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Immune potential and differentiation of equine induced pluripotent stem cells (eiPSC)

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Résumé

Les cellules souches pluripotentes induites (iPSC) ont la capacité de s'auto renouveler et de se différencier en une myriade de types cellulaires, ce qui en fait des outils intéressants pour la thérapie cellulaire et la médecine régénérative. Le but de cette thèse était de déterminer les caractéristiques des iPSC équines (eiPSC) qui peuvent être exploitées pour l'usage potentiel en médecine régénérative vétérinaire. Chez le cheval, une plaie cutanée est souvent cicatrisée par seconde intention et est sujette à de nombreuses complications lorsque située sur le membre, notamment une épithélialisation lente. Ainsi, l'hypothèse globale de cette thèse était que les eiPSC pourraient offrir une solution novatrice de couverture pour de telles blessures. Avant d'envisager l'utilisation d'eiPSC à des fins cliniques, leur immunogénicité doit être étudiée afin de s'assurer que les cellules transplantées seront acceptées et intégrées dans les tissus du receveur.

Le premier objectif de cette thèse était de définir la réponse immunitaire suscitée par les eiPSC. Afin d'étudier l'immunogénicité d'eiPSC, l'expression de molécules du complexe majeur d'histocompatibilité (MHC) des lignes choisies a été déterminée, puis les cellules ont été utilisées dans un modèle de transplantation intradermique développé pour cette étude. Bien que la transplantation allogénique d'eiPSC non différenciées ait induit une réponse cellulaire modérée chez les chevaux d'expérimentation, elle n'a pas provoqué de rejet. Cette stratégie a permis la sélection de lignées d'eiPSC faiblement immunogènes pour la différenciation ultérieure en des lignées d'importance thérapeutique.

Les eiPSC représentent une solution intéressante et qui, par l'entremise du développement d'une lignée de kératinocytes, pourraient servir à la création d'une greffe ayant la capacité de former non seulement l'épithélium manquant mais aussi d'autres structures accessoires de l'épiderme. Le deuxième objectif de cette thèse était donc de

développer un protocole pour la différentiation des eiPSC en lignée de kératinocytes. Un protocole visant cette différenciation fut ainsi développé et ce dernier a démontré une grande efficacité à produire le phénotype attendu dans une période de 30 jours. En effet, les kératinocytes dérivés d'eiPSC (eiPSC-KC) ont montré des caractéristiques morphologiques et fonctionnelles des kératinocytes primaires équins (PEK). En outre, la capacité de prolifération d'eiPSC-KC est supérieure tandis que la capacité migratoire, mesurée comme l'aptitude à cicatriser les plaies *in vitro*, est comparable à celle du PEK.

En conclusion, les eiPSC-KC ont des caractéristiques intéressantes pour le développement d'un substitut cutané à base de cellules souches, ayant le potentiel de régénérer la peau perdue lors de trauma ou de maladie, chez le cheval. Cependant, parce que les eiPSC n'échappent pas totalement à la surveillance immunitaire, malgré une faible expression du MHC, des stratégies pour améliorer la prise de greffe eiPSC-KC doivent être élaborées.

Mots clés: équin /cheval, cicatrisation, médecine régénérative, cellules souches pluripotentes induites, kératinocytes, réponse immunitaire, transplantation, greffe

Abstract

Induced pluripotent stem cells (iPSC) have the capacity to self renew and differentiate into a myriad of cell types making them potential candidates for cell therapy and regenerative medicine. The goal of this thesis was to determine the characteristics of equine iPSC (eiPSC) that can be harnessed for potential use in veterinary regenerative medicine. Trauma to a horse's limb often leads to the development of a chronic non-healing wound that lacks a keratinocyte cover, vital to healing. Thus, the overall hypothesis of this thesis was that eiPSC might offer a solution for providing wound coverage for such problematic wounds. Prior to considering eiPSC for clinical applications, their immunogenicity must be studied to ensure that the transplanted cells will be accepted and integrate into host tissues.

The first objective of this thesis was to determine the immune response to eiPSC. To investigate the immunogenicity of eiPSC, the expression of major histocompatibility complex (MHC) molecules by the selected lines was determined, then the cells were used in an intradermal transplantation model developed for this study. While transplantation of allogeneic, undifferentiated eiPSC elicited a moderate cellular response in experimental horses, it did not cause acute rejection. This strategy enabled the selection of weakly immunogenic eiPSC lines for subsequent differentiation into lineages of therapeutic importance.

Equine iPSC offer a potential solution to deficient epithelial coverage by providing a keratinocyte graft with the ability to differentiate into other accessory structures of the epidermis. The second objective of this thesis was to develop a protocol for the differentiation of eiPSC into a keratinocyte lineage. The protocol was shown to be highly efficient at inducing the anticipated phenotype within 30 days. Indeed, the eiPSC derived

keratinocytes (eiPSC-KC) showed both morphologic and functional characteristics of primary equine keratinocytes (PEK). Moreover, the proliferative capacity of eiPSC-KC was superior while the migratory capacity, measured as the ability to epithelialize *in vitro* wounds, was comparable to that of PEK, suggesting exciting potential for grafting onto *in vivo* wound models.

In conclusion, equine iPSC-derived keratinocytes exhibit features that are promising to the development of a stem cell-based skin construct with the potential to fully regenerate lost or damaged skin in horses. However, since eiPSC do not fully escape immune surveillance despite low MHC expression, strategies to improve engraftment of iPSC derivatives must be pursued.

Keywords: equine / horse, wound healing, regenerative medicine, induced pluripotent stem cells, keratinocytes, immune response, transplantation, grafting

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List of abbreviations

A-MSC - adipose-derived mesenchymal stem cells

APC - antigen presenting cell

AP - alkaline phosphatase

ASC - adult stem cell

bFGF - basic fibroblast growth factor

BLI - bioluminescence imaging

BMP - bone morphogenic protein

BMSC - bone marrow-derived mesenchymal stem cell

CD - cluster of differentiation

CEA - cultured epidermal autografts

C-GAG - collagen-glycosaminoglycan

CM - cynomolgus macaque

C-MYC - myelocytomatosis oncogene

CPP - cell penetrating peptide

CTL - cytotoxic T-lymphocyte

DC - dendritic cell

DMEM - Dulbecco's minimal essential medium

DNA - deoxyribonucleic acid

DSG3 - desmoglein 3

eiPSC - equine induced pluripotent stem cell / *cellules souches pluripotentes induites équines*

eiPSC-KC - equine induced pluripotent stem cell-keratinocyte / *cellules souches pluripotentes induites-kératinocytes*

EB - epidermolysis bullosa

EBS - epidermolysis bullosa simplex

ECM - extracellular matrix

eES - equine embryonic stem cell

EGC - embryonic germ cell

EGT - exuberant granulation tissue

EHK - epidermolytic hyperkeratosis

EPC - endothelial precursor

EpSC - epithelial stem cell

ESC - embryonic stem cell

ESE - equine skin equivalents

FBS - fetal bovine serum

FGF - fibroblast growth factor

GAG - glycosaminoglycan

GFP - green fluorescent protein

$GFP^{+/-}$ - green fluorescent protein positive/negative

GMP - good manufacturing protocol

GVHD - graft versus host disease

HD - Huntington's disease

H&E - hematoxylin and eosin

HG-EGF - heparin-binding epidermal growth factor-like growth factor

hiPSC - human induced pluripotent stem cell

HLA - human leukocyte antigen

HPS - hematoxylin phloxine saffron

HSC - hematopoeitic stem cell

hUCB-MSC - human umbilical cord blood-derived mesenchymal stem cell

ICM - inner cell mass

IFN - interferon

IGF - insulin-like growth factor

IL - interleukin

iPSC - induced pluripotent stem cell / *cellules souches pluripotentes induites*

ISCT - international society for cellular therapy

JAK - janus kinase

KGF - keratinocyte growth factor

KGM - keratinocyte growth medium

KLF-4 - Kruppel-like factor 4

KRT - keratin gene

KSC - keratinocyte stem cell

LIF - leukemia inhibitory factor

LOR - loricrin

LPS - lipopolysaccharide

mDA - midbrain dopamine

MEF - mouse embryonic fibroblast

MFI - mean fluorescence intensity

mHC - minor histocompatibility complex

MHC - major histocompatibility complex/ *complexe majeur d'histocompatibilité*

MKOS - C-MYC, KLF-4, OCT-4, SOX-2

MLR - mixed lymphocyte reaction

MMP - matrix metalloproteinase

MSC - mesenchymal stem cell

MW - molecular weight

NANOG - Nanog homeobox

NK - natural killer

NO - nitric oxide

NSC - neural stem cell

OA - osteoarthritis

OCT-4 - octamer-binding protein 4

OSM - oncostatin M

PANCK - pancytokeratin

PB - piggybac

PBS - phosphate buffered saline

PDGF - platelet-derived growth factor

PEK - primary equine keratinocytes/ *kératinocytes primaires équins*

PGE2 - prostaglandin E2

PHA - phytohaemagglutinin

phESC - parthenogenesis embryonic stem cell

piPSC - porcine induced pluripotent stem cell

PKC - protein kinase C

PRP - platelet-rich plasma

PSC - pluripotent stem cell

RA - retinoic acid

RDEB - recessive dystrophic epidermolysis bullosa

RiPSC - micro RNA derived iPSC

RNA - ribonucleic acid

ROS - reactive oxygen species

rPDGF-BB - recombinant platelet-derived growth factor-BB

RPE - retinal pigment epithelium

SCF - stem cell factor

SCI - spinal cord injury

SCID - severe combined immunodeficiency

SCNT - somatic cell nuclear transfer

SDF - superficial digital flexor

SDS - sodium dodecyl sulfate

SOX-2 - sex determining region Y- box 2

SSEA - stage-specific embryonic antigen

STAT-3 - signal transducer and activator of transcription 3

TA - transit amplifying cells

TCR - T-cell receptor

TGF - transforming growth factor

TIMP - tissue inhibitor of metalloproteinase

TNF - tumor necrosis factor

TRA - podocalyxin-like

UCB-MSC - umbilical cord blood-derived mesenchymal stem cell

VEGF - vascular endothelial growth factor

WB - western blot

Dedication

To my beloved parents for their unending love and support

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I. Introduction

The equine industry in the US and the UK exerts an economic impact of $102 billion and $10 billion per annum, respectively [1]. In Canada, the equine industry contributes more than $19 billion annually to the national economy and supports more than 154,000 jobs for Canadians [2]. Wounds, injuries and trauma are the most common medical condition seen in equids, accounting for 25.7% of all conditions [3]. In equine veterinary practice, the anatomic location most frequently wounded is the distal limb, including the carpus and tarsus, accounting for more than 60% of all wounds [4]. Wounds on horse limbs heal with difficulty compared to those in other areas of the body; characteristic features of limb wounds are chronic inflammation, excess matrix accumulation, poor wound contraction and delayed epithelialization leading to an absence of wound coverage [5]. Impaired healing in horses is a common clinical problem that poses welfare implications [6] and bears a significant financial impact on the equine industry; for example, 7% of injuries leading to the retirement of racehorses are the result of a wound [7].

Stem cell-based therapies hold promise in equine regenerative medicine and could potentially provide the lacking epithelial cover in non-healing limb wounds. Equine induced pluripotent stem cells (eiPSC) may be interesting tools in regenerative medicine, given their capacity to differentiate into a myriad of cell types, and warrant further study. While autologous keratinocytes can be used to engineer skin substitutes, in man they require 3-4 weeks for culture expansion, have low proliferative rates, are fragile [8] and, most importantly, do not regenerate secondary accessory structures of the skin required for normal function [9]. Equine keratinocyte grafts have not yet been developed, and due to the

aforementioned disadvantages relating to the use of primary keratinocytes to manufacture skin substitutes, eiPSC-derived keratinocyte (eiPSC-KC) grafts may be a superior choice. Deriving and testing patient specific iPSC is time consuming and expensive [10], thus allogeneic eiPSC-derived grafts may be a logical alternative and are therefore the focus of my studies.

Literature Review

1. Skin anatomy and physiology

The skin is the largest organ protecting the body from chemical, infectious and mechanical hazards. Depending on age and species, the skin constitutes 12-24% of an animal's body weight [11]. It is the primary organ that retains water and electrolytes thereby protecting the underlying tissues [12]. The skin is composed of two compartments: the epidermis and the dermis **(Fig.1)**.

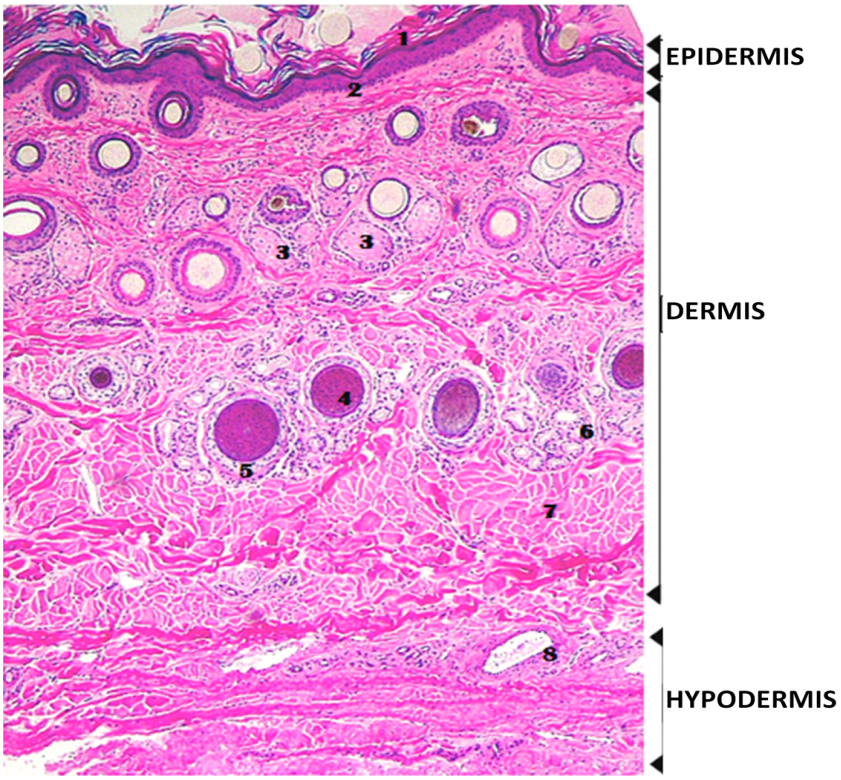

Fig. 1: **Normal histological section of equine forelimb skin showing the various compartments and structures within them**: 1- *stratum corneum*; 2- *stratum basale*; 3- sebaceous glands; 4- hair follicle; 5- outer root sheath; 6- sweat gland; 7- erector pili muscle bundle; 8- blood vessel. 10X magnification; staining with Haematoxylin and Eosin (H&E).

Epidermis: In the adult, the outermost epidermal component consists of 4-5 layers (depending on the species and anatomic location): *stratum basale, stratum spinosum, stratum granulosum*, (*stratum lucidum*) and *stratum corneum* [13]. Additional components, referred to as skin appendages include hair follicles, sweat glands, sebaceous glands and hooves/nails, complete the organ of skin. During fetal development, the epidermis develops from two layers; the outermost is called the periderm while the innermost is the basal layer (*stratum basale)* or germinative layer. The periderm acts as a transient covering for the epidermis during much of the remainder of fetal development [14]. The basal keratinocytes gives rise to the spinous layer (*stratum spinosum),* which in turn forms the granular layer (*stratum granulosum)*. Cells from this layer differentiate to form the outermost keratinized layer of the epidermis known as the cornified layer (*stratum corneum)* **(Fig. 2)**.

Keratinocytes in situ: The keratinocytes of the *stratum basale* are typically columnar and are replete with keratin filaments around the nucleus and hemidesmosomes (junctions between a basal cell and the basal lamina) and desmosomes (junctions between adjacent basal cells) [15]. The *stratum spinosum* is named for the spine-like appearance of the cell borders in histological sections attributed to numerous desmosomes joining adjacent keratinocytes [16]. Next, the *stratum granulosum* is so named because the keratinocytes in this layer possess electron-dense keratohyalin granules [17]. Finally, the outermost *stratum corneum* is formed of flattened polyhedron-shaped cells called corneocytes, uniquely adapted to providing a protective surface.

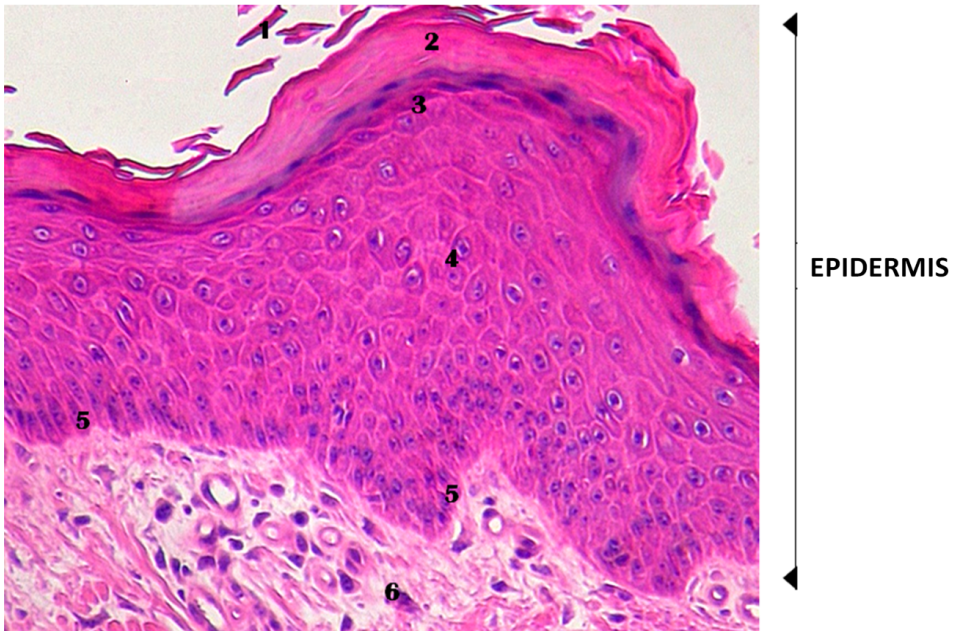

Fig. 2: Epidermis and its strata in equine forelimb skin (40X, H&E stain). 1- squames; 2- *stratum corneum*; 3- *stratum lucidum*; 4- *stratum spinosum*; 5- *stratum basale*; 6- dermis.

