PT: Partial thickness; Ra: Randomized study; Retro: Retrospective study; SSG: Split skin graft; TBSA: Total burned surface area.

filled the criterium 'epidermal substitute'. One of these papers concerned Apligraf® (Organogenesis, Inc., MA, USA) and is described in Table 1. The other three papers described customized dermal substitutes that were combined with epidermal cultured cells [78–80]. All three papers reported an initial reduced graft take, in seven children [80], and 17 [78] and 25 adult patients [79]. This phenomenon is now known to occur frequently after application of a dermal substitute followed by immediate grafting [81]. Separately, a search was carried out on all of the product names as described in Table 1.

Some epidermal substitutes were investigated in clinical trials on other skin diseases than burn wounds. Examples of these products are: BioSeed®-S (BioTissue Technologies AG, Freiburg, Germany) [97], Tiscover® (A-Skin BV, Amsterdam, The Netherlands) [98], EpiDex® (Euroderm, Leipzig, Germany or Modex Therapeutics, Lausanne, Switzerland) [99–101], MySkin® (Altika Ltd, Sheffield, UK, Formerly named Trancell) [102] and Celaderm™ (Advanced BioHealing Inc., NY, USA) [103]. Since this review focuses on application in burn wound healing, these products are not discussed further.

There are several full skin substitutes available today. Apligraf is a bilayer cellular substitute that consists of a type 1 bovine collagen sponge combining human allogeneic neonatal dermal fibroblasts and human allogeneic neonatal keratinocytes. Waymack *et al.* demonstrated effective treatment with cosmetic and functional improvement in a clinical trial in partial- and full-thickness burn wounds [85]. It is worth noting that the original design of the clinical trial protocol showed significant graft loss, because Apligraf was placed directly upon the prepared wound bed and a meshed autograft was transplanted on top of Apligraf. However, in the described clinical trial, the substitute was applied over meshed autografts, resulting in substantially less graft loss.

Hu *et al.* performed a clinical trial in acute partial-thickness donor site wounds in patients who required treatment with a split-thickness skin graft for other purposes than burn injuries [82]. Their aim was to provide knowledge on the role of Apligraf in the mechanisms of wound healing at a molecular level [82]. Eight patients completed the study. Besides confirmation of the safety of this substitute, the described success in wound healing could not be related to the sustained presence of Apligraf-specific cells or on basement membrane restoration by Apligraf.

OrCel™ is another bilayer cellular substitute containing a layer of cultured human allogeneic neonatal dermal fibroblasts and a layer of cultured human allogeneic neonatal epidermal keratinocytes in a type 1 bovine collagen sponge. OrCel is used in epidermolysis bullosa [104,105] and partial-thickness wounds from split-thickness skin graft donor sites [86]. No studies were found including OrCel in treatment of the burn wound itself.

Autologous epidermal cell sprays are described by Wood *et al.* in several reports [93,106–108]. They show that the delivery of cells using a spray method is feasible in porcine wound models. A clinical pilot study including scald burn in children is described in Table 1 [93].

Recently, a study was accomplished in 28 patients with post burn hypopigmentation [109]. A biopsy from unaffected skin was taken and prepared to epidermal cell suspension. In the first group (n = 18) the epidermal cell suspension was sprayed on a debrided wound bed and in the second group (n = 10) the epidermal cell suspension was injected in the hypopigmented skin. A limited effect was shown on pigmentation of hypopigmented skin and the difference between the cell spray and intradermal injection methods was not evident.

In 2011 Gerlach *et al.* published a case report: a 43-year-old male burn patient with a deep partial-thickness defect of 7% TBSA was treated with autologous epidermal cell spray [110]. The authors did not find any complications, but the effectiveness of the cell spray was not proven in this single individual case report.

Keraheal™ is an autologous cell spray containing preconfluent autologous keratinocytes. Two clinical studies reported on the use of this technique in severely burned patients with full-thickness wounds. The first study from Yim *et al.* was a prospective clinical trial that described a two-step procedure [92]. First, early wound excision was achieved and the wound was grafted with cadaveric skin. In the second step the cadaveric skin was removed and the patients received an 1:4–6 meshed split-thickness skin autograft, followed by the application of autologous cultured preconfluent keratinocytes in combination with a fibrin sealant spray. This procedure was performed in 29 patients of whom 13 also received an acellular dermal matrix directly onto the excised wound bed before application of the meshed autograft and cell spray. The authors concluded that the cultured cells in combination with the acellular matrix and the fibrin spray resulted in successful grafting with a take rate of more than 95% after 2 weeks. In a retrospective study Lee reported on 16 severely burned patients treated with Keraheal and stated that transplantation of cultured epithelial cells with wide meshed autograft can preserve donor sites for the severely burned patient, but the clinical value for the patient and wound quality was not proven [91].

Although various studies in animal models of burn wounds were performed to explore the beneficial effects of stem cells on the healing process of burn wounds clinical studies with MSC therapy in burns are sparse.

In 2005 Rasulov *et al.* described the clinical application of allogeneic bone marrow-derived MSCs in a patient with severe burns (40% TBSA and 30% full thickness) [111]. They reported accelerated wound healing of both the burn wound and the split-thickness skin graft donor site, and activation of neovascularization. The accelerated healing of the donor site allowed a second harvesting of a skin graft in this location 13 days after the first transplantation.

The group of Lataillade reported two case studies on the treatment of radiation burns with bone marrow-derived MSCs [112,113]. These wounds are difficult to treat with conventional surgical methods due to inflammatory waves for months or years after the exposure to radiation. These inflammatory waves are responsible for further expansion of the wounded area. Repetitive applications of bone marrow-derived MSCs resulted in long-time stable healing of the wounds, probably through suppression of the inflammatory waves.

To achieve restoration of skin appendage Sheng *et al.* applied the procedure described by their group earlier in a rat model on a patient undergoing scar reconstruction [114]. Bone marrow-derived MSCs were incubated with heat shock-treated sweat gland cells to induce sweat gland cell differentiation of the MSCs. In the patient two scars were excised; one wound was treated with the differentiated MSCs in combination with decellularized allogeneic dermal matrix and an autologous skin graft and the other wound was treated the same but with the exclusion of the differentiated MSCs. Two months later the iodine-starch test revealed that the healed wound treated with the differentiated MSC produced sweat.

These few clinical case studies show that indeed MSC therapy could improve burn wound healing and even regenerate skin appendages. Although most of the studies were performed with bone marrow-derived MSCs there are indications that adipose tissue, possible through the action of adipose-derived MSCs, also improves the outcome of burn wound healing. Application of lipoaspirate in 20 patients with radiotherapy tissue damage showed systematic improvement or even remission of symptoms in all patients [115]. Klinger *et al.* treated patients with hypertrophic scar by injecting subcutaneous fat at the dermohypodermal junction. They show that this lipofilling technique improved scar quality [116].

Although these studies show the potential of the use of MSCs in burn wound treatment, clinical trials have to be performed to prove safety and efficacy. Therefore, it is exciting that the first clinical trial (Phase I/II) for the treatment of large burns with cadaveric bone marrow MSCs has been announced [117].

Most autologous cultured epidermal substitutes for burn wounds are still in the clinical trial phase and are not yet commercially available. Companies that produced such products for the market earlier frequently experienced problems with reaching an acceptable level of funding. The limited commercial volume of the products, high production costs and increasing demands in terms of regulatory issues represent some of the challenges for small biotechnology companies. The quality of most described autologous substitutes, in all the different application techniques, are promising, but the lack of evidence still exists. More research is necessary before cultured human autologous epidermal substitutes are the standard of care for burn patients.

Wound dressing

Application of cell-based therapies, such as cultured keratinocytes, to a wound bed induced new challenges regarding the choice of the perfect wound dressing material. The use of antimicrobial wound dressings is essential, especially for burn wounds, as they are susceptible for microbial contamination and infection. This is not only caused by the lack of a skin barrier function but also because the host defense mechanism in these patients is usually impaired. Application of cultured skin is not the solution for the prevention of wound infection. Although the autologous cultured cells can contribute to a faster epithelialization of the wounds and thereby decrease the time for the pathogens to colonize the wounds, the use of antiseptic wound dressings is unavoidable. An extensive range of different types of antiseptic wound dressings are available for the best treatment of open wounds. However, their affectivity on microorganisms and the wound healing effects might be in conflict [118]. The choice of wound dressing material to be used in combination with cultured skin is of great importance, since most of the antiseptic dressings are also toxic for cultured cells and decrease cell viability, including proliferating keratinocytes and fibroblasts [119,120]. Various studies have been performed to address the cytotoxic effect of topical wound treatment on cells [121–124]. Most of the studies focus on the silver-containing products and dressings because of their wide spread use and their high potential for antimicrobial activity, but they are also highly cytotoxic for human cells. Poon *et al.* even recommend that silver-based products should be avoided if possible as a topical

antimicrobial strategy when using undifferentiated cultured keratinocyte applications [120].

Le Duc *et al.* have shown in an *in vitro* study that most antiseptics used by clinicians in burn care are also cytotoxic to autografts and human skin substitutes [125]. Commonly used antiseptic ointments used to treat acute burn wounds are Betadine® (Viatris Manufacturing BV, Diemen, The Netherlands), Furacine® (Norgine BV, Amsterdam, The Netherlands), Fucidin® (Leo Pharma BV, Breda, The Netherlands), cerium–silver sulfadiazine cream and silver sulfadiazine cream. From those, Fucidin showed the least cytotoxicity effect to autografts and human skin substitutes. In general, nonadhesive dressings such as Acticoat® (Smith & Nephew BV, Hoofddorp, The Netherlands) and Aquacel Ag® (ConvaTec, Woerden, The Netherlands) were found to be less cytotoxic to skin substitutes than creams and ointments [125].