While 90-95% of the cells populating the epidermis are keratinocytes, this compartment also includes melanocytes, Langerhans cells and Merkel cells. Keratinocytes are 6-70 times stiffer than other epithelial cells because of their keratin intermediate filament network [18]. At least 54 keratin (*KRT*) genes have been studied in the human genome and encode proteins classified as either type I (acidic) or type II (basic). Together, these form heterodimers that aggregate to generate the intermediate filaments of the cytoskeleton or of the hard cornifications of epidermis [19]. Thus keratins contribute to the structural integrity and possess a mechanistic role in the functionality of skin.

Keratinocytes of the *stratum basale* are attached to a basement membrane located between the outermost epidermal and underlying dermal compartments. The basement membrane supporting the basal cells is composed mainly of collagen type IV, proteoglycans and nidogen. The basement membrane serves as a scaffold to which basal keratinocytes adhere via α6β4 integrins within the hemidesmosomes [20]. The *stratum*

basale possesses three subpopulations of keratinocytes: Keratinocyte Stem Cells (KSC), Transit Amplifying (TA) cells and post-mitotic differentiating cells [21]. The KSC constitute the most important cell type in the basal layer of the epidermis, and are the mitotic cells giving rise to daughter stem cells and to TA cells. The epidermis' capacity for self-renewal is therefore mediated by KSC of the hair follicles as well as those in the *stratum basale* of the interfollicular epidermis, defined as the region of stratified epidermis flanked by hair follicles [22]. The KSC are responsible for tissue homeostasis and regeneration of the epidermis following injury. After a few rounds of cell division, TA cells permanently exit the cell cycle and undergo stratification or terminal differentiation. Further epidermal maturation occurs when (suprabasal) spinous cells differentiate into granular cells and finally, cornified cell envelopes are assembled by cross-linking of structural proteins and lipids [23].

The spinous cells differentiate and undergo a complex series of morphological and biochemical changes in keratin expression and adhesive properties, lose their organelles, enucleate, become cornified, flatten and eventually are sloughed from the surface of the skin. These corneocytes form the lipid-rich superficial cornified layer referred to as 'squames' responsible for waterproofing the epidermis **(Fig. 2)** [24]. Desquamation leads to a loss of cornified cells superficially while epidermal cells undergo continuous cycling from the basal KSC layer. During keratinocytes' transition from the basal epidermal layer to the granular layer, the nucleus undergoes programmed transformation from a highly active status, associated with execution of the genetic program of epidermal barrier formation, to a fully inactive condition and, through a process of DNA degradation, becomes a part of the keratinized cells of the cornified epidermal layer [21]. Mammalian keratinocyte cornification is a multi-step process initiated by a switch in the expression of particular *KRT* genes, followed by the expression of the keratin-bundling protein filaggrin (FIL) and

proteins such as involucrin (IVL) and loricrin (LOR), which, together with keratins, become cross-linked by transglutaminases to reinforce the formation of a cornified envelope across species [25].

Dermis: The dermal compartment consists of two regions: the papillary dermis and reticular dermis [26]. It is a dense, fibroelastic connective tissue constituting the bulk of the skin, the thickness of which varies according to body site. The epidermis is anchored to it by projections referred to as rete ridges. A network of collagen and elastic fibers provides tensile strength and flexibility to the dermis with some elastin and glycosaminoglycans (GAG) [11]. Collagen type I is the major collagen of the dermis (around 62%) while collagen type III constitutes approximately 15% of the dermis [27]. The fibroblast is the major cell type of the dermis; perivascular mast cells are also found in the connective tissue along with tissue macrophages. The connective tissue supports the fibroblasts, nerve network, epithelial glands, keratinizing appendages and a microcirculatory vascular and lymphatic system.

The subcutaneous tissue (just deep to the skin) is known as the hypodermis (or superficial fascia) and is not considered part of the skin [27]. It contains loose connective and adipose tissue and it anchors the skin to the underlying organs while allowing the skin to move relatively freely. It also acts as a shock absorber and insulates the deeper body tissues from heat loss.

1.1 The role for keratins

The major and universal role of keratin filaments is to act as a resilient yet adaptable scaffold that endows epithelial cells (e.g epidermal cells) with the ability to sustain mechanical and non-mechanical stresses. Keratin proteins significantly impact signaling pathways and metabolic processes and profoundly influence specific aspects of cytoarchitecture [28]. While the actin cytoskeleton is critical for lamellipodial motility of adult keratinocytes and purse-string closure of embryonic epidermal wounds, the keratin cytoskeletal network contributes to essential cell and tissue strength during such strenuous epithelial movements [29].

The process of keratinocyte differentiation in mature epidermis mimics the initial development and maturation of keratinocytes during embryogenesis. Epidermal keratinocytes are derived from the single-layered surface ectoderm and develop when the underlying mesenchyme releases inductive signals [21]. Like other single-layered epithelia, the surface ectoderm in fetuses expresses *KRT 8* and *KRT18* and when the commitment to stratification occurs, *KRT 8* and *KRT 18* expression is downregulated, concomitant with the induction of *KRT 5* and *KRT 14* expression [30]. *KRT 5* and *KRT 14* are expressed only in stratified epithelia or epithelia that have committed to initiate a stratification program **(Fig. 3)** [31]. Basal *KRT* genes are first detected at E9.5 of mice in a highly regional fashion, and surprisingly as early as the single layered ectodermal stage [32]. The transition of the keratinocyte from the basal to the suprabasal layers is accompanied by the repression of basal keratinocyte transcripts *KRT* 5, *KRT* 14, and α4β6 integrin, followed by upregulation of transcripts for the cornified envelope precursor proteins such as IVL, LOR, and FIL [30].

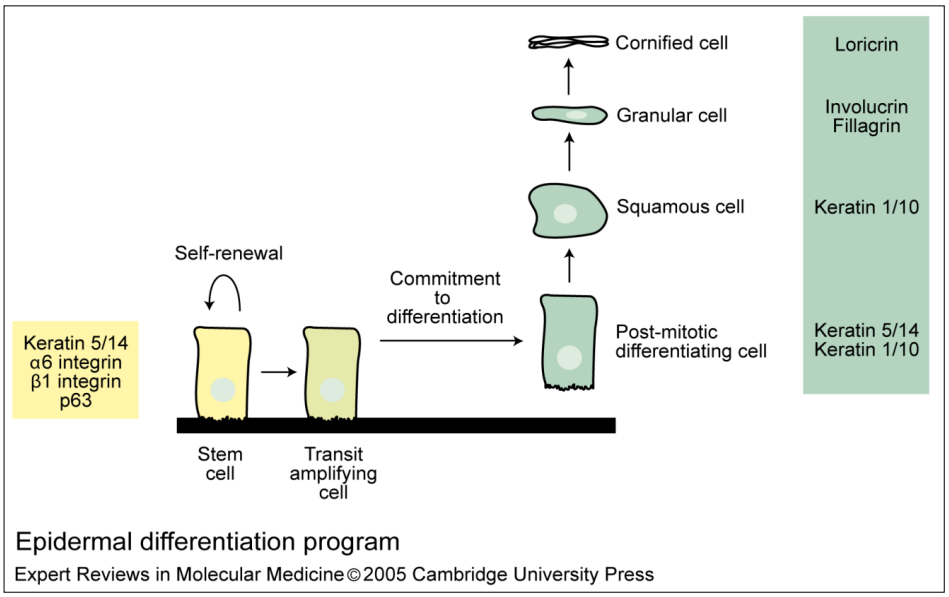

Fig. 3: Epidermal differentiation program: Keratinocyte stem cells (KSC) residing in the basal layer of the epidermis undergo self-renewal, thereby giving rise to another stem cell, or can divide to give rise to transit amplifying (TA) cells, which are rapidly dividing cells that provide the skin with new epidermal basal cells. New epidermal basal cells subsequently detach from the basement membrane and begin the process of terminal differentiation. As cells differentiate, they move up to form the different layers of the epidermis. Stem and TA cells can be distinguished from terminally differentiated cells on the basis of protein expression (yellow box). Each stage of the differentiation program (epidermal layers) can also be distinguished according to the proteins expressed (green box).

In spite of a recent revolution in the discovery of novel genes, the most reliable criterion to define the epithelial nature of a cell remains *KRT* gene expression. The pattern of keratin protein synthesis provides a useful tool for studying progression through complex programs of epithelial differentiation and cycles of epithelial self-renewal [32]. The notions of sequence diversity, pairwise regulation, and differentiation-specific expression of *KRT* genes have largely been conserved throughout evolution, suggesting the existence of a functional link between keratin proteins and the diversity of structure and function of epithelial tissues [33]. Frameshift mutations in epidermal keratins have been associated with ‘classical’ and ‘atypical’ forms of epidermolysis bullosa simplex (EBS), epidermolytic hyperkeratosis (EHK) and a related family of diseases [34]. Thus, there now

is conclusive evidence supporting the involvement of keratins in six broadly defined functions: structural support, cytoarchitecture, stress response, regulation of signaling pathways towards apoptosis and protein synthesis, and organelle/vesicle distribution [28].

1.2 The keratinocyte *in vitro*

Keratinocytes isolated from skin samples can be maintained as a monolayer in culture. These cells have a polyhedral, squamous or cuboidal appearance *in vitro*, similar to that observed histologically in skin sections, with the nucleus containing one or more large nucleoli [35]. The histological characteristics of the various layers of the epidermis are not observed in standard culture conditions but can be induced in 3D culture systems. In monolayers, normal tissue architecture is usually absent and cells organize in flattened, loosely associated layers, synthesize a different pattern of keratin polypeptides and form keratohyalin granules [36]. Studies in man and mouse have shown that cultured epidermal cells can differentiate, in response to factors like high calcium concentrations and high cell density, from a basal proliferating cell to a flattened squamous, enucleated cell of the *stratum corneum* and are eventually sloughed off into the media [37]. Pioneering studies by Rheinwald & Green *et al.* (1977) have shown that low calcium media promotes keratinocyte growth whereas media with calcium concentrations higher than 1.2mM causes arrest of cell growth and acceleration of terminal differentiation [38]. Similarly, terminal differentiation can be induced either by raising the keratinocytes (in 3D culture systems) to the air-liquid interface or by the use of delipidized serum (lacking vitamin A) [39]. Keratinocytes grown in low calcium media can be clonally passaged to differentiate and stratify similarly to the epidermis *in vivo* [40]. *In vivo*, basal keratinocytes are exposed to a low calcium concentration while in the supra basal layers keratinocytes differentiate in the presence of an increasing calcium concentration gradient.

2.0 Physiology and pathophysiology of wound healing

A skin wound occurs when the organ sustains a break compromising its form and function [41]. Tissues of the body are capable of healing by two mechanisms: regeneration and/or repair. The former occurs in fetuses and in some adult tissues like liver, bone and epithelium [4]. Repair is a less efficient process wherein damaged tissue is replaced by connective tissue resulting in the formation of a scar. The objective of skin repair is re-establishment of the protective epithelial cover and recovery of tissue integrity, strength, and function. Wound healing occurs by primary or secondary intention. Surgically induced, clean and non contaminated wounds that can be opposed with sutures or staples are classified as healing by primary intention. Healing by secondary intention occurs in wounds with edges that cannot be approximated, wound infection is common and wound healing is delayed [42]. This occurs through the four coordinated phases that characterize secondary intention healing of full-thickness skin wounds: hemostasis, acute inflammation, cellular proliferation and finally, matrix synthesis and remodeling [43]. It relies on the formation of granulation tissue that fills in the wound gap and over which epithelialization occurs. The process can be associated with substantial scarring.

2.1 Wound healing in the horse

The equine species can be roughly subdivided into horses and ponies, defined by height at the withers of an adult (ponies are <1.48 meters) [5]. The types of wounds commonly encountered in horses are lacerations, abrasions, penetrating or puncture wounds, contusions etc. Most often, traumatic skin wounds in horses are not amenable to primary closure and are forced to heal by secondary intention because of massive tissue loss, excessive skin tension, extreme contamination, or undue time elapsed since injury or the cost of treatment [6].

Wound healing in horses differs from the generic pattern described in humans and other commonly studied species such as rodents. In humans and rodents, the inflammatory phase lasts two to seven days, the proliferative phase is complete in two to three weeks, and the remodeling phase begins at three weeks lasting up to two years [42]. While the three, aforementioned phases are conserved, inflammation is sluggish and prolonged in the horse extending up to approximately four weeks [44]. Surprisingly, the inflammatory response in ponies is stronger and more succinct with a faster initial leukocytic infiltration during the first three weeks of healing. Further, leukocytes of ponies produce more reactive oxygen species (ROS) and higher levels of other inflammatory mediators like tumor necrosis factor (TNF), interleukin -1 (IL-1), chemoattractants, and transforming growth factor beta (TGF-β). The lower initial production of inflammatory mediators in horses renders debridement inefficient, consequently prolonging the inflammatory response and favoring a sustained release of cytokines, particularly TGF-β, that may lead to an exaggerated production of extracellular matrix (ECM) in the subsequent phases [6]. Indeed, it has been shown that the horse activates collagen formation to a greater extent and earlier during wound healing than rodents, predisposing it to the development of Exuberant Granulation Tissue (EGT), also known clinically as 'proud flesh'.

Wound contraction is desirable since it reduces the wound surface area requiring coverage by fragile neoepithelium, therefore leading to better cosmesis and function [5]. However, in horses, wound contraction is slower than in other species due to the imbalance of cytokines and mediators released in the aforementioned phases of healing. Moreover, epithelialization is a relatively slow process, further delaying wound repair in comparison to other species and to ponies [44]. In cases where fibroplasia is excessive, epithelialization is also impaired since keratinocytes are unable to cover the protruding tissue (EGT) that

physically impedes migration and inhibits cell mitosis [46].

In addition to species-related physiological differences in the wound healing process, horses also show different healing patterns according to the anatomical location of the wound. Indeed, while wounds on the head or trunk heal promptly and with few complications, those on the distal limb are subject to a number of complications that may arise due to anatomical traits such as 1- proximity to the ground that favors wound contamination and chronic inflammation; 2- relative deficiency of soft tissue coverage and consequently a scant vascular bed and hypoxic conditions [45]; 3- high motion due to the presence of numerous joints; and 4- poor wound contraction, related to the absence of a *panniculus carnosus* [6] and to the haphazard orientation of myofibroblasts within the granulation tissue [4]. Moreover, wounds on the distal limb must usually heal by secondary intention since the skin in this location is under more tension and consequently difficult to mobilize around a wound and prone to dehiscence following primary closure.

Several studies have compared the healing of experimental wounds created on the body and the limb of horses and shown important differences. For example, limb wounds double in size (due to wound retraction) after two weeks of creation, only returning to their original dimensions after 6 weeks of healing, while body wounds retract to reach a 22% increase in size at one week but quickly contract down to their original size one week later [46]. Indeed, full-thickness wounds on the body heal 70% by wound contraction and only 30% by epithelialization while the pattern is reversed in distal limb wounds and leads to the formation of a fragile, unesthetic epithelial scar. Moreover, the sluggish and weak inflammatory response to wounding leads to the formation of granulation tissue in limb wounds that remains irregular and purulent, further delaying epithelialization that progresses at a rate of 0.09 mm/day as opposed to 0.2 mm/day in body wounds [47]. These differences collectively lead to disparate healing times in wounds of identical size

(6.25cm^2) created in different locations: 6-8 weeks for body wounds versus 10-12 weeks for distal limb wounds [6].

Considering healing as a spectrum, the complications that develop in limb wounds in horses can lead to chronic nonhealing wounds at one end of the healing spectrum while at the other end of the spectrum, fibroproliferative conditions such as EGT ('proud flesh') are found to occur [4]. In both cases, the epithelial cover is lacking.

3.0 Advanced wound management (selected approaches)

Considering the cellular and molecular bases of wound healing, commercial products incorporating recombinant growth factors are being used therapeutically [53]. However these have exerted only a moderate impact on wound healing due to redundancy of the components of the wound repair process (e.g TGF-β [48] and PDGF [49]) or because of rapid degradation of these growth factors at the wound site particularly in chronic wounds where proteinases abound. It was thus proposed that administration of cells and/or tissues that retain the ability to elaborate the full complexity of biological signalling may hold the key to accelerate wound healing processes [50]. This has been accomplished via the grafting of natural or bioengineered skin or the cells composing it.