More knowledge on the effects of combining cultured cells and antimicrobial wound dressings and creams is highly important to enable new developments for cell therapy in burn wound care.

In general, donor site wounds are contaminated to a lesser extent in comparison with acute burn wounds. Campanella *et al.* compared the effectiveness of two nonantiseptic dressings, SurfaSoft® (Taureon, Rotterdam, The Netherlands) and Mepitel® (Mölnlycke Health Care, Gothenburg, Sweden), on partial-thickness donor sites treated with ReCell® (Avita Medical, MA, USA), a spray system for an epithelial cell suspension [126]. Their results suggest that Mepitel because it is pliable, self-adhesive and the fact that it does not adhere to the wound resulting in less-painful removal during dressing changes, should be preferred.

Conclusion

Current clinical applications of cell therapy for burn wound healing are mainly in the epidermal substitute field. The application techniques have changed from fully differentiated epidermal sheets to transfer of undifferentiated, proliferating cells. The cell-transfer techniques have also changed from the fragile, difficult-to-handle full sheets to cell-carrier systems and various forms of cell sprays.

Clinical evidence of the efficacy of these cellular constructs has been gathered predominantly in leg ulcers. For burn wounds, only a limited number of patients have been treated with various forms of cellular constructs.

MSCs are thought to stimulate burn wound healing by their interaction with epidermal cells, and by exerting immunomodulatory effects in the wound area. Their clinical application is still rather limited at the moment.

Experimental developments have demonstrated a proof of principle for the production of prevascularized skin substitutes with seeded adipose-derived stem cells and stimulation of vascularization both by 3D structure and by addition of relevant growth factors. Re-innervation of artificial skin grafts as well as regeneration of hairs and sweat glands are also still in the experimental stage of research, but promising results have been obtained. Regeneration of fully functional and anatomically correct skin seems feasible.

Future perspective

The ultimate goal of regenerative medicine would be to replace and regenerate whole body organs. For skin this would mean the restoration of the complete structures and functions, including hair follicles, sweat glands, nerves and so on.

Many of these aspects are presently not yet available as a clinical treatment modality. However, on the horizon of experimental work, the contours of such new developments can be visualised. Inclusion of melanocytes to produce a correctly pigmented skin substitute has been recently described by several groups [127–130]. Clinical efficacy and safety were demonstrated for transfer of melanocytes to vitiligo patches [131]. Despite these 'proofs of principle', clinical application still requires elaborate safety studies.

For adequate blood supply in and on a (partial) skin substitute, several strategies can be followed. Different approaches towards a vascularised tissue-engineered product were recently reviewed by Auger *et al.* [132]. Introduction of living autologous or allogeneic endothelial cells into a biomaterial is one strategy. The concept relies on formation of new tubular structures during *in vitro* culture [133]. Prevascularization of skin substitutes by seeded adipose-derived stem cells is another variant of this concept. Indeed, Huang and colleagues demonstrated an increased vascularization after treatment of full-thickness wounds on nude mice with a dermal substitute seeded with adipose-derived stem cells [134]. Addition of angiogenic factors such as VEGF to a skin substitute is a different strategy, which stimulates a faster ingrowth and/or sprouting of existing vessels [135]. This can be achieved both by addition of growth factors, but also by choosing a specific composition or 3D structure of the substitute [136,137]. Even 3D printing could prove to be helpful in providing a predefined 3D structure to allow fast vascular ingrowth [138,139].

Research into regeneration of sensory functions after deep burns is still in its infancy. Nerve regeneration has mainly been studied in models of nerve transections. However, as a result of a greater aware-

ness of quality of healing, restoration of sensory skin functions, and correct regulation of temperature and pain are receiving increasing attention [140]. Innervation of newly formed dermal tissue was demonstrated in an experimental wound model in rats [141] as well as after clinical treatment [142], although these latter authors suggested that the use of dermal substitutes for treatment of deep burns might limit the sensory functions. Knowledge on how to stimulate nerve ingrowth, however, is still limited.

Hairs and sweat glands are also very important structures of the skin that are generally lost after deep burn injuries. Presently, hair transplantations and skin expansion provide a limited solution for these kind of pathologies [143,144]. Production of tissue-engineered skin grafts including regenerated hair follicles has been described in animal models (see [145] for a recent review). Potential improvements might also come from research on specific differentiation of stem cells. Currently, successful attempts to generate skin appendages in engineered skin substitutes *in vitro* have been described [146]. In a chimeric culture of murine and human cells, formation of hair follicles with pigmented hairs was demonstrated in skin substitutes grafted on athymic mice [146].

Recently, the influence of FGF9 on hair regeneration was demonstrated in mice [147]. Overexpression of this growth factor resulted in an increase in the formation of new hair follicles after wounding. This could open new therapeutic avenues. Nevertheless, further work is needed to incorporate such factors into a scaffold that allows *in vitro* and *in vivo* development of follicle structures.

Traditionally, the cells used for cell therapy are harvested from specific tissues from the body and most of the time are expanded in tissue culture before they are delivered back to the patient. Because of the potential risks during processing these procedures are bound to very strict and demanding regulations. In addition, *in vitro* expansion may alter the cells. Therefore, endogenous cell recruitment to the site of injury is an alternative that is currently being investigated [148,149]. To be able to recruit sufficient numbers of MSCs and to be able to differentiate them into the specific cell types, the homing and differentiation signals should be elucidated [150]. These signals (e.g., VEGF or FGF9) can then be incorporated into smart (3D) scaffolds that are applied to the wound. Ideally, this *in situ* skin regeneration would also restore other functions of the skin through the creation of appendages and restoration of functions (e.g., sensory).

Financial & competing interests disclosure

This work was supported by the Dutch Burns Foundation project 07.116 and by the European Commission (FP7-HEALTH-2011-1, Grant Agreement no. 279024), project EuroSkinGraft. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Keratinocytes

- The application of a cultured epithelial autograft for the treatment of severe burns is hampered by long culture times, high costs, poor reliability in terms of graft take and disappointing long-term results of scar quality.
- New developments in cultured epithelial cells focus on proliferating cells, administered in sprays or on carriers, and clinical-grade culture systems.
- Goals to achieve in optimizing production requirements for tissue-engineered skin are the elimination of xenobiotic cells and materials as well as the removal of antibiotics in the culture media.

Mesenchymal stem cells

- Mesenchymal stem cells (MSCs) are considered to be very promising for tissue engineering purposes due to their multilineage differentiation capacity and the immune-modulating effects.
- MSCs have been shown to possess the ability to differentiate into different lineages, *in vitro* as well as *in vivo*.
- Experimental research has been performed on a combination of epidermal replacements and MSCs.

Clinical studies with cell-based therapy

- Clinical trials in burns are available on epidermal substitutes, cell sprays and full-skin substitutes. However, the level of evidence is still low as comparative trials with measurable outcome parameters are scarce.
- Regarding the application of cell-based therapy in burn wounds antimicrobial wound dressings are essential as the burn wounds are susceptible to microbial contamination and infection.

Future perspective

- The development of vascularized skin constructs is currently in the experimental stage.
- Future research will focus on the regeneration of pigment, nerves, hairs and sweat glands, and eventually at *in situ* tissue regeneration.

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Research Article

Regenerative Medicine

Induced pluripotent stem cells have similar immunogenic and more potent immunomodulatory properties compared with bone marrow-derived stromal cells *in vitro*

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Aim: To evaluate the in vitro immunogenic and immunomodulatory properties of induced pluripotent stem cells (iPSCs) compared with bone marrow-derived mesenchymal stromal cells (MSCs). **Materials & methods:** Mouse embryonic fibroblasts (MEFs) were isolated from C3HeB/FeJ and C57BL/6J mice, and reprogrammed to generate iPSCs. Mixed leukocyte reactions were performed using MHC-matched and -mismatched responder leukocytes and stimulator leukocytes, iPSCs or MSCs. To assess immunogenic potential, iPSCs and MSCs were used as stimulator cells for responder leukocytes. To assess immunomodulatory properties, iPSCs and MSCs were cultured in the presence of stimulator and responder leukocytes. MEFs were used as a control. **Results:** iPSCs had similar immunogenic properties but more potent immunomodulatory effects than MSCs. Co-culture of MHC-mismatched leukocytes with MHC-matched iPSCs resulted in significantly less responder T-cell proliferation than observed for MHC-mismatched leukocytes alone and at more responder leukocyte concentrations than with MSCs. In addition, MHC-mismatched iPSCs significantly reduced responder T-cell proliferation when co-cultured with MHC-mismatched leukocytes, while MHC-mismatched MSCs did not. **Conclusion:** These results provide important information when considering the use of iPSCs in place of MSCs in both regenerative and transplantation medicine.

Keywords: allogeneic stem cell therapy • immunogenicity • immunomodulatory • induced pluripotent stem cell • mesenchymal stromal cell • transplantation medicine

Induced pluripotent stem cells (iPSCs) were first generated in 2006 and regarded as the most promising stem cell candidate for the clinical application of regenerative therapies [1–3]. iPSCs are pluripotent, unlike mesenchymal stromal cells (MSCs), and can be used in an autologous manner, unlike pluripotent embryonic stem cells (ESCs). Additionally, iPSCs avoid the ethical concerns surrounding the isolation and use of human ESCs [4]. However, many concerns have been raised over the safety of iPSCs in terms of genetic instability, tumorigenic potential and immunogenic potential [4–10]. It has become evident that iPSC lines must be thoroughly screened for stability, safety and efficacy prior to clinical application [7,10]. Such screening, after an already lengthy generation process, makes autologous iPSC use impractical for many of the diseases that would potentially benefit from stem cell therapy. Furthermore, it has been demonstrated that genetic background affects generation of iPSCs, suggesting that autologous iPSC therapy may not be feasible for some patients regardless of timing issues [11]. For these reasons, the immunogenicity of iPSCs is of particular concern as the need for having a bank of previously screened cells has become a reality [7,10,12].

The immunogenic and immunomodulatory properties of MSCs continue to be investigated so that these cells can be available at the time of diagnosis for immediate treatment [13–19]. Both the genetic background and age of the patient affect proliferation and differentiation rates of MSCs, suggesting that allogeneic

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MSC therapy may be required for some patients as for iPSCs [20–22]. Adult MSCs have low immunogenicity when used in an autologous manner and possess significant immunomodulatory properties [14,16,23–26]. Many mechanisms for the immunosuppressive effects of MSCs have been described including inhibition of T-cell proliferation, alteration of dendritic cell maturation, induction of regulatory lymphocytes and apoptosis of $CD8^+$ T cells [16,23,27–29]. Mesenchymal stem cells were initially believed to be immune privileged due to these immunosuppressive properties [30–33], but immune rejection of allogeneic MSCs has also been reported [34–39]. The finding that MSCs are capable of fluctuations in their MHC class I and II expression profiles is likely the cause of these conflicting results. MSCs from many species were originally described as having the phenotype of high MHC class I expression and low or negative MHC class II expression, but MSCs from mice, humans and more recently horses with high MHC II expression levels have also been described [34,35,40,41]. Additionally, both MHC class I and II expression levels on MSCs can be upregulated by proinflammatory cytokines such as IFN-γ [42,43]. These studies suggest that MSCs have a dynamic immunophenotype that can alter their immune status.

Investigation into the immunogenic properties and immune plasticity of iPSCs has just recently begun [7,12,44–47]. It is known that undifferentiated iPSCs, like ESCs, express low or absent levels of MHC class I, and are negative for MHC class II expression [47,48]. Unlike MSCs, iPSC MHC class II expression is not upregulated by differentiation or by stimulation with IFN-γ [47–50]. The extent to which MHC class I expression can change upon iPSC differentiation or stimulation with proinflammatory cytokines, however, is not understood. Several studies have shown increased MHC class I expression in iPSCs with differentiation or IFN-γ stimulation, but often to a level still much less than that of somatic cells [45,47,48]. The consequence of such a change in MHC class I expression is complex as a high expression level of MHC class I could lead to T-cell activation while a continued lack of MHC class I expression could result in iPSCs being targeted by natural killer (NK) cells *in vivo* [6,47]. Conflicting results have been reported for ESCs on this subject, with some groups reporting ESCs as susceptible to NK cell lysis, and others reporting that ESCs are neither susceptible to NK cell lysis nor capable of eliciting T-cell responses [6,51]. It is likely that culture conditions or differences in ESC lines could have affected these results.

It is not surprising that conflicting results have also been reported on the immunogenicity of iPSCs, as iPSCs are in many ways more variable than ESCs, particularly with the discrepancies in reprogramming methods including viral versus nonviral and integrating versus nonintegrating [44–47,49,52,53]. The first report on immunogenicity of iPSCs revealed that undifferentiated autologous (syngeneic) mouse iPSCs were immune rejected in a teratoma model study [44]. Two other reports since then have shown that both undifferentiated and differentiated syngeneic mouse iPSCs are non-immunogenic *in vitro* and *in vivo* [45,46]. To date, no studies have examined the immunomodulatory properties of iPSCs even though it is known that ESCs are capable of immunosuppression through multiple mechanisms including expression of arginase I [49,54], prevention of dendritic cell maturation [55] and upregulation of regulatory T cells [49,56]. When considering the use of iPSCs as an alternative for MSC therapy, this information is critical. The purpose of this study, therefore, was to evaluate the *in vitro* immunogenic and immunomodulatory properties of iPSCs compared with adult bone marrow-derived MSCs using modified mixed leukocyte reactions (MLRs). Our hypothesis, based on prior ESC knowledge, was that undifferentiated iPSCs would have similar immunogenic and immodulatory properties as MSCs.

Materials & methods

A schematic of the study design and methods is shown in Figure 1.

Mice

Male and female mice of the C3HeB/FeJ (MHC H*2* haplotype *k*) and C57BL/6J (MHC H*2* haplotype *b*) inbred strains were purchased from The Jackson Laboratory (ME, USA). For each strain, mice were bred to produce offspring needed to harvest different cell types and perform experiments. NOD.CB17-*Prkdc*^{scid}/J mice, used for teratoma formation assays, were also purchased from The Jackson Laboratory. The use of mice in this study was approved by the Institutional Animal Care and Use Committee of Cornell University.

Mouse embryonic fibroblast isolation

Embryonic day 13.5 C3HeB/FeJ and C57BL/6J embryos were collected and processed to generate mouse embryonic fibroblasts (MEFs) from each strain, as previously described [11]. MEFs were cultured in MEF media (high-glucose Dulbecco's Modified Eagle's Medium [DMEM]), containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml) and cryopreserved at passage 1 (P1) for iPSC generation and at P2 for controls in MLR experiments. MEFs to be used as feeder cells from each strain were culture expanded, irradiated with 30 Gy from a Cs-137 source, and cryopreserved.

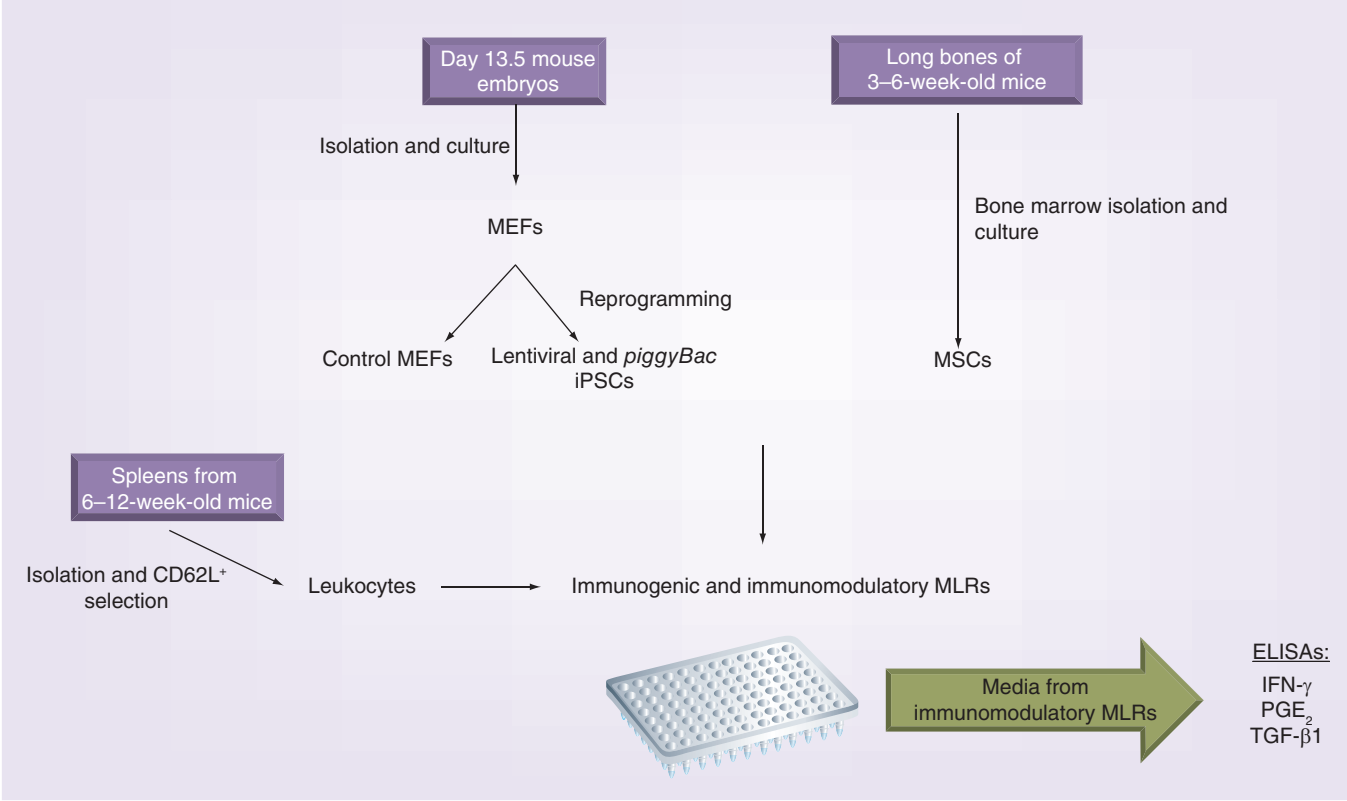

Figure 1. Schematic of the study design and methods used.
iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibroblast; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stem cell.