3.1 Skin grafting

Skin grafting is a technique used for coverage of large full thickness skin wounds to enhance healing and improve cosmetic and functional outcomes [51]. Wounds at or below the carpus and hock in horses are often best treated with a skin graft because wounds in these regions are incapable of significant contraction, leaving large areas to be covered by weak neoepithelium and, if not grafted, may develop EGT [9]. The two basic types of skin grafts are pedicle grafts and free grafts. The former, also referred to as 'axial pattern distant flaps' are not used in horses because relatively few axial pattern flaps have been identified

in horses compared with humans, dogs and cats [52]. A free skin graft, on the other hand, is detached from the donor site and relocated to a distant recipient site where it must create a new vascular connection to the wound bed in order to survive. A free skin graft is defined by the relation of the donor to the recipient. Autografts are taken from one site on a patient and transplanted to another site on the same patient. Isografts are grafts between identical twins or highly inbred strains (rare in horses). Allografts are transplanted from another individual of the same species, whereas xenografts are transplanted from another species. Autografts are the most common type of skin graft used in equine practice because allografts and xenografts are often rejected due to host-mediated immune responses causing necrosis and sloughing of the foreign tissue [53]. Acute rejection of skin grafts is characterized by moderate to severe perivascular inflammation (predominantly neutrophilic) with epidermal and/or adnexal involvement, spongiosis and exocytosis, epidermal dyskeratosis, apoptosis, or keratinolysis and necrosis evident at the transplanted site [54]. Alloreactivity may occur days and weeks after transplantation and cause acute graft rejection. Chronic rejection is characterized by parenchymal fibrosis and intimal thickening that occurs months to years after transplantation, resulting in a gradual loss of graft function [55]. Infiltration of the graft vessels and tissues by macrophages, followed by scarring, are prominent histological features of late graft rejection [56].

The poor cosmetic and functional outcome of skin grafts in horses along with the lack of redundant donor skin are some drawbacks of skin grafts that can potentially be addressed by using tissue engineered skin substitutes. Ultimately, the goal is to generate a skin construct that effects the complete regeneration of functional skin, including all its layers and appendages, as well as an operational vascular and nervous network, with scar-free integration within the surrounding host tissue [9].

3.2 Skin substitutes

The goal of regenerative therapies for wounds is to renew tissues such that both the structural and functional properties of the wounded tissue are restored to pre-injury levels. Skin substitutes should display some essential characteristics which include: ease of handling, conformability (to the wound site), provision of a vital barrier with appropriate water flux, adherence, appropriate physical and mechanical properties, ability to undergo controlled degradation, sterility, absence of toxicity and antigenicity, and minimal inflammatory reactivity [57]. Skin substitutes should incorporate into the host with minimal scarring and pain while facilitating angiogenesis of the construct. Such matrices should model the properties of ECM, with the ability to release a multitude of growth factors, cytokines and bioactive peptide fragments in a temporally and spatially specific event-driven manner. These matrices should consist of naturally occurring substances like collagen or be prepared from biodegradable polymers. In addition, the restoration of skin anatomy must go beyond rehabilitation of structural architecture to include restitution of skin pigmentation, nerves, vascular plexus, and adnexa [58].

Conventionally, tissue engineered skin consists of cells grown *in vitro* and subsequently seeded onto a scaffold or some porous material which is then placed *in vivo* at the site of injury. Cells are derived from a variety of lineages, from stem cells to differentiated somatic cells. These populations can be classified as progenitor, systemic or local. The different cells considered for skin substitutes include keratinocytes, fibroblasts, adipocytes, melanocytes, hair follicle-associated cells, and various progenitor cells. A common multilayered design consists of a highly cellular keratinocyte layer overlying a fibroblast-incorporated matrix to mimic the epidermis and dermis, respectively. The dermal matrix can be derived from natural sources (decellularized human or pig skin), created from

natural proteins (collagens, fibronectin or chitosan), or engineered from synthetic molecules (glycolic acid or polycaprolactone) [59].

Progress has been made in developing biomaterial substrates that contain various growth factors and that can be activated to release stored contents under controlled conditions. Ultimately, successful skin engineering relies on recapitulating the optimal cellular, matrix, biochemical and biophysical cues that drive tissue regeneration. At present there are no commercial skin substitutes available for use in horses, nevertheless, the recent development of a 3D skin culture system consisting of primary equine keratinocytes (PEK) seeded on tissue scaffolds made of porous polystyrene holds promise for the eventual commercialization of such products for horses [60].

3.2.1 Cultured epidermal grafts

The large-scale production of epidermal cultures suitable for the covering of skin defects such as those caused by burn wounds, was developed by a single suspension of disaggregated keratinocytes, seeded onto a feeder layer of irradiated (growth arrested) mouse 3T3 fibroblasts [38]. Autografting of these cultured epidermal autografts (CEA) onto large burn wounds was achieved by detachment of CEA as an intact sheet using the enzyme dispase [61]. Since then, CEA grafts have been the 'gold standard' for wound coverage in burn victims. In addition to treatment of burn wounds, CEA sheets have been used for the treatment of acute and chronic traumatic wounds in human patients. For this purpose, an initial population of clonogenic keratinocytes is amplified by subculturing once or twice to generate several hundred cultured epithelial cells within a three week period [62]. An increased calcium concentration within the culture media (1.8 mM) subsequently supports differentiation and stratification of the cells as confluence is approached, such that an intact stratified sheet can be formed. Ultimately, grafted cultured keratinocytes generate

a normal epidermis over many years and favor the regeneration of a superficial dermis. While CEA benefit from histocompatibility, the availability of normal skin from which to harvest the cells represents a significant limiting factor in massively burned patients. Thus, cultured epidermal allografts offer an ‘off-the-shelf’ skin replacement therapy for immediate application to injured skin in the form of a temporary dressing until CEA become available [57]. Besides cost, the disadvantages of cultured epidermal grafts (both autologous and allogeneic) is the three week waiting time, fragility, poor cosmetic quality of healed zones, variable 'take time', sensitivity to infection [8], spontaneous blistering and avulsions beyond three months of grafting attributed to the lack of underlying deep dermis [63]. Moreover, when keratinocytes achieve confluence, they transform from a highly proliferative state to one of growth arrest and stratification.

4.0 Regenerative medicine

Regenerative medicine was first defined as 'A new branch of medicine that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems' [64]. This definition has since evolved to include the fields of biology, chemistry, engineering, and medicine, encompassing disciplines such as gene-therapy, biomaterials and molecular medicines to provide solutions to some of the most challenging diseases. With the promise of regenerative medicine, cells, tissues and even organs can be grown in the laboratory and implanted back into the patient when the body cannot self-repair its tissues. Regenerative medicine relies on various approaches such as: 1- administration of stem cells, referred to as 'cell therapies'; 2- use of bioactive molecules with or without cells, referred to as 'immunomodulation therapies'; 3- strategies for *in situ* tissue regeneration, focusing on proliferation and migration of host stem or progenitor cells

into target-specific scaffolds and 4- transplantation of tissues or organs, grown in the laboratory, referred to as 'tissue engineering' [65].

Because the studies reported in this PhD thesis relate to the first approach, that of 'cell therapies', the remaining portion of the literature review will focus exclusively on this component of regenerative medicine.

4.1 Veterinary regenerative medicine

A relatively liberal legal and ethical regulation of stem cell research in veterinary medicine has facilitated the development, and in some instances, clinical translation of a variety of cell-based therapies. Volk and Theoret (2013) nevertheless suggest that despite rapid advancement of this field, there is a deficiency of supporting *in vitro* data as well as few evidence-based preclinical animal studies [66]. Thus, controlled and well designed studies must be carried out until accurate and reliable data is obtained for translation to the clinics [67]. Given the paucity of stem cell translational studies in non-human primates, large animal models (that include all companion and farm animals besides rodents) may be the next most relevant models [66]. For this reason, derivation of stem cell lines in veterinary patients may pave the way for the development of future cell-based therapies, disease modeling, drug and toxicity screening, biodiversity preservation, transgenic animal generation, etc [10].

4.2 Stem Cells

A stem cell is defined as an undifferentiated cell that can divide, through mitosis, for an indefinite period of time, with the capacity to give rise to other undifferentiated stem cells (referred to as 'self-renewal') and to more committed descendants through differentiation, including fully functional mature cells. Simply put, stem cells are the self renewing progenitors of specialized body tissues [68]. 'Stemness' is a cellular property

therefore determined by two main hallmarks: 1- the ability of self-renewal and 2- the capability to differentiate into mature cell types. Cell potency is the ability to differentiate into other cell types or a measure of the number and range of phenotypes into which stem cells can develop [69]. The therapeutic potential of stem cells lies not only in their ability to contribute healthy, differentiated cells to a compromised tissue bed, but also in their production of beneficial growth promoting factors and chemotactic recruitment of additional reparative cells.

The following diagram shows the hierarchy of pluripotency from the zygote to the completely differentiated cell:

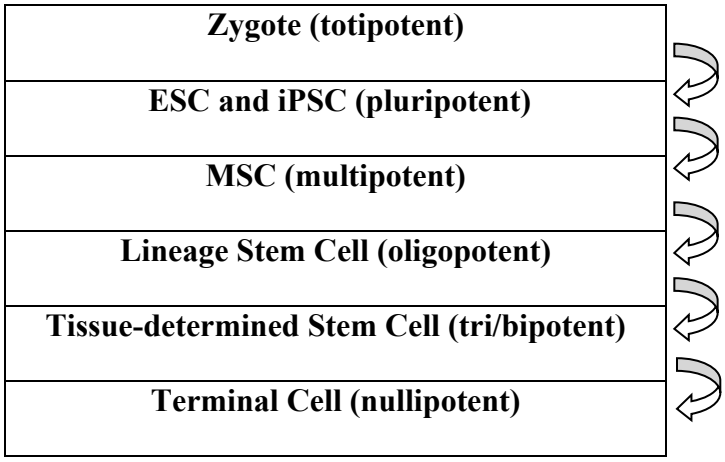

Fig. 4: Hierarchy of potency in stem cells from the zygote to the adult [67]

A totipotent cell sits atop the lineage hierarchy and can generate any type of cell in the body, thereby giving rise to an entire organism. A cell that can differentiate into all cell types, including the placental tissue, is known as totipotent. In mammals, only the zygote and subsequent blastomeres are totipotent [70]. Pluripotent stem cells can give rise to many or almost all cell types, as the prefix 'pluri' means many. Pluripotent cells can be isolated, adapted and propagated indefinitely *in vitro* in an undifferentiated state as embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC). Multipotent stem cells have the ability

to differentiate into a closely related family of cells. Examples include mesenchymal stem cells (MSC) such as hematopoietic stem cells (HSC) that can become red and white blood cells or platelets. Oligopotent stem cells have the ability to differentiate into a few cell types; examples include lymphoid or myeloid stem cells. These cells are restricted to the lineage into which they differentiate and are also called 'lineage stem cells'. For example, epithelial stem cells (EpSC) are considered to be developmentally committed such that they can form the differentiated cells of their own particular tissue type but not those of any other. Tripotent or bipotent cells are tissue determined stem cells and divide into two to three cell types [71]. Examples of these include neural stem cells that can divide into oligodendrocytes, astrocytes and neurons [72]. Bipotent stem cells can divide into two cells, a differentiated cell and another bipotent stem cell. These cells include conjunctival stem cells, hepatic stem cells and mammary epithelial stem cells, among others. Unipotent stem cells can produce only cells of their own type but possess the property of self-renewal, albeit limited, required of stem cells. An example would be (adult) muscle stem cells. All of these examples suggest that steady state cell renewal occurs largely from unipotent stem cells whereas tissue regeneration following damage may occur from multipotent stem cells. This implies that, when regeneration is required, stem cells are activated from their dormant state and divide based on the extent of tissue requirement for repair.

4.2.1 Pluripotent Stem Cells (PSC)

Pluripotent stem cells are derived from preimplantation embryonic cells, the inner cell mass (ICM) of the blastocyst (i.e ESC), or from primordial germ cells of the gonadal ridge (embryonic germ cells, EGC). Derivation of PSC is also possible through parthenogenesis (phESC) from unfertilized oocytes, through somatic cell nuclear transfer

(SCNT) into oocytes, from fusion of ESC with somatic cells, or from somatic cells through induction with pluripotency factors (induced PSC, iPSC) [73; 74].

Embryonic Stem Cells: The most pluripotent stem cell is the ESC derived from the ICM of 4-5 day blastocysts. Common methods to test the pluripotency in an ESC line are: 1- ability to form embryoid bodies *in vitro* and subsequent differentiation of those cells into specific cell types representing the three germ layers; 2- formation of teratomas upon transplantation into an immunocompromised host; 3- derivation of chimeric offspring *in vivo* with a demonstration that the ESC contributed to tissues derived from the endoderm, mesoderm and ectoderm, in addition to the germline; and 4- the most stringent of these, tetraploid complementation, where the ESC alone gives rise to the embryo proper [75]. Numerous human ESC-based therapies have shown promise in animal models and two of them, oligodendrocyte precursors and retinal pigment epithelium (RPE), have recently entered human clinical trials [76]. The development of ESC lines in mice has enabled the production of individual specific ESC lines in domestic and wild animals by adopting protocols used for isolation of murine ESC [75].

Equine ES-like cells have been isolated by Saito *et al.* (2006) [77], Li *et al.* (2006) [78], Guest *et al.* (2007) [79] and Desmarais *et al.* (2011) [80]. Li *et al.* (2006) were successful at culturing, for up to 26 passages, ES-like cells that maintained a normal diploid karyotype and expressed common markers for ESC such as AP, SSEA-1, SSEA-4, OCT-4, TRA-1-60 and TRA-81. The ES-like cells differentiated into three germ layer cells in the absence of mouse embryonic fibroblast (MEF) feeder layers and leukemia inhibitory factor (LIF). However, horse ES-like cells failed to produce any signs of teratoma *in vivo,* a classic assay for pluripotency, therefore preventing their qualification as 'ESC'; they are thus called 'ES-like lines' [81]. Li *et al*. (2006), suggest that the inability of equine ES-like cells to form teratomas in immunopriviledged sites may be a characteristic of this species,

given that teratoma formation was achieved in other farm animals [78]. Because the equine cells used in the teratoma trials were of late passage, it has been suggested that culture-induced changes might have reduced their *in vivo* pluripotency. Suboptimal culture conditions can also inadvertently select and enrich an alternative stem cell line [82]. In ungulates, trophectoderm and primitive endoderm can outgrow cultured ICM even after careful dissection or immunosurgery, and these cells can still form embryoid-like bodies reminiscent of those formed by ESC [83]. Thus, outstanding issues including differences in pluripotency characteristics among the existing ESC lines, preimplantation embryo development, pluripotency pathways as well as culture conditions plague efforts to establish authentic ESC lines from horses.

Induced Pluripotent Stem Cells: iPSC are somatic cells that have been genetically reprogrammed to an ESC-like state by being forced to express genes and factors important for maintaining the defining properties of ESC. The pioneering discovery of iPSC occurred when reprogramming somatic cells by forcing the exogenous expression of specific transcription factors, or 'Yamanaka factors': c-MYC, KLF-4, OCT-4 and SOX-2 (MKOS) [84]. The iPSC can undergo long term self-renewal when cultured *in vitro* with defined culture media or with growth factors released by feeder layers [85]. The irrefutable requirements or 'hallmarks of pluripotency' recommended by the International Stem Cell Banking Initiative (ISCBI) include: 1- pluripotency tests; 2- differentiation tests both *in vitro* and *in vivo*; 3- karyotype analysis to show that the newly generated lines have maintained genetic stability, as it is known that prolonged culture of pluripotent cell lines can result in genetic abnormalities, commonly causing aneuploidy; 4- determination of cell identity, usually performed by DNA fingerprinting and HLA analysis; 5- gene expression profiling via a stem cell array, to detect the expression of a common set of genes expressed

in undifferentiated cells and down-regulated upon differentiation; and 6- microbiological tests to ensure that the cultures are free of any contaminants [86].

Several viral and non viral methods have been used for iPSC reprogramming; the first reprogramming method to be reported was retroviral transduction with the Yamanaka factors, achieving a reprogramming efficiency of 0.01-1% [84]. One of the most efficient non-viral gene delivery systems for iPSC generation makes use of the *Piggybac* (PB) transposon that has the ability to be excised from its integration site without changing the original DNA sequence. The transfected PB transposon carries a single construct containing the coding sequences of MKOS that, upon inducible expression, successfully reprogram the somatic cells to iPSC [87; 88]. Other non-genetic systems explored for iPSC induction are direct delivery of the reprogramming proteins attached to cell penetrating peptides (CPP) that contain a high portion of basic amino acids and enable the proteins to cross the plasma membrane [89]. Use of microRNA was demonstrated to improve the efficiency of reprogramming by use of short RNA molecules that bind to complementary sequences on mRNA and block expression of a gene [90]. Use of synthetic modified mRNA is another technique to generate RiPSC (micro RNA derived iPSC) that are non mutagenic and highly efficient in comparison to the earlier retroviral methods [91].

Irrespective of the induction technique, iPSC have been assessed therapeutically in murine models of spinal cord injury, Parkinson's disease, sickle cell anemia, hemophilia, limb ischemia, acute myocardial infarction, peripheral vascular disease, diabetes and liver regeneration [92]. Additionally, trials in humans for treatment of macular degeneration [93], spinal cord injury [94] and recessive dystrophic epidermolyis bullosa (RDEB) are underway [95]. While human iPSC technology has been rapidly advancing, iPSC lines have also been successfully developed from numerous domestic and wild animal species (horse [87], pig [96], sheep [97], goat [98], dog [99], cattle [100], buffalo [101], rhinoceros and

snow leopard [102], thus making them more accessible for studies in veterinary research, especially since authentic ESC lines have yet to be established in these veterinary species. Moreover, given the aforementioned limitations of ESC, iPSC represent a promising alternative.