Lentiviral reprogramming of MEFs

Lentiviral supernatant generation and reprogramming of MEFs was performed as previously described by our laboratory [11] using vectors for doxycycline-inducible transgene expression of the mouse factors *Oct4*, *Sox2*, *Klf4* and *c-Myc*. All plasmids were purchased from Addgene (MA, USA). Briefly, P1 MEFs from each strain were thawed and cultured in MEF media for 24–48 h, after which they were trypsinized and counted. The P2 MEFs were seeded onto gelatin-coated six-well tissue culture plates at a density of 6.75×10^3 cells/cm^2 in MEF media and allowed to adhere for 24 h. Culture media was replaced with fresh MEF media supplemented with viral supernatant for an additional 24 h. Following incubation with viral media, MEFs were trypsinized and passaged onto 60 mm tissue culture plates seeded with feeder cells of the same strain. Culture media was changed to ESC media (KnockOut™ DMEM (Gibco, NY, USA) supplemented with 15% KnockOut™ Serum Replacement (Gibco), recombinant LIF, MEM non-essential amino acids solution (100 µm), 2 mM GlutaMAX™ (Gibco), 0.1 mM 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 µg/ml) and doxycycline (2 µg/ml; Sigma, MO, USA). Media was refreshed daily during reprogramming.

piggyBac reprogramming of MEFs

Passage 2 MEFs were transfected with the Nucleofector® II electroporation device (Amaxa Biosystems, MD, USA) set on program A-023. Each electroporation was performed in a 2-mm cuvette (Amaxa Biosystems) with 2×10^6 cells and a DNA mixture of 1 µg each of the *piggyBac* plasmids PB-TET-MKOS, PB-CAG-rtTA and PB-CAG-GFP (kindly provided by the laboratory of Dr Nagy [57]), as well as 1 µg of the transposase expression vector pCyL43 (Wellcome Trust Sanger Institute, Cambridge, UK) in a total volume of 100 µl Ingenio® electroporation solution (Mirius Bio, WI, USA). Following electroporation, cells from each cuvette were seeded onto a 100-mm tissue culture plate in MEF media. After 24 h, culture media was changed to ESC media.

iPSC line generation

Lentiviral and *piggyBac* iPSC colonies were picked with pipette tips and culture expanded on feeder cells in ESC media, as previously described [11]. Lentiviral iPSC colonies were picked on day 7–11 of reprogramming, while *piggyBac* iPSC colonies were picked on day 17–22 post-transfection. Doxycycline was removed from media around P7 and doxycycline-independent cell lines were then further expanded (P10-P12) in

order to reach cell numbers necessary for teratoma formation assays and cryopreservation of stock from each strain. In preparation for MLR experiments, iPSC cell lines from each strain were further cultured in modified RPMI 1640 media containing 10% FBS, 0.1 mM 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 µg/ml), and ESGRO® LIF (1 µl/ml; Millipore, MA, USA). Following transition to modified RPMI 1640 media, teratoma assays were again performed.

Teratoma formation & histological analysis

iPSC lines from each strain were trypsinized, pelleted and suspended at 1×10^7 cells/ml in a 1:3 solution of Matrigel™ (BD Biosciences, CA, USA) to MEF media. Of this cell suspension, 150 µl (1.5×10^6 cells) was injected subcutaneously into the flank of a NOD. CB17-*Prkdc*^{scid}/J mouse [11]. For each cell line, a total of two to four injections were performed. A total of 4–5 weeks post injection, tumors were surgically dissected, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. All histologic sections were reviewed by a board-certified veterinary pathologist (Teresa L Southard) for teratoma formation.

Bone marrow harvest & isolation of MSCs

Ten female mice 3–6 weeks of age from each strain were euthanized, prepared with ethanol and processed for bone marrow harvest according to a protocol kindly provided by the laboratory of Dr Rocky S Tuan [Pers. Comm.]. Hindlimbs were skinned, disarticulated from the pelvis, and placed in a petri dish with MEMα media with nucleosides and L-glutamine (Gibco) containing 10% FBS, penicillin (100 units/ml), streptomycin (100 µg/ml) and Fungizone® (0.25 µg/ml; Gibco). All muscle and tissue was removed from the bone using a scalpel blade and placed in a second petri dish with phosphate-buffered saline (PBS). Next, the ends of the long bones were cut off so that the marrow cavity was exposed. The marrow cavity of each bone was flushed with MEMα media using a 27-g needle and 12-ml syringe into an empty petri dish. The bone marrow cell suspension was then passed through a 70-µm cell strainer (BD Biosciences, CA, USA), pelleted, resuspended in red blood cell lysis buffer (0.84% NH_4Cl), and incubated for 2 min on ice. Following the incubation, cells were washed with MEMα media, counted and seeded at 25×10^6 cells/ml onto 100-mm tissue culture plates with MEMα media. After 5 h of incubation, media was removed, plates were gently washed to remove non-adherent cells, and new MEMα media containing FGF-2 (5 ng/ml) added. For the next 72 h, media exchange occurred every 12 h; afterwards media exchange occurred every 72 h. When cells were approximately 80% confluent, they were trypsinized at room temperature for 2 min, counted, and seeded at 5000 cells/cm^2 onto T-75 flasks with MEMα media containing FGF-2 (5 ng/ml). Cells were expanded to P2 and P3, and stocks were cryopreserved for immunophenotyping and MLR experiments.

Immunophenotyping of MEFs, iPSCs & MSCs

Mouse embryonic fibrobasts (MEFs), iPSCs and MSCs were immunophenotyped for expression levels of MHC class I and II. MSCs were additionally phenotyped for a panel of positive (CD44, CD29) and negative (CD45, CD117) markers using flow cytometry [58–60]. Leukocytes were used as a control cell type. MHC class I (rat anti-mouse; PE-conjugated), MHC class II (rat ant-mouse; PE-Cy5-conjugated) and CD29 (hamster anti-mouse; PE-conjugated) antibodies were purchased from eBioscience (Affymetrix, CA, USA). CD44 (rat anti-mouse; FITC-conjugated), CD45 (rat anti-mouse; PerCP-Cy5.5) and CD117 (rat anti-mouse; APC-conjugated) antibodies were purchased from BD Biosciences. Cells were pelleted in aliquots containing approximately 1×10^6 cells on 96-well V-bottom plates and treated with a 10-min blocking step using anti-mouse CD16/CD32 (Fc Block™; BD Biosciences) at 1:100 in PBS. Cells were pelleted and resuspended in conjugated primary antibody and incubated for 45 min at 4°C. Cells were then washed, resuspended in PBS and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, CA, USA) flow cytometer equipped with 488-µm argon and 635-µm red diode lasers and BD Cell Quest™ analysis software (BD Biosciences). Cells exposed to appropriately conjugated rat or hamster IgG were used as negative isotype controls. Data were collected on 2×10^4 cells for each sample.

Splenocyte isolation & leukocyte purification

Spleens were aseptically harvested from C3HeB/FeJ and C57BL/6J female mice 6–12 weeks of age and dissociated in RPMI 1640 media (Gibco) using a cell dissociation sieve equipped with a 40-mesh screen (Sigma-Aldrich). The resultant splenocyte suspension was passed through a 100-µm cell strainer (BD Biosciences), pelleted, resuspended in red blood cell lysis buffer (0.84% NH_4Cl) and incubated for 5 min at room temperature with rocking. Following red blood cell lysis, the suspension was washed with PBS, pelleted and purified using Lympholyte®-M density gradient centrifugation (Cedarlane Laboratories, NC, USA) according to the manufacturer's directions to obtain leukocytes. Cells destined to be stimulator leukocytes in MLRs were aliquoted at this time. The remaining leukocyte suspension was plated onto

100-mm tissue culture plates in RPMI 1640 medium (Gibco) containing 10% FBS, 0.1 mM 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 units/ml). After 2 h, nonadherent cells were removed from plates, pelleted and counted. Nonadherent leukocytes were then positive selected for CD62L, a naive T-cell marker [61–64], using MACS CD62L microbeads and LS columns (Miltenyi Biotec, CA, USA), according to manufacturer directions. Adherent leukocytes (containing antigen-presenting cells [APCs]) were dissociated using Accumax® cell dissociation solution (Innovative Cell Technologies Inc, CA, USA), counted and aliquoted. All leukocytes were used fresh in MLRs.

Modified one-way MLRs

Modified one-way MLRs were performed in duplicate in 24-well tissue culture plates using MHC-matched and -mismatched C3HeB/FeJ responder leukocytes and C3HeB/FeJ and C57BL/6J stimulator leukocytes, MEFs, iPSCs and MSCs. In order to assess immunogenic potential of cells, MEFs, iPSCs and MSCs were used as stimulator cells for responder leukocytes. In order to assess the immunomodulatory properties of cells, MEFs, iPSCs and MSCs were cultured in the presence of stimulator and responder leukocytes. MHC-matched stimulator leukocytes were used to establish baseline T-cell proliferation and MHC-mismatched stimulator leukocytes were used as positive MLR controls. MEFs were considered the negative control in immunomodulatory potential studies. C3HeB/FeJ responder leukocytes were labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE [0.13 µg/ml], Sigma-Aldrich) and examined at four different concentrations (2×10^5, 4×10^5, 8×10^5 and 1.2×10^6 cells/well). The proliferative ability of responder cells was verified via mitogen stimulation with phytohemaglutinin (PHA-P [5 µg/ml], Sigma-Aldrich). Stimulator MEFs, iPSCs and MSCs were plated 24 h prior to addition of responder leukocytes in their appropriate media such that all cells would be approximately 80% confluent by the end of the experiment. MEFs were seeded at 1×10^4 cells/well, iPSCs (removed from feeders) at 7.5×10^4 cells/well, and MSCs at 3×10^4 cells/well. Stimulator leukocytes were irradiated with 9 Gy from a Cs-137 source to inhibit proliferation and plated at 1.6×10^6 cells/well. Responder APCs (adherent cells during isolation) were plated at 1×10^5 cells/well. Importantly, responder leukocytes and APCs were mixed with stimulator leukocytes prior to plating due to concern that the 3D nature of the iPSC colonies could interfere with responder and stimulator cell contact. The resultant ratios of responder:stimulator cells was based on previously published experimental protocols and determined to be optimal for these studies in preliminary experiments [65–67]. Cultures were maintained for 5 days with modified RPMI 1640 media (1.5 ml/well) containing 10% FBS, 0.1 mM 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 µg/ml), and ESGRO® LIF (1 ul/ml; Millipore, MA, USA). Media were not exchanged over the 5 days. Following culture, leukocytes were aspirated from wells and stained with a hamster anti-mouse APC-conjugated CD3 antibody (Abcam, MA, USA). The antibody staining process for flow cytometry analysis was performed as described above for immunophenotyping.

Proliferation of gated CFSE-labeled $CD3^+$ responder T cells was evaluated via CFSE attenuation using flow cytometry. Cells were first gated on FL4 so that only $CD3^+$ cells (T cells) were then examined on FL1 for CFSE attenuation. Nonstimulated responder T cells were used to set the boundary of nonproliferating cells such that all cells to the left (lower fluorescence intensity on FL1) of that boundary were determined to be proliferating. Because the number of cell counts in the proliferating T-cell gate was measured, data was collected on the entirety of each sample.

MLRs were performed in a total of three separate experiments. MEFs from two different embryos of each strain were tested in addition to three iPSC lines (two lentiviral and one *piggyBac*) from each strain and batched MSCs from each strain. Due to naturally occurring variation in leukocyte responses between mice and experiments, the relative T-cell proliferation was reported as the fold change from that of MHC-matched MLR for the immunogenic potential experiments (i.e., looking for an increase from baseline T-cell proliferation potential if immunogenic) and as the percentage proliferation of MHC-mismatched MLR for the immunomodulatory potential experiments (i.e., looking for a decrease from positive control T-cell proliferation if immunomodulatory).

Measurement of cytokine concentrations in MLR media

Media from immunomodulatory MLR experiments were harvested after centrifugation to pellet leukocytes for flow cytometry and stored at -80°C in aliquots with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche, IN, USA). Media from control MHC-mismatched MLRs and MHC-mismatched MLRs cultured in the presence of matched or mismatched MCSs or iPSCs were pooled according to experimental group and assayed for active IFN-γ, prostaglandin E_2 (PGE_2), and TGF-β1 concentrations using the IFN-γ Quantikine ELISA kit (R&D

Systems, MN, USA), the Prostaglandin E2 ELISA kit (Abcam, MA, USA) and the TGF β1 Quantikine ELISA kit (R&D Systems), respectively.

Statistical analyses

Mixed leukocyte reaction (MLR) data for lentiviral (average of the two lines tested) and *piggyBac* iPSC lines were first compared using two-sample t-tests. All MLR data were normalized by log transformation and analyzed with analysis of covariance (ANCOVA), with experiment as a covariate, followed by a Tukey multiple comparisons test. All ELISA data were determined to be normally distributed via the Shapiro–Wilk test and were analyzed by analysis of variance (ANOVA) followed by a LSD multiple comparisons test. Analyses were performed using Statistix 9 software (Analytical Software, FL, USA) and significance was set at $p \leq 0.05$.

Results

iPSC line generation & validation

Multiple doxycycline-independent lentiviral and *piggyBac* iPSC lines were established from each strain and early passage stocks were cryopreserved. Two doxycycline-independent lentiviral iPSC lines and one doxycycline *piggyBac* iPSC line from each strain was tested after expansion in ESC media and then again after expansion in modified RPMI 1640 media. These lines were capable of producing teratomas in NOD.CB17-*Prkdc*^{scid}/J mice by 5 weeks post injection (Supplementary Figure 1; please see online at www.futuremedicine.com/doi/full/10.2217/rme.14.29), thereby confirming pluripotency and lack of alteration due to the change in media.

Immunophenotyping

MEFs had a phenotype of MHC class I positive (low) and MHC class negative, while iPSCs had a phenotype of both MHC class I and II negative (Supplementary Table 1). Mesenchymal stromal cells (MSCs) were positive for expression of MHC class I, CD44 and CD29, and negative for expression of MHC class II, CD45 and CD117 (Supplementary Figure 1).

Modified one-way MLRs

There were no significant differences in responder T-cell proliferation when stimulated by lentiviral iPSCs or *piggyBac* iPSCs, or when stimulated by MHC-mismatched leukocytes in the presence of lentiviral iPSCs or *piggyBac* iPSCs. Cells were therefore considered one group and are collectively referred to as iPSCs for the remainder of the results.

As predicted based on MHC class II expression, all cell types tested (MEFs, iPSCs and MSCs) had low immunogenicity when either MHC-matched or MHC -mismatched with responder leukocytes (Figures 2 & 3). MHC-mismatched MSCs resulted in the highest levels of responder T-cell proliferation compared with MEFs and iPSCs, but these levels still did not reach those of

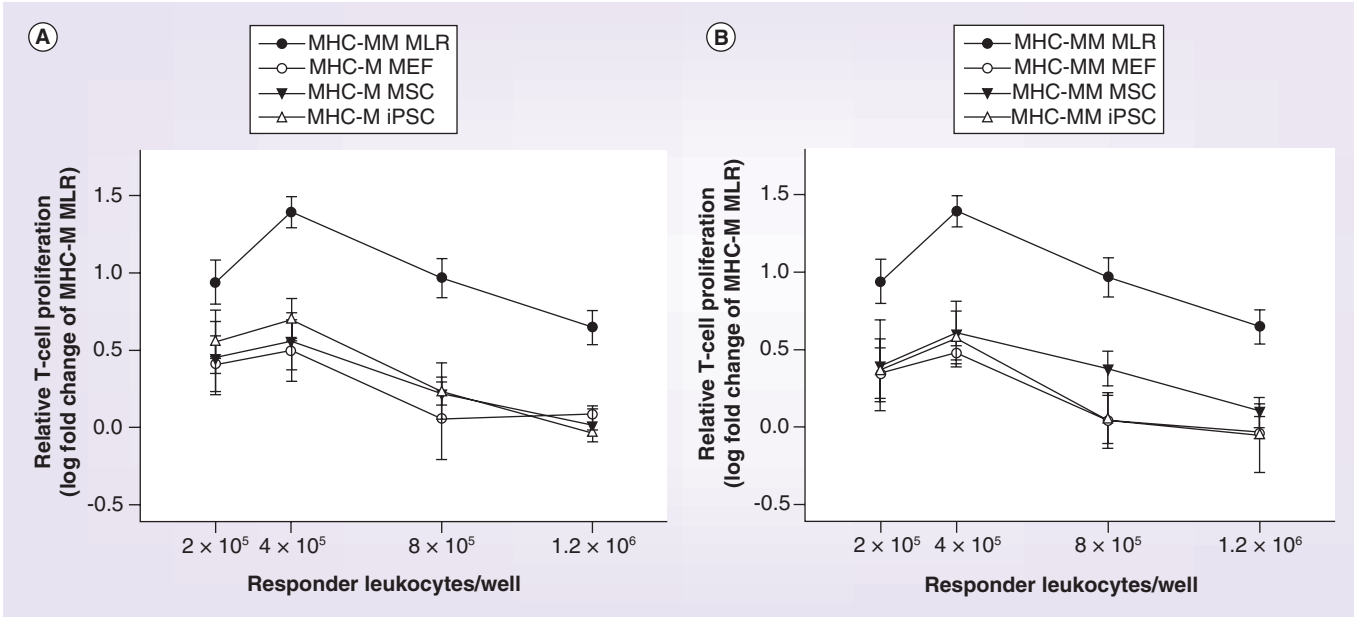

Figure 2. Immunogenicity (A) of MHC-matched, and (B) MHC-mismatched MEFs, MSCs and iPSCs as determined by responder T-cell proliferation in modified one-way mixed leukocyte reactions. Data are presented as the log fold change of MHC-matched MLR, which was considered the baseline responder T-cell proliferation value. Bars represent mean ± SD from a total of three separate experiments performed with multiple cell lines.
MHC-M: MHC-matched; MHC-MM: MHC-mismatched; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stromal cell.