Specific to the horse, blastocysts can be efficiently produced *in vivo* and flushed out for ES-like cell isolation however the derivation of true ESC from equine blastocysts is challenging due to high costs and the inefficient protocols for the *in vitro* production of embryos [103]. The generation of equine iPSC (eiPSC) could be a promising alternative to equine ES-like cells. The pioneering work by Nagy and Smith who were the first to develop iPSC from equine fetal fibroblasts using a tetracycline-inducible transposon-based delivery of four factors (MKOS), has paved the way for eiPSC research. Equine iPSC maintain a diploid karyotype during *in vitro* culture over 26 passages while colonies exhibit morphology similar to that of human iPSC and express AP, OCT-4, NANOG, SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81[87]. Embryoid body and teratoma formation containing derivatives of the three germ layers is also observed. Equine iPSC have since been successfully derived from adult equine fibroblasts via retroviral transduction with three transcription factors (OCT-3/4, KLF-4, SOX-2) [104; 105] and also from equine keratinocytes, by the same group [1].

Several challenges hinder the clinical translation of iPSC, one of which concerns the donor cell age and differentiation status [106]. For example, aging cells harbor higher levels of genes that limit the efficiency and fidelity of the reprogramming process [107]. Other factors to consider prior to translation are the challenges with the efficiency of transduction based on the technique used for reprogramming [108]. Genomic insertion of viral transgenes, reactivation of transgenes and subsequent tumor formation in chimeric animals make viral derived iPSC highly unreliable for therapeutic purposes [109]. Other

viral-based methods use lentiviruses, retroviruses and sendai virus; all run the risk of insertional mutagenesis due to genomic integration. Besides transgene integration, these viral methods also require repeat transduction to maintain transgene expression and are less effective in certain cell types [110] and may also cause cells to sit in an intermediate stage of reprogramming [111].

4.2.1.1 Teratoma formation by PSC

The teratoma assay is key to ascertaining pluripotency [112]. Studies have shown that teratocarcinoma-like tumors arising upon transplantation of PSC contain malignant cells, differentiated tissues and normal immature precursor cells, confirming the close relationship between pluripotency and tumorigenicity [113]. The assay is performed by injecting cells into the kidney capsule, muscle, subcutaneous space, peritoneal cavity, testis, liver or the epididymal fat pad of severe combined immunodeficiency (SCID) mice [114]. Teratoma formation is strongly dependent on the site of transplantation/engraftment, differentiation status of the cells as well as purity and concentration of the cell population, among other factors [115]. Disparate growth patterns of teratomas have also been attributed to the environmental cues that influence stem cell behavior [116].

Some sites are considered 'immune privileged' because they prevent the occurrence of inflammation within an immune mediated environment. These sites are of importance in tissue allografting because they affect the survival rate of a transplanted graft [117]. For example, teratomas developed in immune privileged sites of the brain, testes and liver rapidly produced large tumors containing predominantly immature cells whereas subcutaneous implants were significantly slower growing and eventually formed tumors composed of differentiated tissues [116].

4.2.1.2. **iPSC derivatives**

In a clinical setting, iPSC would first be converted into specific types of differentiated cells before transplantation [118]. Directed differentiation of PSC into specific cell types is controlled by application of extrinsic signals in a precise temporal sequence that mimics development [119]. Recently, iPSC have been differentiated into several cell types for patient-specific therapy: neural lineages for generation of midbrain dopamine (mDA) neurons intended to treat Parkinson's disease [120], hepatocyte-like cells for treatment of liver disease [121], insulin producing cells for treatment of diabetes [122], germ cells and oocyte-like cells [123] for treatment of infertility, and keratinocytes for grafting onto burn victims and for treatment of epidermolysis bullosa (EB) [124] are some examples. All of these studies documented long-term *in vivo* survival as well as functional improvement in at least one relevant animal model of disease. Moreover, iPSC-derived retinal epithelium has been approved for clinical trials in human macular degeneration, implying that iPSC-derived grafts are not far from the clinic [125].

A major breakthrough of iPSC in dermatology was achieved through the generation of keratinocytes capable of populating 3D skin equivalents from normal and RDEB-diseased humans [126]. In EB and family of diseases, adult KSC are exhausted because of the continuous need to repair blister wounds [127]. Results showed that iPSC can be differentiated into KSC and skin-like structures, with the potential of treating EB. In mice, iPSC-derived p63/K14^{+} cells could form a stratified epidermis as well as hair follicles and sebaceous glands when grafted into the skin as a cell suspension mixed with normal skin fibroblast and dermal papilla cells [128]. Another recent study differentiated human iPSC to epithelial precursor cells (EPC) [129]; upon transplantation of human iPSC-EPC with trichogenic mouse dermal papilla cells into immunodeficient mice, hair follicle reconstitution, due to enhanced epithelial-mesenchymal interactions, resulted. Nissan *et al*.

(2011) worked on the differentiation of human iPSC to melanocytes, which were shown to integrate appropriately into organotypic epidermis reconstructed *in vitro*, as a potential treatment of hypopigmentation disorders [130]. Another study developed human ESC/iPSC-derived keratinocytes that could generate human epidermal equivalents in an air/liquid interface culture exposed to a sequential high-to-low humidity environment [131]. Although, the complexity of *bona fide* skin was not entirely reproduced, the essential components for a pigmented 3D skin equivalent have recently become available from the aforementioned iPSC studies.

Only a few reports of iPSC derivatives differentiated from domestic animals have been published. Pig iPSC (piPSC) were differentiated *in vitro* into rod photoreceptors [132], endothelial cells [133], neural cells [134] and hepatocyte-like cells [121]. A single report using eiPSC demonstrated neural differentiation cells with features of cholinergic motor neurons, including the ability to generate action potentials *in vitro*, were generated from eiPSC in hopes of one day using them to treat equine grass sickness and/or equine motor neuron diseases [1].

4.2.1.2.1 **Immunogenicity of iPSC derivatives**

Allogeneic cells and tissues face the challenge of the immune barrier, while autologous PSC are anticipated to engraft into recipients without the requirement for immune suppression [135]. However, deriving and characterizing patient-specific (autologous) iPSC is a time-consuming and expensive process (current estimates are 6 months and tens of thousands of pounds) [10]. A general assumption is that autologous PSC will be immune-privileged but this point has been contradicted by Zhao *et al.* (2011) [136]. Their study demonstrated immune rejection of syngeneic iPSC-derived teratomas in mice (derived by retroviral and episomal reprogramming) with T-cell infiltration. There

was overexpression of tumor-related genes, including Hormad and Zg16, in teratomas derived from syngeneic iPSC. This study is however critiqued since retroviruses have been shown to cause vector-driven genotoxicity and immunogenicity and because it made use of undifferentiated iPSC, consequently limiting clinical interpretation[137]. The immunogenicity of iPSC, whether autologous or allogeneic, has been attributed to retention of developmental antigens, acquisition of xenogeneic epitopes, and/or expression of aberrant antigens over the course of long-term culture to generate iPSC and differentiated cells [138].

Two recent reports have contradicted Zhao's findings by showing no immunogenicity of *in vitro* differentiated syngeneic iPSC derivatives. Araki *et al.* (2013) studied the immunogenicity of terminally differentiated skin and bone marrow tissues derived from integration-free plasmid borne iPSC and ESC lines. They found no differences in the rate of success of transplantation when skin and bone marrow cells derived from iPSC were compared with ESC-derived tissues; moreover, both skin grafts and bone marrow were very rarely rejected in the syngeneic setting [118]. Specifically, there was limited or no immune response such as T-cell infiltration, for tissues derived from either iPSC or ESC, and no increase in the expression of the immunogenicity-causing *Zg16* and *Hormad1* genes in regressing skin and teratoma tissues. Skin samples from the tails of the chimeric mice were prepared and then transplanted onto the backs of syngeneic GFP^- allogeneic mice. All of the iPSC differentiated and GFP^+ transplanted grafts could be sustained over a 10-month period.

A similar study showed that lentivirally reprogrammed murine iPSC, episomal iPSC (EiPSC) and ESC, when terminally differentiated *in vitro* into endothelial cells, hepatocytes and neuronal cells then transplanted syngeneically, led to 100% graft survival with no evidence of immune rejection as measured by T-cell infiltration [139]. These findings

suggest that iPSC-derived endothelial cells, hepatocytes and neuronal cells are potentially non immunogeneic and thus immuneprivileged.

4.2.2 Adult Stem Cells (ASC)

Adult stem cells are undifferentiated cells, found throughout the body after development, that multiply by cell division to replenish dying cells and regenerate damaged tissues [140]. Adult stem cells are post natal stem cells, also known as somatic stem cells, and can be found in juvenile as well as adult mammals. Examples of ASC are EpSC, HSC, mammary stem cells, intestinal stem cells, endothelial stem cells, neural stem cells, olfactory stem cells, neural crest stem cells, testicular stem cells and MSC [141].

4.2.2.1 Mesenchymal Stem Cell (MSC)

Mesenchymal stem cells are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts, chondrocytes, and adipocytes. Due to the current controversies in the definition of MSC, the International Society for Cellular Therapy (ISCT) has suggested some phenotypical and functional characteristics for identification of MSC. These include 1- plastic adherence; 2- expression of the cluster of differentiation (CD) markers CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules; and 3- multipotency differentiation capacity [142]. The ISCT suggests that plastic-adherent cells generally described as MSC should be retermed 'multipotent mesenchymal stromal cells', while the term MSC should be reserved for cells with *in vivo* demonstrations of long-term survival with self-renewal capacity and tissue repopulation with multilineage differentiation [143]. An exception to the classification of MSC based on plastic adherence, are non-adherent HSC that differentiate into myeloid and lymphoid lineages [144].

Progenitors are proliferative cells with a limited capacity for self-renewal (short term) and are often unipotent, whereas stem cells self renew throughout the lifetime of the animal (long term) [145]. Perinatal and midtrimester MSC have shown advantageous growth and plasticity properties over adult MSC. These fetal MSC were readily expandable and senesced later in culture than their adult counterparts attributable to their longer telomeres and greater telomerase activity [146].

Based on the source MSC are called bone marrow-derived MSC (BMSC), adipose-derived MSC (AMSC), umbilical cord blood-derived (UCB)-MSC [67]. These have lately been gaining therapeutic relevance for several clinical conditions [66]. The BMSC have the advantage of being relatively noninvasively obtained and have a greater capacity to differentiate into tissue types of the musculoskeletal system in comparison with other MSC [147]. Therefore, despite the wide diversity of tissue sources from which to harvest MSC, many investigators employ BMSC for the treatment of musculoskeletal afflictions [148]. The drawback of culture-expanded BMSC is the time lag of three to six weeks from bone marrow aspiration until readiness for treatment. In addition, BMSC are more difficult to isolate with increasing donor age and show reduced plasticity and growth as a factor of both increasing donor age and number of *in vitro* passages [146].

In the skin wound context, experimental studies have shown that BMSC enhance healing in normal and diabetic mice by significantly accelerating wound closure, via increases in cellularity, angiogenesis and the rate of epithelialization [149; 150]. In a rat model of burn wounds, injection of BMSC significantly decreased the rate of apoptosis in dermal cells in and around the initial wound [151]. In human burn patients, administration of BMSC resulted in mild pain relief and decreased plasmarrhea after 30 min, neovascularization and epithelialization was improved and skin grafts were accepted better than previous cases of allogeneic skin grafting [152; 153].

In conclusion, MSC are currently being used therapeutically in various organ systems in humans, based on the promising results obtained in various animal models. However MSC have disadvantages such as limited capacity to proliferate, rapid loss of differentiation potential, differentiation into few selected lineages and are thus unlikely to regenerate the entire organ of the skin, contrary to what has been achieved by iPSC studies [126].

4.2.3 Immunogenicity of MSC

An intriguing characteristic of MSC is their ability to act as immunomodulators and pro-angiogenic agents [154]. This is potentially mediated through the secretion of a variety of trophic signals that favor engraftment and/or endogenous regeneration [155]. The therapeutic use of MSC has been encouraged by low expression of MHC-I and absence of expression of MHC-II molecules that endows MSC with the potential to escape recognition by alloreactive $CD4^+$ T-cells [154]. Under non-inflammatory conditions, human and mouse MSC are MHC-II^-, suggesting they are hypoimmunogenic through the control of alloantigen expression [156].

Equine regenerative medicine has evolved rapidly with the use of MSC for musculoskeletal disorders, given their immunomodulatory properties [67]. A study comparing MSC from equine bone marrow, adipose tissue and umbilical cord was conducted to determine the best source for banking of equine MSC for allogeneic therapy. All types of MSC showed low or negative expression of MHC-II (**Table I**). A preclinical safety study in 16 horses investigated autologous and allogeneic placentally-derived MSC injected intra-articularly. While MSC were MHC-I^+, MHC-II^- and $CD86^-$, there were no significant differences in the degree and type of inflammation elicited between self and non-self-MSC [157]. Nevertheless, while the potential immunomodulatory effect of MSC

on various disease states is promising, the lower differentiation potential of MSC in comparison to iPSC implies a weaker regenerative capacity.

SOURCE	TISSUE	STEM CELL	IMMUNE RESPONSE
Embryonic	Blastocyst (5-7 days)	ESC	Low expression MHC-I and MHC-II antigens [158]
Fetal	Fibroblasts	iPSC	Low expression MHC-I and MHC-II antigens [159]
Newborn	Umbilical cord blood Whartons Jelly Placenta	UCB-MSC	Absence of MHC- II [160]
Adult	Bone marrow Adipose	BMSC A-MSC	MHC-I^{+}, absence of MHC-II [154]
	Others: Epidermal (skin), Neuronal, Muscle, etc	iPSC NSC	Low expression MHC-I and MHC-II [161]

Table I: Stem cell types, sources and immune response they generate

4.3 Immunological hurdles in stem cell transplantation

The major problem in cell and tissue transplantation is that the recipient's immune system may cause rejection of the graft. In the absence of immunosuppression upon transplantation, the immune system of the recipient mounts a rejection response towards foreign cells and tissues, while transplantation of autologous cells / tissues are recognized by the host as its own [162]. Transplant rejection, as mediated by the MHC complex, is of one of three types: hyperacute, acute or chronic [54]. Rejection is initiated by recognition of MHC alloantigens by the T-cells of the immunocompromised recipient. In humans, the MHC is referred to as the human leukocyte antigen (HLA) while in horses it is referred to

as the equine leukocyte antigen (ELA). Specifically, HLA/ELA refers to the protein molecule whereas the MHC is the region on the genome that encodes for this protein [55].

Adequate compatibility of donor and recipient MHC, ensured through prior typing and matching, is crucial to enhance graft acceptance [163]. Nevertheless, although MHC matching significantly improves the success rate of tissue and cell transplantation, it does not, in itself, prevent rejection. This is because MHC typing is imprecise, owing to the polymorphism and complexity of the MHC gene [164]. In addition to MHC-I molecules, all cell types express mHC antigens that are derived from mitochondrial and H-Y gene products. While they are less critical to acceptance of cell transplants than are MHC antigens, mHC antigens can nevertheless initiate allograft rejection, particularly when many mHC antigens act together [165].

In mice, MHC-I molecules were not detected by flow cytometry on ESC nor on undifferentiated iPSC [161]. Similarly, in Mauritian cynomolgus macaques (CM), the MHC-I expression by undifferentiated iPSC was found to be very low while MHC-II expression was undetectable. Deleidi *et al*. (2011) determined that the MHC genetic diversity in CM is distributed among seven haplotypes and that specific haplotypes can be identified by genetic screening using a panel of microsatellite markers, permitting MHC-matched iPSC transplantation [166]. Likewise, ELA haplotyping, using microsatellite PCR, has been characterized by Tseng *et al*. (2010) [167]. However, there is currently no data on the immunogenicity of eiPSC despite the recent development of eiPSC by other groups [105]. Undifferentiated porcine iPSC were found to express only low levels of MHC-I and moderately increased levels of MHC-I on their differentiated derivatives, whereas MHC-II was rarely expressed on both undifferentiated and differentiated porcine iPSC [168].

Possible solutions to prevent allorecognition and rejection include the development of mild immunosuppressive regimens (e.g. monoclonal antibodies targeting NK cells and/or T-cell subsets) sufficient to induce tolerance to allogeneic iPSC-derived cells [169]. Other proposed strategies for inducing tolerance in stem cell grafts are based on clinical efficacy in treating graft versus host disease (GVHD). Inhibition of signaling pathways (tyrosine kinase, JAK, PKC) through pharmacological approaches has been achieved using imatinib mesylate, sotrastaurin etc. [170]. Suppression of neovascularization by inhibition of VEGF has also been shown to ameliorate symptoms of GVHD [171]. Immunotherapy by blockade of lymphocyte molecules like CD2, CD20 [172], co stimulatory molecule blockade of CD28, CD40, and inhibition of cytokines like TNF-α, IL-1, IL-10 are other suggested strategies to encourage graft acceptance [173]. Induction of tolerance has been shown by use of regulatory T-cells, DC, NK and MSC [174]. However, any level of immunosuppression would likely increase the risk that rare undifferentiated PSC in an iPSC-derived population will form teratomas [158].