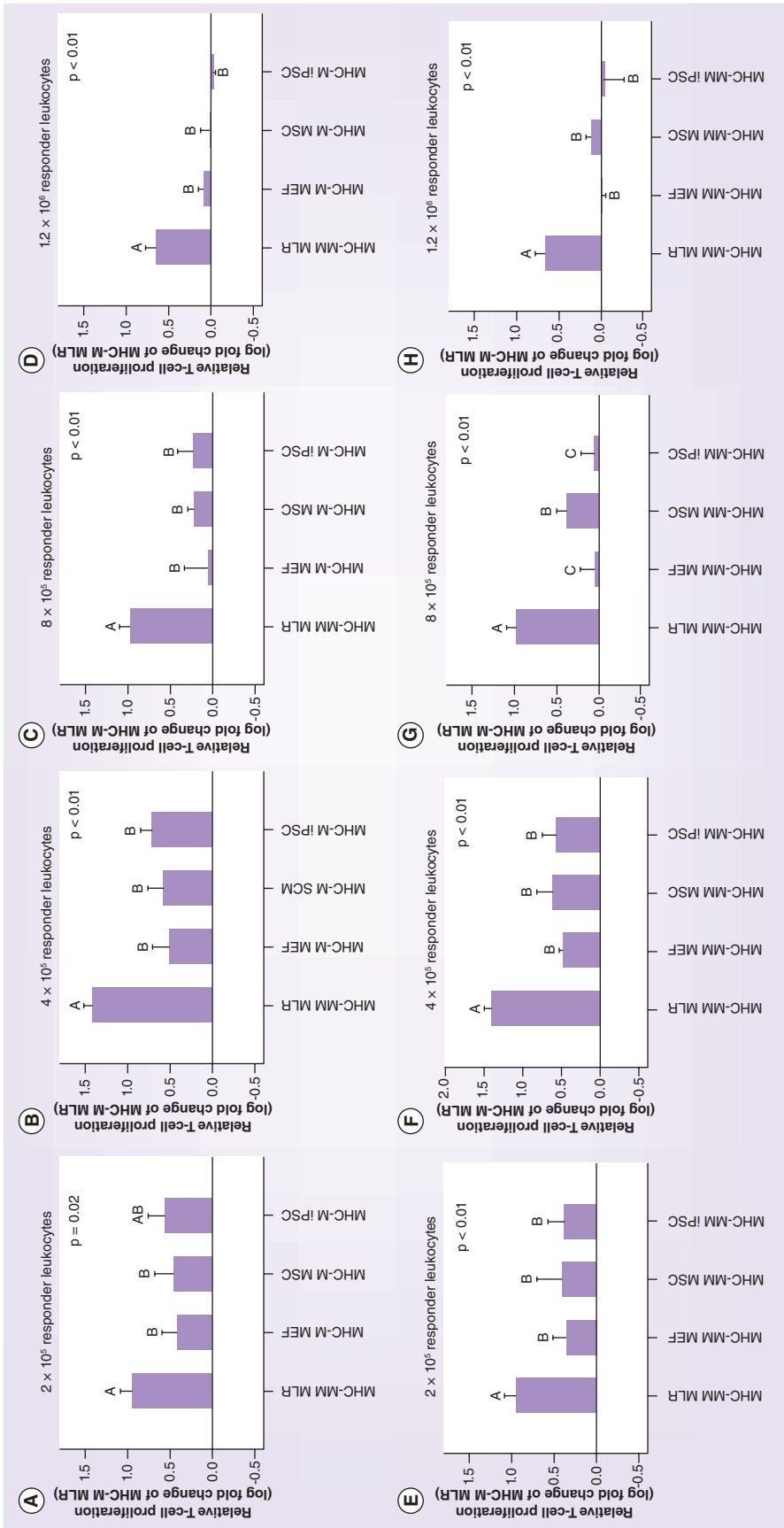

Figure 3. Immunogenicity of mouse embryonic fibroblasts, mesenchymal stem cells and induced pluripotent stem cell as determined using modified one-way mixed leukocyte reactions and with data reported as the relative T-cell proliferation, which was calculated as the log fold change from MHC-matched MLR. MLR results as shown in Figure 2 are displayed here for MHC-matched cells **(A–D)** and MHC-mismatched cells **(E–H)** at the different responder leukocyte concentrations tested. Bars represent mean ± SD from a total of three separate experiments performed with multiple cell lines. Superscript letters indicate significant differences between groups by analysis of covariance, with experiment as a covariate, followed by a Tukey multiple comparisons test, $p \leq 0.05$.
iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibrobast; MHC-M: MHC-matched; MHC-MM: MHC-mismatched; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stromal cell.

the positive control of MHC-mismatched leukocytes (Figure 3E–H).

At the majority of responder leukocyte concentrations tested, both iPSCs and MSCs cultured in the presence of MHC-mismatched responder and stimulator leukocytes (MHC-mismatched MLR) resulted in a reduction of responder T-cell proliferation from that observed for the MHC-mismatched MLR baseline value (Figures 4 & 5). Importantly, MEFs cultured in the presence of MHC-mismatched responder and stimulator leukocytes were unable to reduce responder T-cell proliferation in any of responder leukocyte concentrations tested. Reduction of responder T-cell proliferation was greatest when iPSCs and MSCs were MHC-matched with responder leukocytes compared with when MHC-mismatched with responder leukocytes. MHC-matched iPSCs resulted in significantly decreased responder T-cell proliferation compared with both MHC-mismatched MLR and the negative control of MEFs at the three highest responder leukocyte concentrations (Figure 5B–D). While MHC-matched MSCs resulted in statistically equivalent responder T-cell proliferation compared with iPSCs for the same three responder leukocyte concentrations, mean T-cell proliferations were greater than for iPSCs and in some cases also equivalent to MHC-mismatched MLR and/or the negative control of MEFs (Figure 5B–D). When MHC-mismatched cell types were evaluated, only iPSCs were able to reduce responder T-cell proliferation significantly from MHC-mismatched MLR at the responder leukocyte concentration of 8×10^5 cells (Figure 5G). Once again, while MHC-mismatched MSCs resulted in statistically equivalent responder T-cell proliferation compared with iPSCs at this concentration, the mean T-cell proliferation was greater than for iPSCs and also equivalent to that of MEFs and the positive control of MHC-mismatched leukocytes (Figure 5G).

Measurement of cytokine concentrations in MLR media

Based on the immunomodulatory results described above, only media from the immunomodulatory MLR experiments at the responder leukocyte concentrations of 4×10^5 and 8×10^5 cells were assayed. At both responder leukocyte concentrations tested, there was a significant reduction in the concentration of IFN-γ in the MLR media when MHC-mismatched leukocytes were cultured in the presence of matched MSCs, matched iPSCs or mismatched iPSCs. While the presence of matched MSCs resulted in the lowest mean IFN-γ for all groups tested, the presence of mismatched MSCs had a significant but lesser effect at the responder leukocyte concentration of 4×10^5 cells and no significant effect at the responder leukocyte concentration of 8×10^5 cells (Figure 6A & D). At both

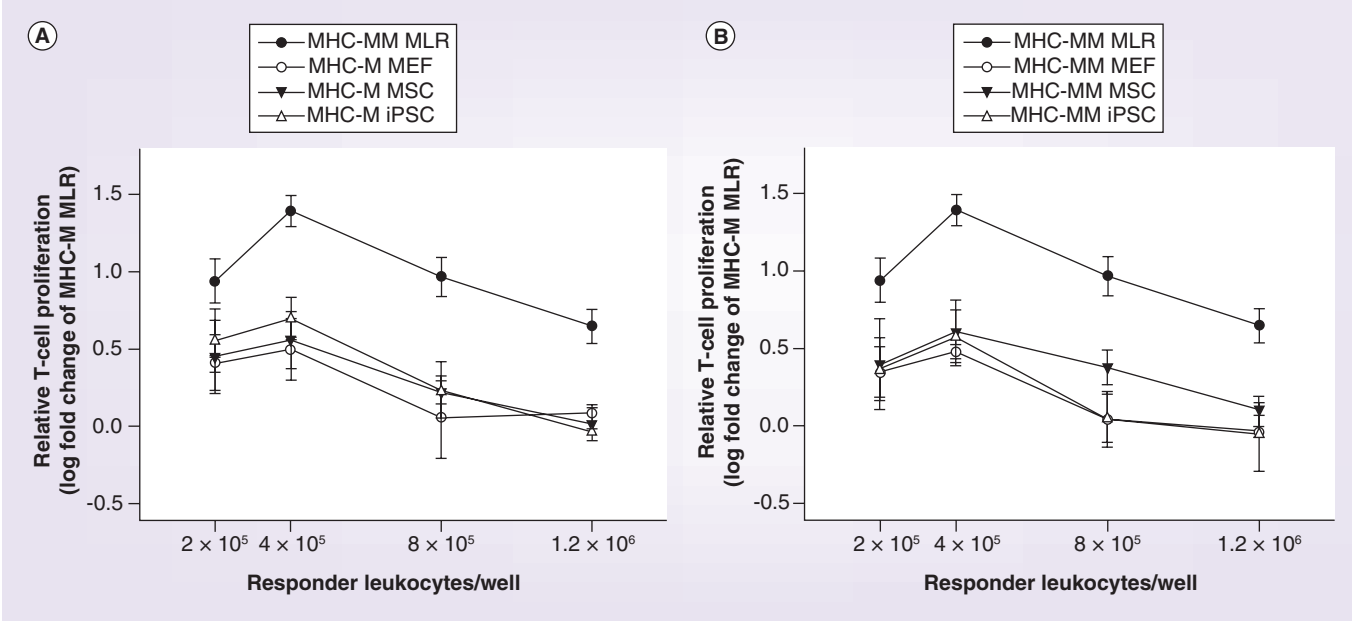

Figure 4. Immunomodulatory potential of (A) MHC-matched and (B) MHC-mismatched MEFs, MSCs and iPSCs as determined by responder T-cell proliferation in modified one-way mixed leukocyte reactions in which MHC-mismatched leukocytes were cultured in the presence or absence of these cells. Data are presented as the percentage proliferation of the control MHC-mismatched MLR as a decrease in proliferation is indicative of immunomodulation. Bars represent mean ± SD from a total of three separate experiments performed with multiple cell lines. A reference line has been placed at 100% to denote the positive control of the MHC-mismatched MLR. iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibrobast; MHC-M: MHC-matched; MHC-MM: MHC-mismatched; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stromal cell.