4.4 Cell and tissue based skin therapy

As a result of the aberrant healing pattern of skin wounds in horses, particularly those located on the limbs, a large area of scar tissue covered by fragile neoepidermis devoid of essential adnexal structures typically develops. Current treatments are only partially effective in accelerating healing and ensuring an outcome that is both cosmetic and functional. Skin grafts and cultured epidermal grafts are the gold standard for coverage of large wounds and burns in humans. Presently, the preparation of CEA requires a sample of donor tissue followed by extensive cell expansion lasting about three weeks before transplantation is possible [8], while CEAl only suffice as a temporary dressing [63]. Moreover, the isolation technique is difficult to standardize and the tissue-derived primary

cells are consequently heterogeneous [65]. Furthermore, CEA used for the treatment of human burn patients only provide wound coverage by epithelial sheets of keratinocytes lacking the capacity to form all the other cell types of the skin, especially the underlying dermis that is crucial to cosmesis and stability of grafts [175]. Although KSC are the most therapeutically relevant cell type for skin grafting within CEA, other stem cell sources have recently been exploited. Adult stem cell (i.e ADSC) have been shown to differentiate *in vitro* into both mesenchymal and non-mesenchymal lineages and a few studies have shown that MSC can transdifferentiate into keratinocyte-like cells and develop stratified epithelium when seeded on decellularized dermis [176] but are unable to regenerate the secondary accessory structures of the skin such as sweat glands and hair follicles.

Pluripotent stem cells such as iPSC represent an important stem cell source for dermatology because of their ability to differentiate into almost all types of cells in the body, providing a renewable source of cells with unlimited proliferative capability [177]. Although autologous iPSC would constitute the ideal 'personalized therapy', the logistics of achieving this on a large scale are daunting, owing to the relatively low efficiency and, consequently, the high cost of inducing pluripotency [158]. Thus, allogeneic iPSC-derived skin substitutes would appear to offer a potential 'off-the-shelf' solution. The obvious advantages of an allogeneic approach are that the donor can be selected ahead, qualified by HLA matching, and tested for absence of different disease organisms, such that the cells are prepared in advance so they are immediately available when needed by a patient [178].

Recent evidence suggests that iPSC can be differentiated into adnexal structures as well as nervous, vascular, immune and pigment components of the skin [175]. Similarly, eiPSC are anticipated to differentiate into multiple cell types, in response to appropriate differentiation protocols, ultimately enabling restoration of the equine skin's stratified epithelium, pigmentation, nervous and vascular plexuses, as well as adnexa (hair follicles,

sweat and sebaceous glands) that together constitute the functional organ of the skin. The concept of integrating different populations of iPSC-derivates to create complex tissue structures may prove more applicable along with the use of 3D scaffolds [59].

Nevertheless, two crucial issues must be resolved before such specifically differentiated cells can be exploited in a clinical setting. First, determining the immune response of undifferentiated iPSC and their derivatives; second developing standardized differentiation protocols to generate iPSC-derived keratinocytes to be used in future applications of wound grafting.

II. Rationale

Horses frequently suffer from chronic non-healing wounds lacking an epithelial cover and thus might benefit from skin grafts to provide much-needed coverage. Because of a paucity of donor skin in this species, it is interesting to consider tissue-engineered skin substitutes such as those available in human medicine. Ideally, the cells used to construct these substitutes should ensure regeneration of all the components of normal skin, including its specific adnexal structures as well as nervous and vascular plexuses. Pluripotent stem cells, such as the iPSC recently engineered in horses, seem promising candidates on account of their properties of unlimited self-renewal and potential to differentiate into most cell types of the body. Prior to considering eiPSC for therapeutic purposes, their immunogenicity must be studied to predict whether eiPSC-derived grafts would be accepted following transplantation. It was originally assumed that autologous iPSC grafts would circumvent the need for tissue matching prior to transplantation but a recent study challenges this assumption [136]. Moreover, significant economic and time constraints are associated with the derivation of autologous (patient-specific) iPSC. Allogeneic grafts might therefore be more appealing since they theoretically should provide an off-the-shelf product. My first study thus investigates the immune potential of allogeneic eiPSC both *in vitro* and *in vivo*. The subsequent study aims to develop an efficient protocol to drive eiPSC to commit to a keratinocyte lineage (eiPSC-KC) in view of generating the cells required to engineer a stem-cell based skin construct for horses.

III. Aims and Hypotheses

The main hypothesis of this thesis was that equine iPSC (eiPSC) have characteristics that can be harnessed for potential use in veterinary regenerative medicine.

SPECIFIC HYPOTHESES

1. Allogeneic equine iPSC weakly express MHC-I and II molecules and therefore will not elicit a rejection response when injected intradermally in horses.

2. Equine iPSC can be induced to differentiate into cells of the keratinocyte lineage (iPSC-KC) with morphological and functional characteristics resembling those of primary equine keratinocytes.

OBJECTIVES

The long-term objective of this research program is to develop a novel approach to engineer a functional skin substitute to be used for the management of wounds or skin disease in horses.

Short-term objectives:

1. To measure the expression of MHC molecules on allogeneic eiPSC and describe their immunogenicity using intradermal testing in horses.

2. To develop an efficient method to drive the differentiation of eiPSC into a keratinocyte lineage (iPSC-KC).

Article 1

Immune potential of allogeneic equine induced pluripotent stem cells

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Summary

Reasons for performing study: Induced pluripotent stem cells (iPSC) have brought immense hope to cellular therapy and regenerative medicine. However, the antigenicity of iPSC has not been well documented and remains a hurdle for clinical applications. Expression of major histocompatibility (MHC) molecules by human and murine iPSC is downregulated, making these cells potentially safer for transplantation. No such data is available for any large animal model.

Objectives: The aim of this study was to measure expression of MHC molecules on eiPSC and describe their antigenicity using intradermal testing. We hypothesised that allogeneic equine iPSC (eiPSC) weakly express MHC molecules and would not elicit a rejection response when injected intradermally.

Study Design: Experimental study involving both in vitro and in vivo components.

Methods: Two green fluorescent protein positive (GFP^+)-expressing eiPSC lines were analysed by flow cytometry for MHC expression. One line was then transplanted intradermally, along with appropriate controls, into two unrelated experimental horses. Blood was collected pre- and 7 days post-transplantation. The wheals formed at the sites of injection were measured and at regular intervals beginning at 0.25 h until 4 weeks. Tissue samples of the injected sites were obtained at 2, 3, 7 and 30 days post-transplantation and analysed by histopathology and immunofluorescence.

Results: Both eiPSC lines weakly expressed MHC molecules. eiPSC were detectable up to 7 days following allogeneic transplantation and elicited no apparent systemic response. Injection of eiPSC caused small wheal formation at the skin surface. Skin sections revealed $CD4^+$ and $CD8^+$ mononuclear cells up to 30 days post-transplantation.

Conclusions: These data suggest that while transplantation of allogeneic eiPSC elicits a moderate cellular response, it does not cause acute rejection. The feasibility of banking allogeneic iPSC for regenerative medicine applications should be explored.

Introduction

Regenerative medicine is evolving rapidly due to advances in the use of stem cells for research and therapeutics. Evidence-based clinical trials in veterinary models like horses provide tremendous opportunity for assessing both efficacy and safety of stem cell therapies for all species. Mesenchymal stem cells (MSC) are used therapeutically for equine musculoskeletal disorders, given their ability to differentiate into mesodermal lineages [1]. Significant drawbacks with MSC are low cell survival, limited life span and the lack of sustained engraftment, as well as the potential development of fibrosarcomas and carcinomas [2]. Pluripotent stem cells, on the

other hand, present a limitless capacity for self-renewal and the ability to differentiate into cells of all 3 germ layers. However, attempts to establish embryonic stem cells (ESC) from veterinary species have proven elusive due to loss of pluripotency over a short number of passages and failure to form teratomas in immunodeficient mice [3]. The main advantage of iPSC is that they may offer an off-the-shelf therapy that would considerably reduce the time between diagnosis of disease and implantation. Induced pluripotent stem cells may also offer the opportunity to have fully characterised cell lines that are optimal for different clinical indications.

Induced pluripotent stem cells (iPSC) provide a renewable source of cells with ESC-like properties but which circumvent ethical issues since they are engineered without destroying an embryo. Reprogramming to a pluripotent state was initially achieved through viral transduction; alternative non-genomic integration methods were recently developed [4]. In 2011, equine iPSC (eiPSC) were developed by a transposon system; these lines were propagated for extended periods *in vitro* without losing normal diploid karyotype and were capable of forming teratomas in immunocompromised mice [5].

Since iPSC can be generated from adult cells, patient-specific (autologous) stem cells may be produced, which should obviate the need for immunosuppression and/or tissue matching prior to transplantation. However, the ability to generate the required cell type for each patient is unrealistic due to the time needed for reprogramming and prohibitive cost. Consequently, allogeneic iPSC are expected to be the first choice in future clinical trials and thus, their immunogenicity must be addressed since rejection is a significant hurdle in stem cell transplantation. Allogeneic MSC are well tolerated in horses because they have the capacity to modulate immune activation despite expressing MHC-I molecules [6]. Equine ES-like cells, upon injection into damaged superficial digital tendons, do not produce signs of cell-mediated

immune response [7]. No such information is currently available for eiPSC, though it is reported that even autogenous mouse iPSC, weakly expressing MHC-I molecules, can be targets for cytotoxic T-lymphocytes, leading to their rejection following transplantation [8; 9]. This immunogenicity appears greater when iPSC are established using a viral system. Therefore, the immunogenicity of eiPSC, engineered using a transposon system, must be studied before clinical application is envisaged in horses.

The aim of this study is to determine the *in vivo* immune response of horses to dermally transplanted allogeneic eiPSC, without immunosuppressants, to predict their therapeutic value in regenerative medicine. We postulated that eiPSC express low levels of MHC-I and -II, rendering them weakly antigenic, allowing successful transplantation *in vivo*. This approach will help select lowly immunogenic eiPSC lines for differentiation into specific lineages for eventual clinical applications.

Materials and Methods

In vitro portion of the study

Equine iPSC

Equine iPSC lines were obtained by transfection of equine fetal fibroblasts with a *PiggyBac* transposon system containing the tetracycline inducible Yamanaka factors and constitutively controlled Green Fluorescent Protein (GFP) marker, then propagated in culture conditions as described [5]. Two eiPSC lines (line 1 and line 2) expressed the key pluripotency markers Oct-4, Sox-2, Klf-4, c-Myc in addition to SSEA1, SSEA4, TRA-1-60 and TRA-1-81.

Flow Cytometry

Equine iPSC were prepared in PBS[a], blocked with 0.5% goat serum[b], then incubated with primary equine specific antibodies (MHC-I: CVS22; MHC-II: CVS20)[c] followed by staining with secondary antibody (R-PhycoErythrin (RPE)-conjugated sheep anti-mouse IgG)[d]. A minimum 20,000 events were analysed by flow cytometry (Cell Lab Quanta SC Beckman Coulter)[e] and the percent positivity as well as mean fluorescence intensity (MFI) for specific markers calculated based on the respective isotype controls. Alongside the eiPSC lines, the fibroblast parental line was analysed for expression of MHC-I and -II.

In vivo study

Animals

Two mares, an 8 year old Standardbred and an 18 year old Arabian, were used to study dermal transplantation of eiPSC lines. The animals were routinely vaccinated and dewormed 2 weeks prior to entry into the study. A full physical exam, complete blood count and biochemical profile were performed 7 days prior to the study; all parameters were within normal limits. Horses were kept in standing stalls for the duration of the study and allowed *ad libitum* access to grass hay and water. Horses were examined daily for signs of discomfort and systemic illness. Animals were assessed for potential adverse reactions throughout the study. The systemic response was assessed by monitoring the animals' physiological status (temperature, respiratory rate and heart rate) and general disposition/signs of discomfort (e.g. anorexia and pain at the sites of injection).

Preparation of eiPSC for transplantation

Early passage (P8-P9) allogeneic eiPSC (lines 1) were cultured routinely up to 70% confluency. Forty-eight hours prior to intra-dermal injection the cells were removed from the factors that maintain pluripotency as well as doxycycline[d] and cultured in inhibitor-free media[b]. Twenty-four hours prior to injection, eiPSC were cultured in equine serum[d] rather than FBS to permit elution of absorbed bovine proteins. On the day of injection, eiPSC were separated from the mouse feeder layers (MEF), then pelleted and resuspended in 0.1 mL sterile saline[f] for injection. Equine iPSC were separated from MEF by permitting selective attachment of MEF to gelatin-coated plates for 30 min, as described previously [10]. Equine iPSC were prepared no more than 2 h prior to injection and maintained in tuberculin syringes[g] on ice until injection (cell viability was verified immediately prior to transplantation).

Cell transplantation procedure

A grid model, adapted from a previous study [6], was used as a design for the transplantation of eiPSC along with appropriate controls. The hair was clipped from a rectangular zone (35 cm x 20 cm) on the lateral neck, on both sides of each horse. 4 x 4 grids were traced within each of the clipped areas. Prior to injection, the horses were sedated using detomidine hydrochloride (Dormosedan)[h] (0.01 mg/kg i.v.) and butorphanol tartrate (Torbugesic)[i] (0.01 mg/kg i.v.) and the sites aseptically prepared. Within each horizontal row, each box received 0.1 mL of one of 3 substances injected intradermally using a tuberculin syringe: 1- 0.9% sterile saline solution (cell diluent, negative control); 2- 0.1 mg phytohemagglutinin (PHA)[d] in sterile saline (positive control); 3- allogeneic eiPSC line 1 (1×10^6 cells suspended in 100 µL sterile saline). Injection order was randomised in each horizontal row and clinicians blinded to substance identity. One horizontal row served for each sampling time (described below). The 2 sides of the neck provided duplicate samples for the study.

Physical examinations were performed on Days 1, 2, 3, 7 and 30 post-injection. Complete blood count and biochemical profile were repeated at Day 7. Injection sites were evaluated at 0.25, 4, 24, 48 and 72 h, 7 days post-injection and then twice weekly until 4 weeks post-injection. The diameter of any developing wheals was measured using Vernier calipers.

Tissue sampling

Under sedation and infiltration of 2% lidocaine (Lurocaine)[j], full-thickness skin samples of the injected sites were collected using an 8 mm skin biopsy punch[k] at 2, 3, 7 and 30 days post-injection. The bottom horizontal row served for Day 2 sampling; the second from bottom row for Day 3 sampling, the second from top row for Day 7 sampling and the top row for Day 30 sampling.

Histology

Morphologic features were evaluated on 5 μm paraffin sections stained with hematoxylin phloxine saffron (HPS)[l]. Tissue sections were graded, in a blind fashion, for perivascular dermatitis, eosinophilic infiltration and haemorrhage on a semi-quantitative numerical scale from 0-3, with 0 lacking a response and 3 showing the maximum response. Sections were examined at 40X to quantify the size of the inflammatory foci using an epifluorescent microscope (Zeiss Axio Imager)[m].

Fluorescent and immunofluorescent imaging

Frozen tissue blocks were cryosectioned to a thickness of 4 μm and mounted on Superfrost slides[n]. Slides were stored at -80 °C and thawed to room temperature when ready to be viewed. Unfixed, unstained sections were evaluated for the presence of GFP^+ eiPSC, detectable by direct

fluorescence, using an epifluorescent microscope.

Immunofluorescence was used to detect $CD4^+$ and $CD8^+$ cells in 5 μm paraffin sections of tissue samples. Sections were deparaffinised and rehydrated followed by enzymatic antigen retrieval then were blocked in goat serum for 1 h and stained with primary equine specific antibodies (CD4: HB86A IgG1)[o] and (CD8: ETC142B1A IgG2a)[o]. Secondary antibodies were Cy3 labelled goat anti-mouse IgG[q] and (AlexaFluor 488) goat anti-mouse IgG[b]; nuclei were counterstained with Dapi[d]. Isotype (negative) controls served to assess background staining while positive controls consisted of equine lymph node. Images were recorded using Zeiss MRm camera using AxioVision software. Entire biopsy sections were observed at 40X using an epifluorescent microscope. Five high power fields with representative infiltration were selected and the number of $CD4^+$ and $CD8^+$ cells was counted and compared to the total number of cells in the field.

Data Analysis

Ordinal scores were analysed using the Cochran-Mantel-Haenszel test for repeated measures. Quantitative variables were analysed using a repeated-measures linear model with treatment and time as within-subject factors. *A priori* contrasts were used to compare pairs of means adjusting alpha level for each comparison using the Bonferroni sequential procedure. Results are presented as mean ± standard deviation. Statistical significance is set at $P<0.05$.

Results

Low expression of MHC-I and MHC-II by eiPSC

Surface expression of MHC molecules by eiPSC lines was measured to predict the *in vivo* immune response and thus suitability for eventual transplantation. **Table 1** summarises MHC

184 expression by the 2 allogeneic eiPSC lines, parental fibroblasts and peripheral blood leukocytes
185 **(Fig 1)**.

186 ***Persistence of eiPSC in tissue sections following transplantation***

187 The GFP^+ eiPSC were detected on account of their inherent fluorescence in unfixed, unstained
188 tissue sections. Equine iPSC were found in sections of the injected sites 3 and 7 days following
189 transplantation **(Fig 2b** and **2d)** however no fluorescent cells were detected in 30 day samples.
190 Approximate colocalisation images show neutrophil predominant inflammatory infiltrates at day
191 3 **(Fig 2a)** and lymphocyte predominant infiltrates at Day 7 **(Fig 2c)**, around the site of eiPSC
192 transplantation.

193 ***Response to intradermal allogeneic eiPSC transplantation***

194 Intradermal injection of test solutions elicited no apparent systemic response throughout the
195 study period (data not shown). Because of insufficient volumes of eiPSC, a single animal
196 received a full dose of allogeneic eiPSC in the complete grid design. In this animal, some square
197 of the grid lacked duplicate injections such that statistical analyses could not be computed on
198 these data after 48 h. Nevertheless, these injection sites provide preliminary data on the chronic
199 response to transplantation of allogeneic eiPSC.

200 **I) Gross local response**: All test solutions caused the formation of wheals, the smallest of which
201 resulted from the injection of saline (negative control). The maximum dermal response was
202 caused by injection of PHA; a measurable response was noted within 30 min of injection, with
203 heightened induration and maximal elevation within 72 h **(Fig 3)**.
204

A repeated-measures analysis revealed a statistically significant effect of treatment on the mean wheal diameter ($P = 0.01$) and an interaction between treatment and time ($P = 0.007$), suggesting that the effect of treatment differed from one time period to another (**Fig 4)**. *A priori* contrasts revealed no statistically significant differences among treatments with respect to the mean diameter at 30 min and 2 h. At 4 h, 24 h and 48 h, mean diameter was significantly larger in the PHA treatment than in the allogeneic eiPSC group and the saline group ($P<0.002$).

II) Histologic response: Histopathology is one of the key means to determine acceptance or rejection of allogeneic tissues. Notable responses in this study included perivascular dermatitis, eosinophilic infiltration and haemorrhage, though in all cases inflammation was focal in nature and restricted to the dermis, excluding the epidermis and adnexa. None of the test solutions caused any severe local manifestations normally associated with graft rejection such as atrophy, acanthosis, necrosis or sloughing of the skin at the site of transplantation.

Based on semi-quantitative analyses, PHA injection caused maximal perivascular dermatitis compared to other treatments (**Supplementary Item 1)**. Maximum response was seen 24-48 h following injection, consistent with the measured diameter of the wheal **(Fig 4)**. A nonspecific immune response at PHA sites consisted of clustering of inflammatory cells around blood vessels in the dermis **(Fig 5)**. PHA resulted in subacute/chronic active type inflammation, with macrophages, neutrophils and eosinophils. The leukocytic exudate became lymphocytic 7 days post injection with resolution of inflammation by Day 30. Saline sites had no leukocytic infiltration. The allogeneic eiPSC site had a moderate score of perivascular inflammation with focal inflammation in the superficial dermis, initially leukocyte prevalent, becoming lymphocytic after 7 days **(Fig 2, Fig 5)**. Vessel walls showed no signs of endothelial swelling. The area of perivascular lymphocytic infiltration became progressively larger between Days 7 and 30 of the

study **(Fig 5)**. The distribution of scores for eosinophilic infiltration varied significantly among treatments (P<0.0001). PHA elicited the maximum response with large numbers of eosinophils. Equine iPSC did not differ statistically from the saline group. Large numbers of erythrocytes were noted in PHA-injected sites while all other groups had only a few.

Allogeneic eiPSC cause activation of CD4$^+$ lymphocytes

Leukocytic infiltration at the sites of injection was further characterised by CD4 and CD8 immunostaining. Data suggests that CD4$^+$ and CD8$^+$ cell in PHA injected sites were predominantly high on Day 2 that reduced after 30 day. Whereas in iPSC injected sites CD4$^+$ and CD8$^+$ cells were found to increase over 30 days **(Fig 6) (Supplementary Item 2)**.

Discussion

Induced pluripotent stem cells hold great promise in regenerative medicine by virtue of their ability to form any tissue in the body. For pluripotent stem cells to fulfill their initial promise to revolutionise modern medicine, the immunogenic barrier must be successfully and comprehensively addressed. Although autologous iPSC would be the ideal controls with which to compare the immunogenicity of allogeneic lines; the present study investigated the immunogenicity of allogeneic eiPSC to determine whether they might eventually be used therapeutically in horses. This pilot study is unique in that it reports the *in vivo* transplantation of allogeneic iPSC in a large animal model increasingly used for translational studies [11].

In appraising the suitability of eiPSC for transplantation, flow cytometry determined that MHC-I expression by the parental fibroblasts was reduced from 96% to 4% by reprogramming. Equine

iPSC were found to express low MHC Class II suggesting they would be less prone to rejection. Given that eiPSC contain potentially oncogenic reprogramming factors, doxycycline was withdrawn for 48 h prior to transplantation in order to prevent tumour formation. As expected exogenous transcripts were substantially downregulated or not expressed at all while transcripts of the equine pluripotency markers remain present, indicating that the transplanted iPSC had not undergone differentiation. Furthermore, no change in MHC expression was found after 48 h on either eiPSC lines (data not shown). The near negligible *in vitro* expression of MHC-I and II in the tested cell lines suggested that eiPSC were lowly immunogenic and unlikely to elicit rejection by the host when transplanted *in vivo*.

Equine iPSC showed heterogeneity with respect to cell size, shape and GFP expression. Nevertheless eiPSC showed continued pluripotency gene expression even after 48 h in inhibitor free media. Equine leukocyte typing was done (by the Baker Institute, Cornell University) to rule out the possibility of matched haplotypes between the experimental animals **(Supplementary Item 3)** [12]. The horses were found to be allogeneic to the transplanted eiPSC line as none of the samples shared any previously identified MHC haplotypes.

Transplantation of undifferentiated, allogeneic eiPSC nonetheless attracted $CD4^+$ cells, forming inflammatory foci within the dermis. *In vivo* immunogenicity of iPSC may surpass that predicted *in vitro* due to the antigenicity of GFP [13], existing parent cell mutations, epigenetic abnormalities or culture-induced aberrations [14]. Moreover, following transplantation, cell death and release of antigens could lead to indirect antigen presentation to $CD4^+$ T-cells and subsequent immune activation. To rule out the possibility of xenogeneic components like MEF, quantification of mouse DNA in the transplanted eiPSC was analysed and was found to be <2% (data not shown).

Indeed, the undifferentiated eiPSC transplanted into dermis lacked the stimulatory cues present *in vitro*, such as doxycycline and inhibitory molecules, possibly required to sustain them [5], which may have resulted in sluggish activity or cell death. Alternatively, cells might have migrated away from the site of transplantation. These postulates are supported by the absence of GFP^+ eiPSC at transplantation sites after 7 days following injection.

While allogeneic eiPSC did not elicit overt graft rejection, a focal inflammatory response was evident. Surprisingly, undifferentiated autologous iPSC have been shown to be immunogenic in mice, due to *in vivo* differentiation that induced T-cell-dependent immune responses even in syngeneic recipients [9]. Another hypothesis as to why allogeneic eiPSC were more immunogenic *in vivo* than anticipated, is the upregulation of MHC antigens and costimulatory factors by differentiating eiPSC [15; 16]. The lymphocytic infiltration persisting to Day 30 might be attributed to mismatched MHC antigens resulting in recognition of allogeneic eiPSC as foreign by the host immune system, (as confirmed by equine leukocyte haplotype matching). Nevertheless, this will need to be verified with a larger sample size. While eiPSC expressed negligible MHC-II, $CD4^+$ T-cell infiltration was observed at the site of transplantation possibly because MHC-II molecules on stem cells are targets for immune recognition since they present foreign antigen fragments to T-cells. However, eiPSC-injected sites seem to develop a chronic inflammatory response up to Day 30, based on the presence of inflammatory foci characterised by persistent $CD4^+$ and $CD8^+$ cells. Notably, one year following allogeneic eiPSC transplantation, the animals used in this study show no tumour growth at the injected sites.

In conclusion, we report the immune response to allogeneic eiPSC of immunocompetent horses following transplantation in their undifferentiated state. Before considering the therapeutic use of

eiPSC, standardised protocols must be established to enable directed differentiation into specific lineages since it is unlikely that the *in vivo* environment will suffice to guide differentiation [17]. Screening of such lowly immunogenic eiPSC lines will be useful in the clinical setting prior to further differentiation into specific lineages since the differentiated derivates are also likely to be lowly immunogenic. Whereas it is commonly assumed that, as iPSC ascend the differentiation pathway their MHC expression is upregulated, studies have shown negligible immunogenicity of terminally differentiated cells derived from murine iPSC [18]. Further work is thus warranted in an effort to rigorously examine immunogenicity of eiPSC to eventually be used clinically.

Authors' declaration of interests

No competing interests have been declared.

Ethical Animal Research

The study was approved by the ethics review committee (Comité d'éthique pour l'utilisation des animaux) of the Université de Montréal (license #13-Rech-1665).

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Authorship

C. Aguiar contributed to study execution, data collection, and data interpretation. C. Theoret contributed to study design, data collection, and data interpretation. O. Smith and P. Lemire contributed to data collection. M. Segura contributed to data interpretation. L. Smith contributed to study design, data collection, and data interpretation. C. Theoret and L.C. Smith contributed equally to the work. C. Aguiar and C. Theoret contributed to the preparation of the manuscript.

Manufacturers' addresses

[a]Life Technologies, California, USA.

[b]Invitrogen Corporation, Ontario, Canada.

[c]AbDSerotec, North Carolina, USA.

[d] Sigma-Aldrich Corporation, Missouri, USA.

[e]Beckman Coulter, California, USA.

[f]Baxter Corporation, Ontario, Canada.

[g]Terumo Medical Corporation, Maryland, USA.

[h]Pfizer Inc., New York, USA.

[i]Vétoquinol Inc., Québec, Canada.

[j]Wyeth Animal Division, Ontario, Canada.

[k]Acuderm Inc., Florida, USA.

[l]Haematoxylin - Fisher Scientific Company LLC, Michigan, USA; Eosin Y- EMD Chemicals Inc., New Jersey, USA; Phloxine B - Fisher Scientific, New Jersey, USA; Spanish Saffran - Chaptec Inc., Montreal, Canada.

[m]ZEISS, Jena, Germany.

[n]Fisher Scientific, Ontario, Canada.

[o]Monoclonal Antibody Centre, Washington State University, USA.

[p]Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, USA.

[q]Invitrogen Corporation, Ontario, Canada.

Table 1: Surface expression of MHC molecules as measured by flow cytometry, on control lines of parental fibroblasts, peripheral blood leukocytes and 2 lines of eiPSC.

Figure legends:

Fig 1: Flow cytometry analysis of MHC expression by: A- equine fibroblast parental line; B- peripheral blood leukocytes; C- line 1 eiPSC; D- line 2 eiPSC. Black - isotype control; Red - MHC-I; Blue - MHC-II.

Fig 2: Colocalisation of eiPSC in HPS stained and unfixed, unstained skin sections. a- HPS stained section of allogeneic eiPSC-injected site 3 days after transplantation, showing neutrophils in the inflammatory infiltrate. b- Presence of GFP^+ cells in unstained, unfixed sections of allogeneic eiPSC injected site 3 days after transplantation. c- HPS stained section of allogeneic

eiPSC injected site 7 days after transplantation, showing lymphocyte predominant inflammatory infiltrate. d- Presence of GFP^+ cells in unstained, unfixed sections of allogeneic eiPSC injected sites 7 days after transplantation. Figures were captured using a Zeiss MRm camera on AxioVision acquisition software. Bar 100µm.

Fig 3: A rectangular zone on the lateral neck with 4x4 grid design. Within each horizontal row, each box received one of 3 substances injected intradermally: 1- sterile saline solution (negative control); 2- PHA (positive control); 3- allogeneic eiPSC. Injection order was randomised in each row. Positive response to PHA shows large wheals 24 h after injection.

Fig 4: Mean wheal diameters of injected sites as measured by vernier calipers. Although significant differences were found with the PHA group, the diameter of the wheals formed in response to injection of allogeneic eiPSC was not statistically different from that of wheals formed in response to the negative control (saline). Only values up to time 48 h were considered for standard deviation analysis. Data are representative of mean ± s.d. of the 2 horses, and the 3 treatment groups (with treatment and time as within-subject factors). Significance of differences between series of results was assessed using linear repeated-measures test. $*P = 0.0005$, $**P = 0.001$, $***P<0.0001$, $****P = 0.0006$.

Fig 5: HPS stained skin sections of saline, PHA and eiPSC transplanted sites at 2, 7 and 30 days of the study. Saline injected sites showed no inflammation at Days 2, 7 and 30 (**a, b, c**). PHA sites showed subacute inflammation throughout the dermis on Days 2 and 7 (**d, e**) that mostly resolved by Day 30 (**f**). Equine iPSC transplanted sites were characterised by diffuse inflammatory foci in the dermis on Days 2 and 7 (**g and h**) that persisted up to day 30 (**i**).

Black line indicates approximate separation of the superficial dermis from the deep dermis.

Figures were captured using a Zeiss MRm camera on AxioVision acquisition software.

Bar 50 µm.

Fig 6: Graph on the top panel shows CD4+ cells in sections of skin sites injected with eiPSC. On the bottom panel is the distribution of CD8+ cells in sections of eiPSC transplanted sites. Data are representative of cell count means ± s.d..

Supplementary Items:

Supplementary Item 1: Stock graphs showing the distribution of Histology scores in transplanted eiPSC sites.
Supplementary Item 2: CD4 and CD8 cells in tissue sections.
Supplementary Item 3: Results of microsatellite typing for eiPSC and transplanted hosts.

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Supporting Information:

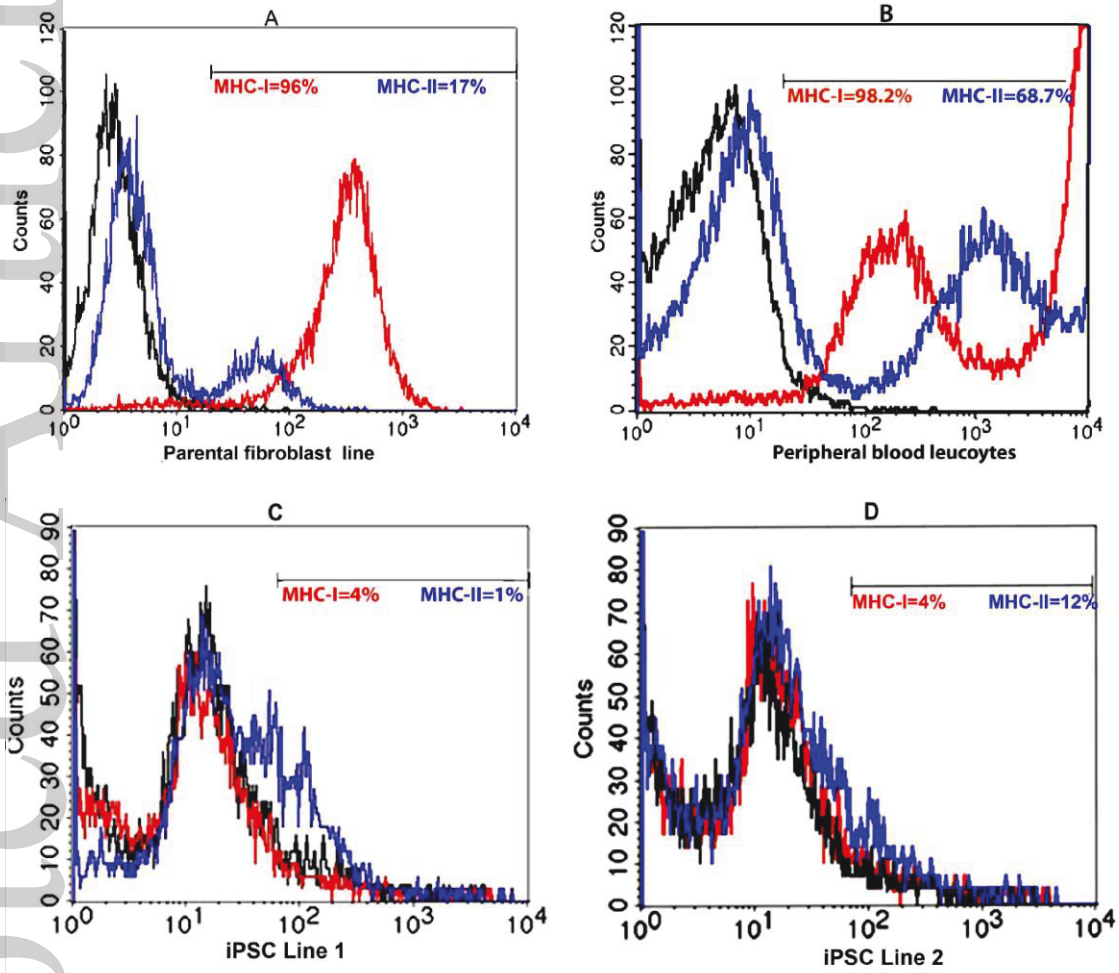
Supplementary Item 1: Immunolocalisation of CD4 and CD8 proteins during the inflammatory response to intradermal transplantation of allogeneic eiPSC at days 2 and 30.

Blue – Dapi; Red – CD4; Green – CD8. Bar 50 μm.

Supplementary Item 2: Results of microsatellite typing indicated that none of the samples share any previously identified MHC haplotypes between individuals. The Arabian mare is heterozygous for MHC Class I region and has the ELA type COR 6 and A3 (at MHC Class II region). The Standardbred mare possesses homozygous alleles for MHC Class I and two sub-haplotypes of ELA-10 at MHC Class II. The allogeneic eiPSC line possesses ELA-10 at MHC Class II on one microsatellite locus while the other is undetermined.

Supplementary Item 3: Stock graphs showing the distribution of Histology scores in transplanted eiPSC sites. Perivascular dermatitis was found to be highest in the PHA group, with moderate scores in eiPSC injected sites. Haemorrhage occurred in the PHA-injected sites but was

absent from the eiPSC-injected sites. Oedema scores were moderate for eiPSC with a tendency of higher scores in the PHA-injected sites. Similarly, PHA injections induced the highest scores for eosinophils while eiPSC induced moderate scores.