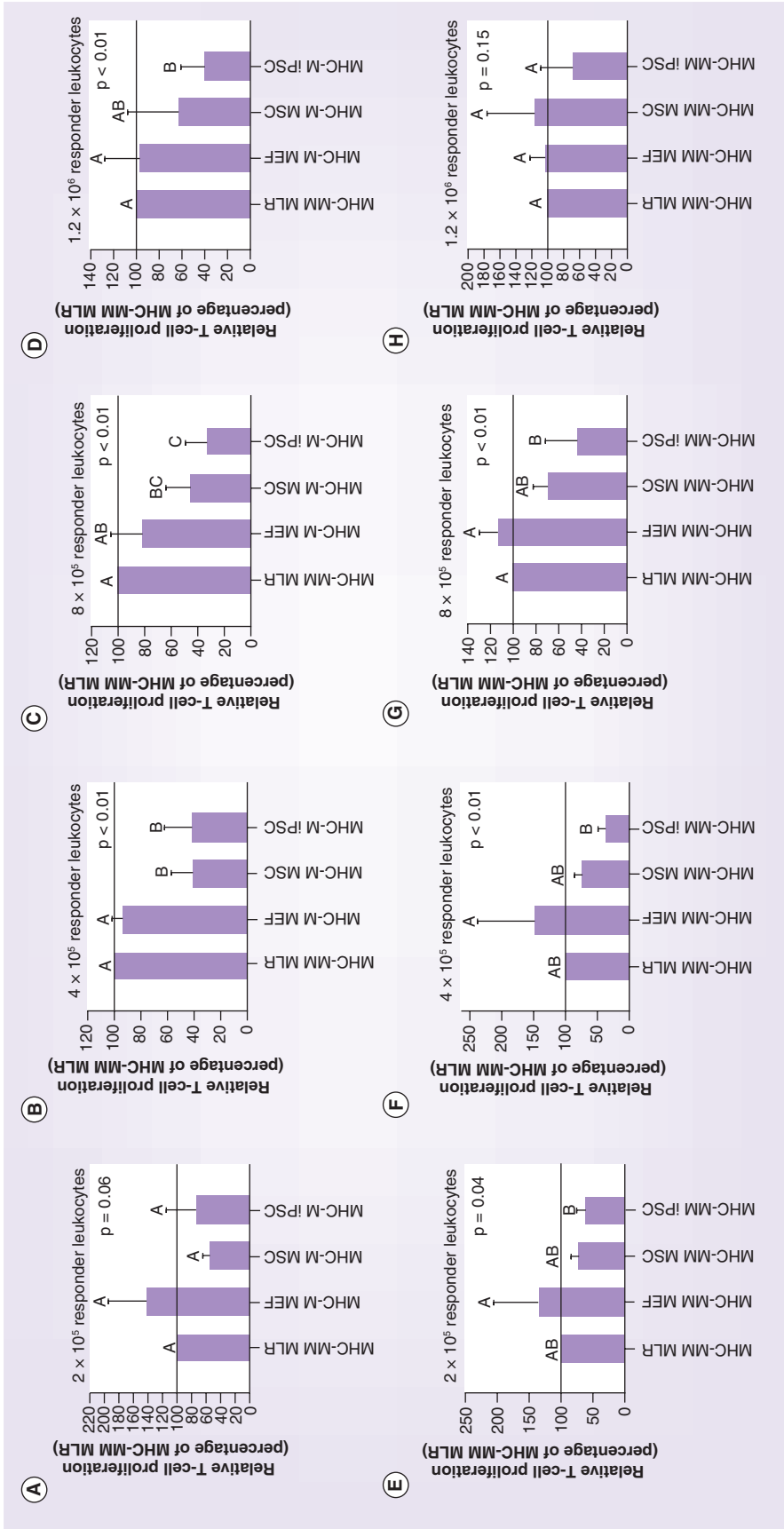

Figure 5. Immunomodulatory potential of mouse embryoinc fibroblasts, mesenchyamal stem cells and induced pluripotent stem cell as determined using modified one-way mixed leukocyte reactions in which MHC-mismatched leukocytes were cultured in the presence or absence of these cells. Data are reported as the relative T-cell proliferation, which was calculated as the percentage proliferation of the MHC-mismatched MLR alone. MLR results as shown in Figure 4 are displayed here for MHC-matched cells **(A–D)** and MHC-mismatched cells **(E–H)** at the different responder leukocyte concentrations tested. Bars represent mean ± SD from a total of three separate experiments performed with multiple cell lines. Reference lines have been placed at 100% to denote the positive control of the MHC-mismatched MLR. Superscript letters indicate significant differences between groups by ANCOVA, with experiment as a covariate, followed by a Tukey multiple comparisons test, $p \leq 0.05$.
iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibrobast; MHC-M: MHC-matched; MHC-MM: MHC-mismatched; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stromal cell.

responder leukocyte concentrations tested, the presence of either matched or mismatched MSCs resulted in the highest mean PGE_2 concentrations compared with control and both matched and mismatched iPSCs. For mismatched MSCs, however, such concentrations were only significantly greater than control at responder leukocyte concentration of 4×10^5 cells. Although mean PGE_2 concentrations were higher in media from MHC-mismatched MLRs cultured in the presence of either matched or mismatched iPSCs compared with control MHC-mismatched MLRs, these results were not statistically significant (Figure 6B & E). No significant differences in media TGF-β1 concentrations were found for any of the cell types tested (Figure 6C & F).

Discussion

In this study we directly compared iPSCs to MSCs in terms of immunogenicity and immunomodulatory capability using MLRs. Our comparisons revealed that iPSCs generated through both lentiviral and *piggyBac* reprogramming methods have similar immunogenic properties as MSCs and may possess more potent immunomodulatory properties than MSCs *in vitro*. Co-culture of MHC-mismatched leukocytes with MHC-matched iPSCs resulted in significantly less responder T-cell proliferation than observed for MHC-mismatched leukocytes alone at more responder leukocyte concentrations tested than was observed for co-culture of MHC-mismatched leukocytes with MHC-matched MSCs. In addition, MHC-mismatched iPSCs were able to significantly reduce responder T-cell proliferation at the responder leukocyte concentration of 8×10^5 cells when co-cultured with MHC-mismatched leukocytes while MHC-mismatched MSCs were not.

None of the cells (MEFs, iPSCs or MSCs) tested in this study were irradiated for use in MLRs due to the fact that iPSCs died following even very low doses

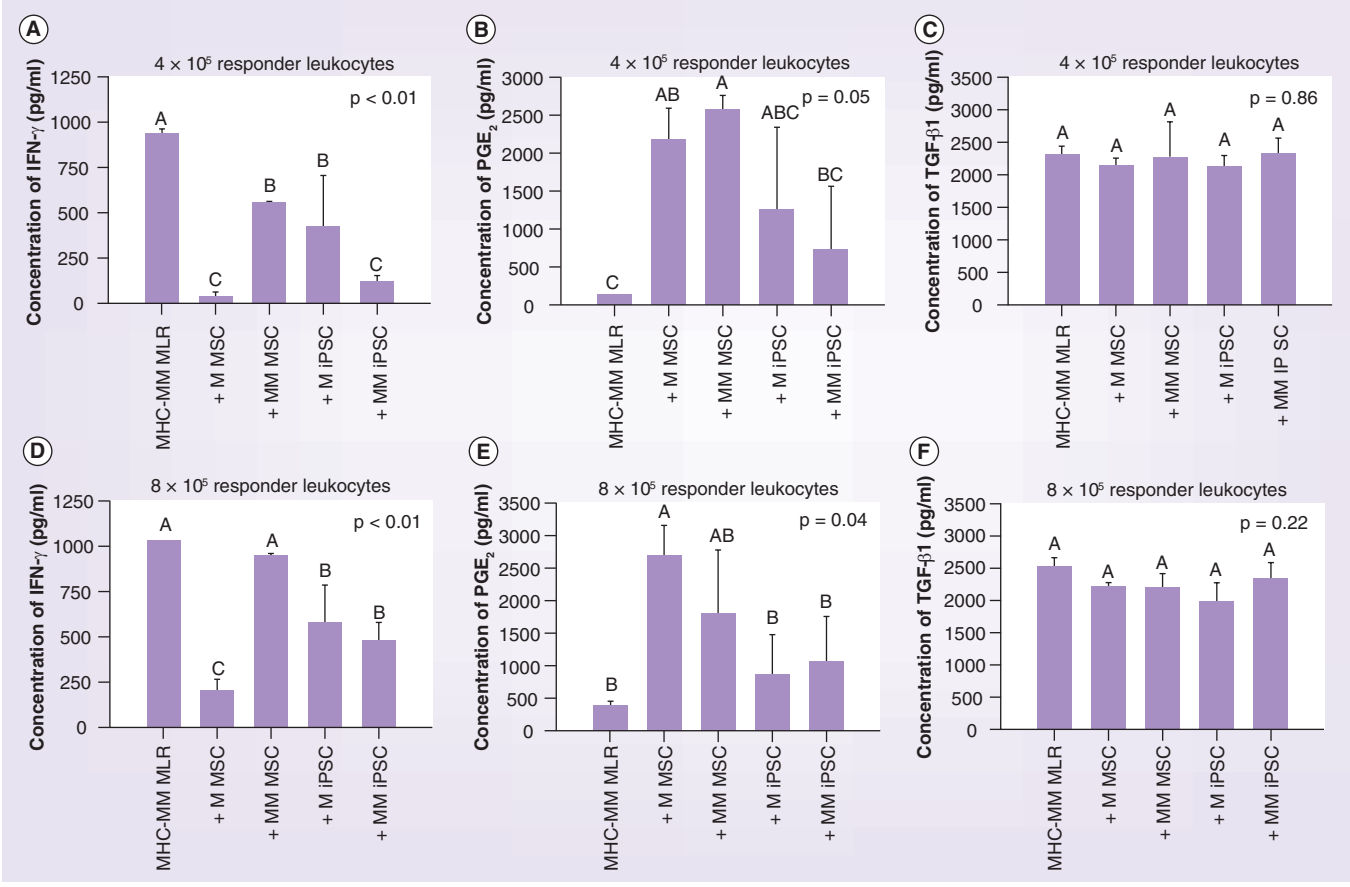

Figure 6. Immunomodulatory mixed leukocyte reaction experiment media concentrations of (A & D) IFN-γ, (B & E) PGE_2 and (C & F) TGF-β1 for control MHC-mismatched MLRs and for MHC-mismatched MLRs cultured with matched or mismatched MSCs or iPSCs. Media from experiments using responder leukocyte concentrations of 4×10^5 cells **(A–C)** and 8×10^5 cells **(D–F)** were examined based on the significant immunomodulatory results found at these concentrations as shown in Figures 4 & 5. Bars represent mean ± SD from a total of three pooled samples of each cell type from each MLR experiment. Superscript letters indicate significant differences between groups by ANOVA, followed by a LSD multiple comparisons test; $p \leq 0.05$.
iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibrobast; MHC-M: MHC-matched; MHC-MM: MHC-mismatched; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stromal cell; PGE_2: Prostaglandin E_2.

(100–200 rads) of gamma irradiation. This finding suggests that iPSCs undergo p53-independent apoptosis in response to DNA damage as described for ESCs [68–70] rather than p53-mediated cell cycle arrest as is well described for somatic cells [71]. For this reason, preliminary experiments were performed to determine the proper seeding density of all cell types such that they were approximately 80% confluent on the 5th (final) day of MLR culture. This method is different than most previously described for MSC immunology studies examining MSC effects in MLRs in which MSCs are irradiated and then plated at different ratios to responder leukocytes [26,42]. It is possible that because the cells were growing that they could have either not reached or surpassed the optimal cell:leukocyte ratio for immunomodulation in some instances. The fact that both MHC-matched iPSCs and MSCs were able to significantly downregulate responder T-cell proliferation to as much as 25–30% of that of the MHC-mismatched MLR, however, suggests a broad enough range of responder leukocyte cell concentrations was covered to confidently determine whether the cells were causing immunomodulation.

Due to the nature of MLRs, it must also be considered that T-cell proliferation may have been falsely diminished due to T-cell competition with growing cells for nutrients in the media. At no point during the experiments, however, did the media appear exhausted in color. More importantly, co-culture with MEFs, which were rapidly growing and just as confluent as the other cell types by the end of the experiments, did not result in reduced T-cell proliferation. This argues against nutrient competition or depletion as a reason for the reduced responder T-cell proliferation observed in MLR co-cultures with iPSC and ESCs. Thus, MEFs were a critical experimental control for these immunomodulatory experiments. Another potential concern is that the leukocyte media used included leukemia inhibitory factor (LIF), which has been shown to have a role in MSC-mediated immunosuppression [24]. LIF is commonly used in iPSC media to maintain pluripotency and prevent differentiation [1,2,11]. It was used in these experiments for that reason and also to avoid use of feeder cells with iPSCs culture, which would have further complicated experimental design and interpretation [72]. The same leukocyte media with LIF was used for all MLR experiments, which should have prevented any biases between cell type comparisons and against MHC-matched and mismatched MLR controls. Again, the finding that control MEFs did not cause significant downregulation of responder T-cell proliferation argues against this concern.

The finding that iPSCs and MSCs that were MHC-matched with respect to the responder leukocytes resulted in a greater reduction in responder T-cell proliferation compared with iPSCs and MSCs that were MHC mismatched is interesting. Engraftment studies evaluating the effect of MSCs have previously demonstrated similar findings, with only syngeneic (MHC-matched) MSCs resulting in enhanced engraftment [17,38]. Previous MLR studies evaluating the immunosuppressive effects of ESCs, however, have found no difference in using MHC-matched or mismatched ESCs with the responder leukocytes [73]. The reasons for this discrepancy between iPSCs and MSCs is unclear, but could be due to specific immunosuppressive mechanisms employed by cells or due to differences in MHC antigen expression between cell types with iPSCs expressing very low or negligible levels of MHC class I and MSCs expressing high levels of MHC I. Of note is the fact that although MLR co-cultures with mismatched MSCs resulted in high levels of PGE_2 in the media that were fairly consistent with that of matched MSCs, mismatched MSCs were unable to produce the same decrease in IFN-γ concentration as was observed in media from MLR co-cultures with matched MSCs. This result suggests that perhaps the immune stimulus of mismatched MSCs was strong enough to cause responder T-cell IFN-γ secretion despite the expected downregulation or inhibition of IFN-γ by PGE_2 secreted from the MSCs [16]. Future studies evaluating the kinetics of responder T-cell proliferation during such MLR co-cultures may prove useful for distinguishing between increased immunogenicity and decreased immunomodulatory potential of the cells.

It is also an important finding that the significant decrease in media IFN-γ concentrations in MLRs co-cultured with iPSCs did not correlate well with the very modest increases in PGE_2 concentrations in the same media. This suggests that iPSCs are inhibiting responder T-cell IFN-γ secretion via a mechanism other than PGE_2. Further studies must be performed to elucidate this result, including examination of other soluble factors potentially expressed by iPSCs and MSCs into the media during MLRs such as TGF-β2, IL-6, IL-10, IDO and LIF [13,14,16,24,32,47,74], as well as examination of arginase-I expression by iPSCs, which could be responsible for the inhibition of responder T-cell IFN-γ secretion as previously described for ESCs [49,54]. Evaluation of the gene expression levels of these soluble factors by different cell types would also be of great interest because media additives such as LIF could be affecting protein expression and because proteins could be rapidly degrading in the culture system. While this would be difficult to perform in the MLR co-culture system described in this study in which all cell types are mixed together within a well, it is possible that the cell types could be sorted at the end of the culture system. Once suspect factors have been identified, the next essential study would be

to inhibit these factors and determine whether or not responder T-cell proliferation is restored in the MLR co-culture system.

Perhaps the greatest question raised by this study is whether or not iPSCs can retain their immunogenic and immunomodulatory properties upon differentiation as it is unlikely that undifferentiated iPSCs will be used in human clinical applications due to concerns of teratoma formation. Follow-up studies must focus on differentiating iPSC lines into specific cell types and then re-evaluating their MHC class I and II expression in addition to their immunogenic and immunomodulatory properties in MLRs. Soluble factor release into the media by these cells during MLRs must also be re-assessed and compared with levels pre-differentiation. Once these *in vitro* studies have been completed, *in vivo* studies evaluating the immunogenic and immunomodulatory effect of iPSCs outside the controlled environment of the MLR must be performed. Such studies will be critical for consideration of iPSC use in the place of MSCs for both regenerative medicine and transplant medicine [7,12,52].

Author contributions

All authors contributed to the study design. LV Schnabel, CM Abratte, JM Cassano and JA Cross performed the experiments. TL Southard carried out histologic assessments on the teratoma assays. All authors contributed to data analysis and interpretation. LV Schnabel and LA Fortier were responsible for drafting the manuscript. All authors revised the manuscript and approved the final version.

Acknowledgements

The authors would like to thank Robert Munroe for his help with animal care and for his contributions to the experimental design. The authors would also like to thank Gina Kemp for her help with animal care and handling.

Financial & competing interests disclosure

This work was supported by Empire State Stem Cell Fund contract no. C024400 (LA Fortier and JC Schimenti) and NIH grant no. 1K08AR060875-01 (LV Schnabel). Finally, the authors would like to acknowledge use of the Cornell University Irradiator Core Facility and support from National Center for Research Resources grant #S10RR023781. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Introduction (rationale & aim)

- The immunologic properties of induced pluripotent stem cells (iPSCs) require investigation as it has become evident that banked iPSCs will be needed for most clinical applications due to cell generation time and time associated with screening for both efficacy and safety.
- The aim of this study was to evaluate the *in vitro* immunogenic and immunomodulatory properties of iPSCs compared with adult bone marrow-derived MSCs using modified mixed leukocyte reactions.

Materials & methods

- In order to assess immunogenic potential, iPSCs and MSCs were used as stimulator cells for responder leukocytes. In order to assess immunomodulatory properties, iPSCs and MSCs were cultured in the presence of stimulator and responder leukocytes. MEFs were used as a control.

Results

- iPSCs generated through both lentiviral and *piggyBac* reprogramming methods had similar immunogenic properties and more potent immunomodulatory properties than MSCs *in vitro*.
- Co-culture of major histocompatibility complex (MHC)-mismatched leukocytes with MHC-matched iPSCs resulted in significantly less responder T-cell proliferation than MHC-mismatched leukocytes alone at more responder leukocyte concentrations tested than was observed for co-culture of MHC-matched leukocytes with MHC-matched MSCs.
- MHC-mismatched iPSCs were able to significantly reduce responder T-cell proliferation at the responder leukocyte concentration of 8×10^5 cells when co-cultured with MHC-mismatched leukocytes, while MHC-mismatched MSCs were not.
- A significant decrease was found in media IFN-γ concentrations in MLRs co-cultured with iPSCs; however, this decrease did not correlate well with the modest increase in prostaglandin E_2 concentration in the same media.

Discussion & conclusion

- iPSCs are presumably inhibiting responder T-cell IFN-γ secretion via a mechanism other than prostaglandin E_2 as for MSCs.
- Further studies must be performed in order to determine whether iPSCs retain their immunogenic and immunomodulatory properties upon differentiation into specific cell or tissue types.
- This information is critical when considering the use of iPSCs in the place of MSCs for both regenerative medicine and transplant medicine.

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