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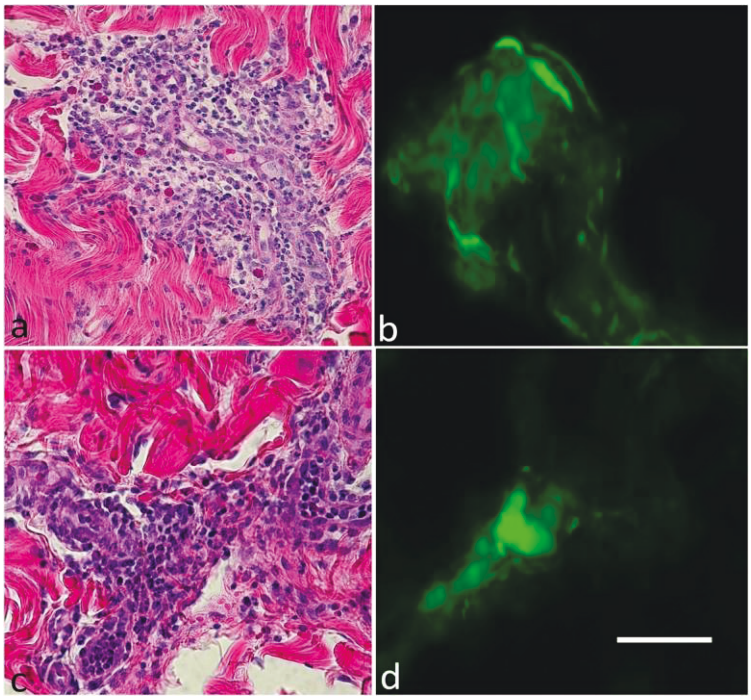

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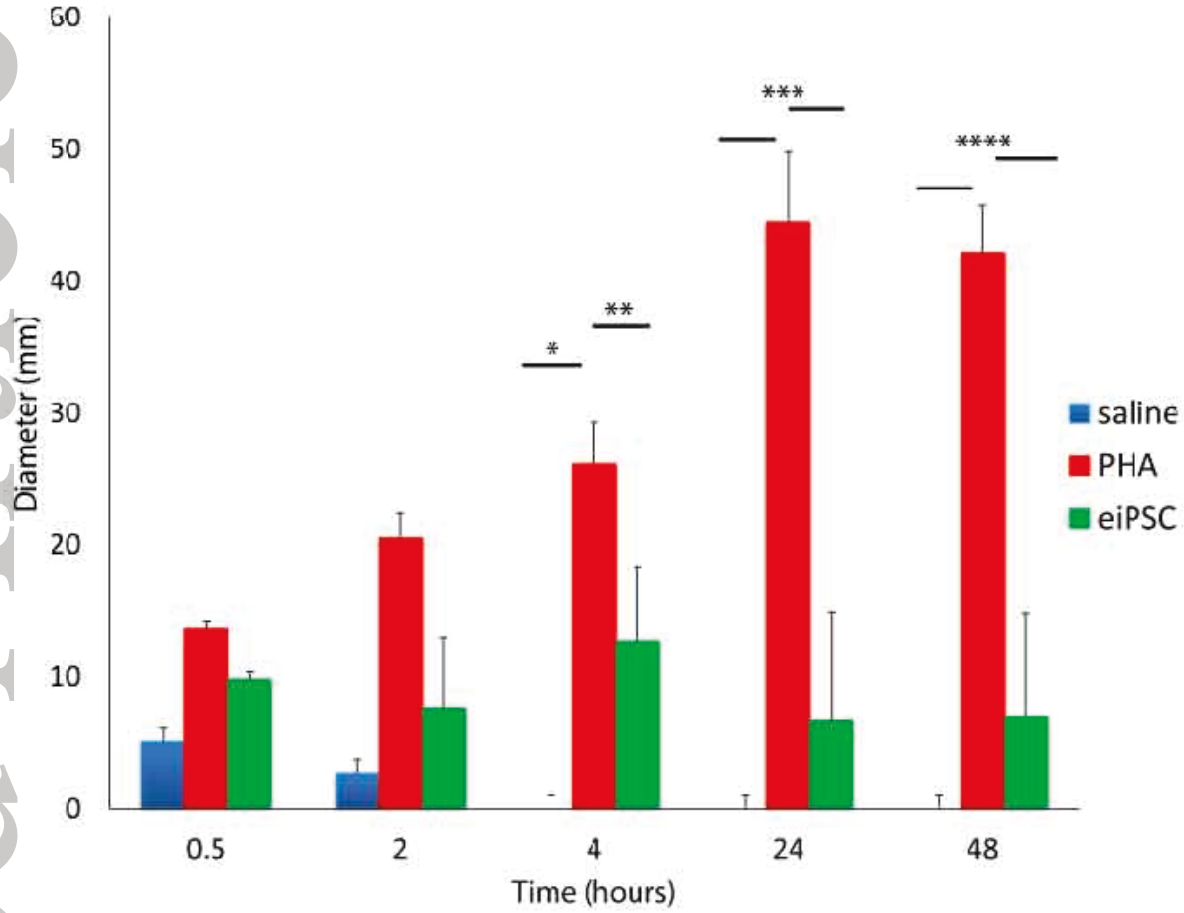

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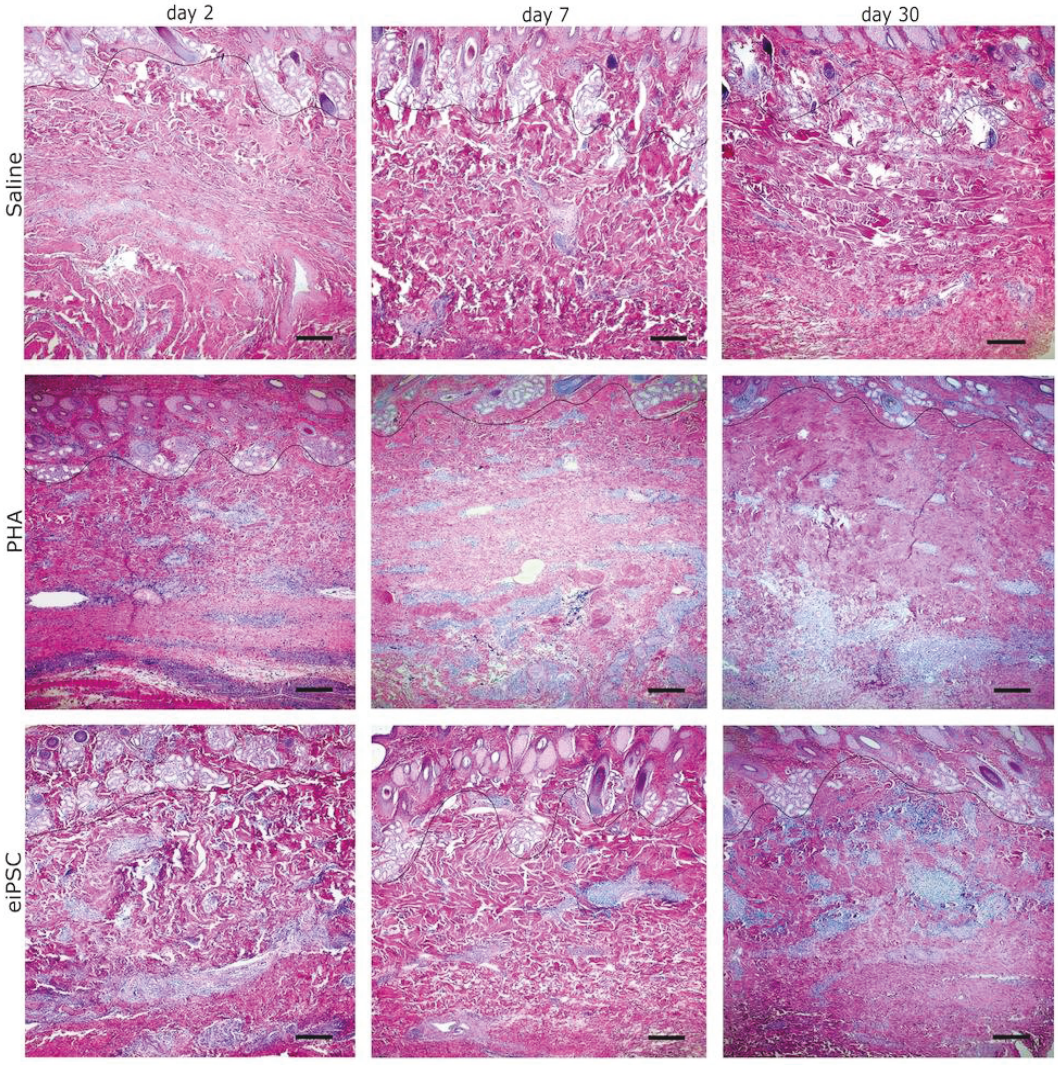

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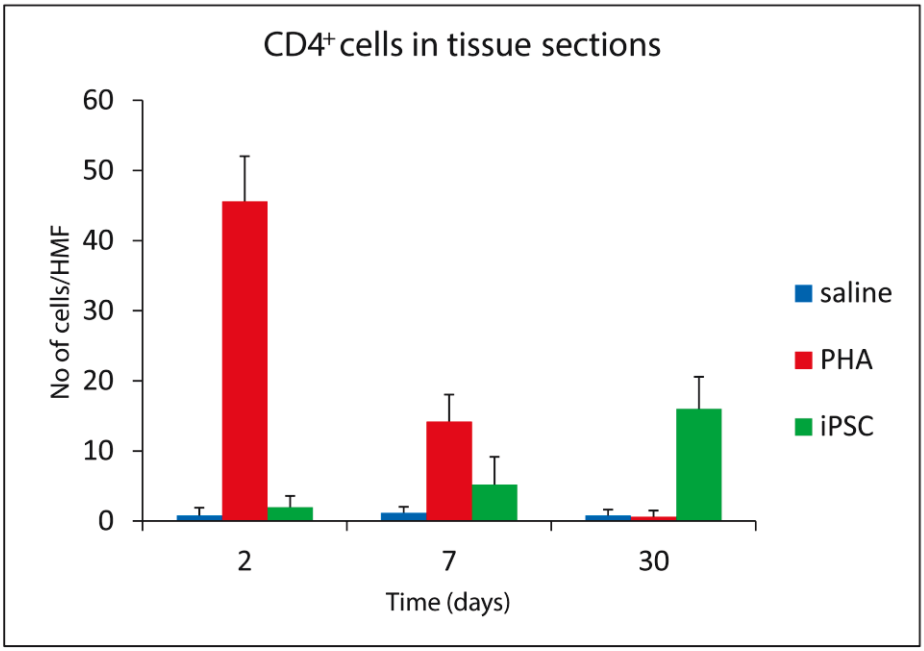

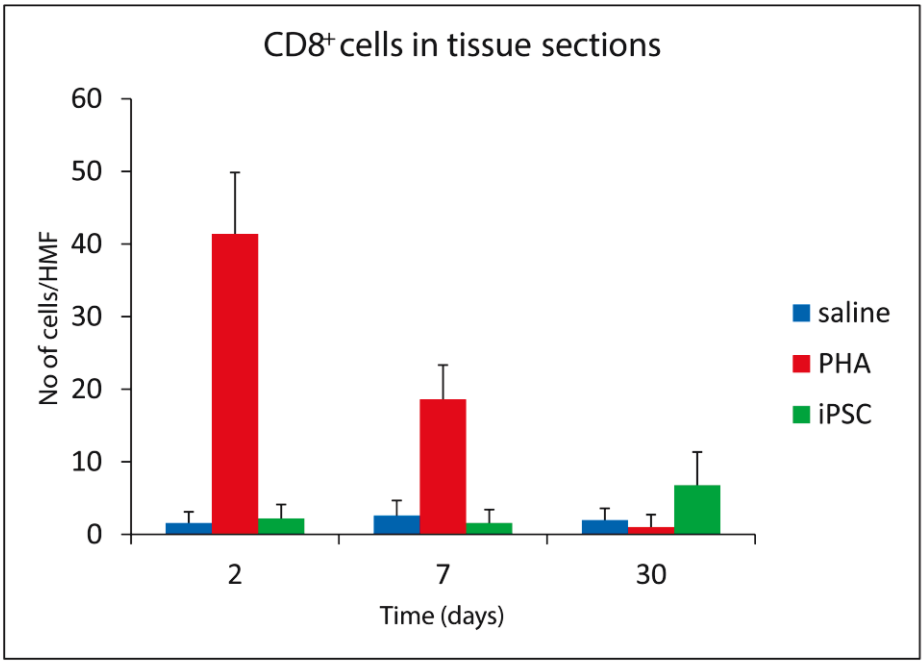

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Cell lines	MHC-I (%)	MHC-II (%)
Parental Fibroblast line	96	17
Peripheral blood leukocytes	98.2	68.7
eiPSC line 1	4	1
eiPSC line 2	4	12

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Article 2

Original Article

DIFFERENTIATION OF EQUINE INDUCED PLURIPOTENT STEM CELLS INTO A KERATINOCYTE LINEAGE

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Key words: horse; induced pluripotent stem cells; keratinocytes; wound healing; regenerative medicine

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Conflict of interest statement

The authors declare no conflict of interest.

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Authorship

Christie Aguiar: study execution, data collection, data analysis, interpretation and preparation of the manuscript

Jacinthe Therrien, Paul Lemire, Mariela Segura: data collection, interpretation of data

Lawrence Smith: study design, interpretation of data and preparation of the manuscript

Christine Theoret: study design, interpretation of data and preparation of the manuscript

Declarations

None

DIFFERENTIATION OF EQUINE INDUCED PLURIPOTENT STEM CELLS INTO A KERATINOCYTE LINEAGE

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Summary

Reasons for performing study: Skin trauma in horses often leads to the development of chronic non-healing wounds that lack a keratinocyte cover, vital for healing. Reports in mouse and man confirm the possibility of generating functional keratinocytes from induced pluripotent stem cells (iPSC),thus presenting a myriad of potential applications for wound management or treatment of skin disease. Similarly, differentiation of equine iPSC (eiPSC) into a keratinocyte lineage should provide opportunities for the advancement of veterinary regenerative medicine.

Objectives: The purpose of this study was to develop an efficient method for the differentiation of eiPSC into a keratinocyte lineage. It was hypothesised that eiPSC can form differentiated keratinocytes (iPSC-KC) comparable to primary equine keratinocytes (PEK) in their morphological and functional characteristics.

Study Design: Experimental *in vitro* study.

Methods: Equine iPSC established using a non-viral system, were treated for 30 days with retinoic acid and bone morphogenic protein-4 to induce directed differentiation into iPSC-KC. Temporospatial gene and protein expression by eiPSC-KC was measured at weekly intervals of differentiation and in response to calcium switch. Proliferative and migratory capacities of eiPSC-KC were compared to those of PEK.

Results: Equine iPSC, upon directed differentiation, showed loss of pluripotency genes and progressive increase in pancytokeratin expression indicating ectodermal specification into

keratinocytes. High differentiation efficiency was achieved, with 82.5% of eiPSC expressing keratin 14, a marker of epidermal-specific basal stem cells, after 30 days of directed differentiation. Moreover, the proliferative capacity of eiPSC-KC was superior while the migratory capacity (measured as the ability to epithelise *in vitro* wounds) was comparable to that of PEK.

Conclusions: This proof of concept study suggests that eiPSC can successfully be differentiated into equine keratinocytes (eiPSC-KC) with features that are promising to the development of a stem cell-based skin construct with the potential to regenerate lost or damaged skin.

Introduction

A large study by the National Animal Health Monitoring System found that injuries are the primary medical condition affecting horses, more common than lameness, colic or respiratory disease; moreover, 20% of euthanasias are due to skin wounds [1]. When a wound is located on the limb, as is the case 60% of the time [2], it tends to heal with difficulty, often leading to a chronic non-healing wound lacking an epithelial cover [3].

Traditional skin grafting, while considered the "gold standard" for managing wounds, achieves limited success in horses due to the scarcity of donor skin [2]. Consequently, there is a need to engineer functional skin, including all its layers and appendages, with pigment and operational vascular and nervous networks, that will integrate into surrounding host tissue in a scar-free manner [3]. At present, no model of artificial replacement skin completely replicates the uninjured organ, either in human or veterinary medicine. Importantly, traditional cultured epidermal autografts lack the accessory adnexal structures of skin [3]. Hence, scientists have

turned to stem cells, capable of multi-lineage differentiation, to populate tissue-engineered constructs [4].

Recent breakthroughs in the generation of induced pluripotent stem cells (iPSC) provide a novel renewable source of cells with embryonic stem cell (ESC)-like properties and that circumvent ethical issues since they are engineered without destroying an embryo. Moreover, immunological rejection is minimised since autologous iPSC can be generated. Reprogramming to a pluripotent state was originally achieved by viral transduction using the Yamanaka factors [5]. Induced pluripotent stem cells have been generated by transduction with reprogramming factors using retroviral [5] or adenoviral methods [6], non viral methods [7], protein transduction [8], small molecules [9] etc. Nagy and Smith recently reported the establishment of equine iPSC (eiPSC) using a non-viral, *PiggyBac* system which was used in the study described herein [10]. While the eiPSC used in the current study were derived from fetal donors, protocols are available for deriving eiPSC from adult cells thereby enabling the production of autologous lines [11]. In parallel, progress has been made in differentiating iPSC into several cell types in view of eventual therapeutic use. Following specific protocols, iPSC have been differentiated into hepatocytes [12], pancreatic cells [13], cardiomyocytes [14], adipocytes [15] etc. that have proven functional in experimental models and are being used for the treatment of various diseases [16]. More recently, eiPSC have successfully generated cells with features of cholinergic motor neurons [17]. Murine iPSC have also been differentiated into keratinocytes capable of reconstituting a stratified epidermis and its appendages when grafted onto mice [18], generating much excitement in the field of cell-based therapies for chronic non-healing wounds. To fully exploit the promise of eiPSC derivatives in equine medicine, reproducible and efficient differentiation protocols are now necessary. Therefore , it was hypothesised that eiPSC can be

differentiated into keratinocytes using the procedure developed for murine iPSC [18]. The aim of the current study was to develop a protocol to differentiate eiPSC to a keratinocyte lineage for eventual grafting onto skin wounds in horses. Here we demonstrate that eiPSC can successfully be differentiated into keratin-expressing cells (eiPSC-KC). Their phenotype resembles that of primary equine keratinocytes (PEK) that express markers such as *KRT 18*, *KRT 14* and integrins when within the basal layer of skin. Upon commitment to stratification the keratinocytes express markers of epidermal differentiation such as *KRT 1* and *KRT 10* and, finally, terminally differentiate and express involucrin (*IVL*) markers [19].

Given the pluripotent nature of iPSC, our 'proof of concept' study represents an important advance for equine regenerative medicine since, in principle, eiPSC would provide an inexhaustible source of cells that might be used to engineer a fully functional skin substitute for wound management or skin disease in horses.

Materials and Methods

Cell cultures

Horse skin was obtained during castration surgery at the Centre hospitalier universitaire vétérinaire of the Université de Montréal. Primary equine keratinocytes (PEK) were isolated and cultured as previously described [20] except using a 2% serum[a] formulation for maintenance. The equine iPSC line used in these experiments (line H2A) was obtained by transfection of fibroblasts obtained from a day-40 fetus using a *PiggyBac* transposon system containing the tetracycline inducible Yamanaka factors and constitutively controlled Green Fluorescent Protein (GFP), then propagated in culture as described [10]. Equine iPSC media consists of DMEM high glucose[a], 2 mM GlutaMax[a], 0.1 mM non-essential amino acids[a], 0.1 mM betamercaptoethanol[b], 1

mM sodium pyruvate[a], 50 U/ml penicillin/streptomycin[a] and 15% fetal bovine serum[a], 1000U/ml leukaemia inhibitory factor (LIF)[c], 10 ng/ml bFGF[d], 1.5 µg/ml doxycycline[b], 3 µM GSK inhibitor[e], 0.5M MEK inhibitor[e], 2.5 µM TGF inhibitor[e], 5µM thiazovivin[e], and 25 µM ALK receptor inhibitor[e].

Keratinocyte differentiation media consists of DMEM/F12[a], 2% FBS[a], HEPES buffer[a], 25µg/ml gentamicin[a], 100X penicillin-streptomycin[a], 0.5 µg/ml amphotericin B[a], 10 ng/ml recombinant mouse EGF[a], 5 µg/ml recombinant human insulin[a] and 30 µg/ml bovine pituitary extract[a].

Directed differentiation of eiPSC into a keratinocyte lineage

Upon characteristic colony formation, eiPSC **(Fig. 1A)** expressing key pluripotency markers *OCT-4*, *SOX-2*, *KLF-4* and *NANOG* were separated from mouse feeder layers (MEF) cultured in media[a] free of LIF[c] and doxycycline[b] to permit differentiation and loss of pluripotency marker expression **(Fig. 1B)**. The cells were cultured for two weeks to enable the formation of embryoid bodies (EB) **(Fig. 1C)**. The EB were treated with 1 µM retinoic acid (RA)[b] for three days followed by 25 ng/ml of bone morphogenic protein 4 (BMP-4)[b] for three days, as previously described [18]. Upon EB formation, the media was changed to a differentiation media that permits selection of ectodermal differentiated cells by their attachment to collagen[a] coated plates[f] [21]. The attached cells were cultured in keratinocyte differentiation media and analysed for gene and protein expression at 0, 7, 14 and 30 days of differentiation. The latter time was when the morphology of differentiated eiPSC matched that of PEK **(Fig. 1D; Fig. 1E).** Once differentiation was complete, eiPSC-KC and PEK were treated with 1.8mM $CaCl^{b}$-supplemented keratinocyte media to induce stratification [22] and studied at 0, 24, 48 and 72 hours.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde[b] and blocked with 5% goat serum[a] for 1h. Incubation with mouse anti-pancytokeratin (PANCK) antibody (MA1-82041[g], 1:100) was carried out overnight at 4°C. Goat anti-mouse Cy3 (115-001-003[h], 1:400) was then added for 1h at room temperature and nuclei were counterstained with 10 mg/ml DAPI[b]. Primary and secondary antibodies were verified for species specificity and cross reactivity using Western blot (data not shown). Negative control of equine fibroblasts were used for primary and secondary antibodies (Supplemental figure 2). Immunostaining was visualised by fluorescence microscopy (Axio Observer Z1)[i]. Digital images were acquired using the image-analysis software Zen[i] and immunoreactivity was quantified using a plugin of Image J, NIH software[j] in a minimum of five random high power fields, as previously described [23]. Data are representative of three independent differentiation experiments performed using the same cell line approximately two months apart.

Reverse transcription and quantitative real-time PCR (qRT-PCR)

Equine iPSC samples were collected in triplicate following 7, 15 and 30 days of directed differentiation, to confirm loss of pluripotency genes and to measure changes in the expression of keratin (*KRT) 10, 14, 18* and involucrin (*IVL*) genes in response to ectodermal morphogens (RA and BMP-4). Fibroblasts were used as a negative control for keratin expression while primary keratinocytes were used as positive controls.

Total RNA was extracted using the RNeasy mini kit[k], then reverse transcribed to mRNA and cDNA using the PCR T3000 Thermal cycler[l]. Reverse transcription was performed using QuantiTect Reverse Transcription kit (Qiagen) [k] with the RT primers and mix provided by the

kit. The reaction was performed at 42° C for 30 min. For all samples, a negative RT was used as a control, consisting of an RT reaction omitting the reverse transcriptase. Quantitative RT-PCR was performed on cDNA using the Quantitect Probe PCR kit[k] in a Rotorgene Q PCR cycler[k] under the following amplification conditions: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. Equal PCR efficiency of all primer pairs was validated by serial cDNA dilution. Primers were designed to amplify only equine-specific target genes and the housekeeping genes *GAPDH*, *RPL-32* and *SDHA*: see **Table 1** for primer details.

Flow Cytometry

Equine iPSC were prepared in PBS, blocked with 0.5% goat serum[a], then incubated with mouse anti-KRT14 primary antibody (ab 7800[m], 1:100) followed by staining with secondary antibody R-PhycoErythrin (RPE)-conjugated sheep anti-mouse IgG (115-116-146 [h], 1:100 [24]). A minimum 20,000 events were analysed by Accuri C6 cytometer[n] and the percent positivity as well as mean fluorescence intensity (MFI) for the specific marker calculated based on the respective isotype controls. Primary equine keratinocytes served as a positive control and equine fibroblasts as negative control. The iPSC were not gated by GFP expression but were sorted using the primary antibody followed by the RPE conjugated secondary antibody.

Scratch wound healing assay

Equine iPSC-KC and PEK were seeded in 35mm, collagen-coated dishes, at a density of 4 x 10^5 cells/cm^2. Upon confluence the monolayer cell cultures were treated with mitomycin-C[b] (50ug/mL) to arrest cell division. The following day, cell cultures were scratched with a sterile 200uL tip to simulate a wound *in vitro* [25]. The cells were incubated in a 2% low serum medium and migration of eiPSC-KC in comparison to PEK across the scratch wound was measured from wounding up to complete coverage of the scratch. The cells were photographed

under phase-contrast microscopy[i] and scratch wound areas were measured using NIH Image J software[j]. The mean scratch wound area was calculated from three independent experiments at each time point for both cell types. Scratch wound coverage was calculated as a percentage of the original wound area, as previously described [26].

Population Doubling

Day 30 differentiated eiPSC (eiPSC-KC) and second passage PEK were seeded in 24-well culture dishes at a density of 2 x 10^4 cells/cm^2. Every other day until day 18, cells were trypsinised[a] then counted using Invitrogen Cell countess[a]. The cells were expanded for two days and trypsinised once confluency was achieved, then replated at the same density. Population Doubling value was calculated as log (N/N_0) x 3.322, where N = number of cells in the dish at the end of a period or growth, N_0=number of cells plated in the dish [27]. Data are representative of three independent experiments.

Statistical Analyses

The temporal variation in keratin markers was analysed using a repeated-measures linear model with time as a within-subject factor followed by Tukey's post-hoc testing to compare the means at different time points. For the temporal expression of PANCK, a linear model was used on log-transformed data with time as a factor controlling for the unequal variances at different time points, followed by Tukey's post-hoc testing to compare the means at different time points. *A priori* contrasts were used to compare treatment means at different time points, and means at different time points for each cell type while adjusting alpha level for each comparison using the Bonferroni sequential procedure. For log-transformed cell counts, the same model was used

except the contrasts were limited to a comparison of the treatment means at each time point. A similar analysis was used for log-transformed wound area data. Results are presented as mean ± SD of independent technical replicates. Statistical analyses were carried out with SAS v. 9.3^{o} and the level of statistical significance was set at $P<0.05$.

Results

Differentiated eiPSC-KC adopt a polyhedral / cobblestone morphology similar to PEK

Undifferentiated eiPSC form distinctive rounded colonies with well-defined borders and high cell density characteristic of iPSC cultures on MEF **(Fig. 1A)**. Moreover, undifferentiated eiPSC express pluripotency markers that are lost upon differentiation into eiPSC-KC **(Fig. 1B)**. Upon ectodermal differentiation, eiPSC adhere to collagen-coated culture dishes and display initial heterogeneous cell populations containing cuboidal epithelial forms and smaller circular cells. After two weeks in the keratinocyte differentiation media and further passaging, differentiated eiPSC-KC adopt a homogeneous, polygonal or 'cobblestone' epithelial morphology resembling that of PEK **(Fig. 1A)**.

Differentiated eiPSC-KC show increase in pancytokeratin expression

Equine iPSC began expressing PANCK, a specific marker of epithelium, after 7 days of ectodermal specification, with peak expression after 30 days of differentiation **(Fig. 2A)**. A gradual, 2.5 fold increase in the first week followed by a 4.8 fold increase at day 30 was measured in response to the directed differentiation protocol ($P<0.001$) **(Fig. 2B)**. Statistically significant differences were found between days 0 and 7, between 7 and 15 and 0 and 30 days of treatment ($P<0.001$). At day 30, PANCK expression levels in differentiated eiPSC were not

different from those of PEK, indicating their similarity to the keratinocytes originated from adult skin epithelia.

Differentiated eiPSC upregulate the expression of epidermis-specific genes

In response to ectodermal morphogens, eiPSC showed an increase in expression of *KRT18* from day 7 of ectodermal specification. Statistically significant upregulation was found at 7, 14 and 30 days of differentiation ($P<0.0001$) **(Fig. 3A)**. A statistically significant upregulation of *KRT14* was observed both at days 7 and 14 ($P=0.003$), with peak expression after 14 days of differentiation **(Fig. 3B)**. Further, expression of epidermal stratification markers *KRT10* and *IVL* was upregulated in response to the differentiation protocol. Expression of *KRT10* increased in a statistically significant manner between days 0 and 30 **(Fig. 3C)**. Involucrin, expressed in terminally differentiating epidermal cells, was significantly upregulated in eiPSC at 7 ands and 14 days of directed differentiation ($P=0.005$) **(Fig. 3D)**.

Differentiated eiPS-KC express the keratinocyte stem cell marker KRT14

Keratin 14 is expressed by keratinocyte stem cells, from which the supra basal layers of the epidermis develop. To determine the efficiency of the directed differentiation protocol, the percentage of the cell population expressing this basal keratinocyte marker was calculated. Flow cytometric analysis revealed that 82.5% of eiPSC expressed KRT14 after 30 days of directed differentiation **(Fig. 4)**, in comparison to 90.9% of PEK. These data suggest that a high purity of epidermal basal stem cell population has been obtained.

Equine iPSC-KC migrate to cover the scratch wound and have a greater proliferative capacity than PEK

Equine iPSC-KC are able to heal an *in vitro* scratch wound by exhibiting forward migration **(Fig. 5A)**. The wound created in mitomycin-treated monolayers of eiPSC-KC was found to be covered at 96h, at which time complete wound coverage was achieved in PEK scratched monolayers. Data are presented as mean ± SD **(Fig. 5B).** The means for percent wound coverage at 72h and 96h were significantly larger than the means at 0h, 24h and 48h of wound coverage in both eiPSC-KC and PEK ($P<0.0001$). There were no statistically significant differences in percent wound coverage between eiPSC-KC and PEK throughout the study.

Equine iPSC-KC were found to have a greater proliferative capacity compared to PEK **(Fig. 5C)**. While PEK enter the stationary phase of cell growth after 8-9 passages when cultured in low calcium media, eiPSC-KC continue to proliferate for more than 35 passages (data not shown). Statistically significant differences were found between eiPSC-KC and PEK at days 12 ($P = 0.0002$), 14 ($P < 0.0001$), 16 ($P = 0.006$) and 18 ($P = 0.0007$) of culture.

Equine iPSC-KC respond to a high calcium medium similarly to PEK

Equine iPSC-KC proliferated in low calcium medium similarly to PEK, as evidenced by immunoreactivity to Ki67 **(Fig. 6)**. Basal keratinocytes can be induced to terminally differentiate when exposed to high levels of extracellular calcium [28]. In this study, both cell types underwent growth arrest in response to an elevation in extracellular calcium concentration, as evidenced by loss of immunostaining.

Discussion

Differentiation of iPSC into cells of the keratinocyte lineage should contribute to the development of customised therapies to regenerate skin lost to injury or disease. In this study, ectodermal morphogens RA and BMP-4 used sequentially and at specific concentrations successfully induced stepwise progression through appropriate intermediate developmental stages prior to generating a differentiated keratinocyte [29; 30]. The combined use of BMP4 (25 ng/ml) along with RA (1 μM) in specific concentrations has been shown to block the neural fate and allow the development of surface ectoderm [18; 31]. The exact role of BMP4 signaling in ectodermal development remains unclear however, prior studies have suggested synergism of RA and BMP signalling during directed epithelial differentiation of human ESCs [32].

Indeed, the directed differentiation protocol caused a 4.8 fold increase in expression of PANCK, a protein specific to epithelium, after 30 days. Moreover, sequential expression of *KRT* genes upon eiPSC differentiation correlated with their expression in keratinised epidermis of the fetal vertebrate [33]. During embryonic development *KRT18*, then *KRT14*, are expressed by the basal cells of the epidermis. Similarly, eiPSC began expressing these ectodermal markers upon directed differentiation and, eiPSC-KC finally expressed key markers of epidermal stratification, *KRT10* and *IVL* [34]. Although keratins are also expressed by simple epithelia, epidermal keratinocytes can terminally differentiate into *stratum corneum* and express epidermis specific markers *KRT 10* and *IVL*, as shown in this study. Since *IVL* is absent in simple mucosal epithelia and expressed only in keratinised epidermis having the capacity to stratify, our results confirm the generation of eiPSC-KC comparable to epidermal cells *in vivo*.

Directed differentiation yielded a population of basal eiPSC-KC of high purity similar to that obtained in a recent study [35]. Indeed, FACS revealed that 82.5% of the eiPSC population

expressed KRT14 specifying the epidermal basal cell subtype. Such a population is ideal for cell grafting since it can give rise to the supra basal layers of the epidermis due to its high proliferative potential and mitotic capacity [36]. Importantly, no pluripotent cells remained in culture after 15 days of directed differentiation, confirming the absence of heterogeneous (undifferentiated) iPSC that could, in principle, lead to teratocarcinoma formation [37].

Epidermal cells can be induced to differentiate and stratify, with concurrent changes in morphological characteristics [38], by increasing the calcium concentration of the media [39]. In high calcium media eiPSC-KC assumed a flattened cuboidal morphology similar to that of PEK, indicating their ability to form corneocytes as seen *in vivo* [40]. Moreover, while both eiPSC-KC and PEK continued to form monolayers in low calcium media, their ability to proliferate was compromised by high extracellular calcium. These results suggest that eiPSC-KC are functionally similar to PEK.

In addition to their capacity for self-renewal, a significant advantage of iPSC derivatives over PEK is their proliferation potential. In horses, PEK have a low proliferative capacity [41], undergo stratification and exit the cell cycle earlier than eiPSC-KC making them inferior to populate tissue-engineered constructs. Moreover, the *in vitro* wound healing assay used in this study confirms the migratory ability of eiPSC-KC [42]. Previous studies suggest that immature / semi differentiated cells undergo maturation in the microenvironment of the graft [43]. While the eiPSC-KC derived in this study show morphology and marker expression characteristic of keratinocytes, their true identity and function remain to be determined via functional assays. Should *in vivo* experiments involving transplantation of these eiPSC derivatives into experimental equine wound healing models confirm their capacity to survive, engraft and integrate into host tissues, these abilities of eiPSC-KC would be of benefit in the regeneration of

functional skin lost to injury or disease. Interestingly, studies in other species have shown that iPSC-derivatives, both autogenous and allogeneic, are lowly immunogenic [44]. Undifferentiated allogeneic eiPSC were similarly found to be weakly antigenic upon intradermal transplantation in horses [45], suggesting that allogeneic eiPSC (and their derivatives) might eventually provide an 'off-the-shelf' product for wound management.

An exciting new study reports the isolation and characterisation of bipotent epithelial stem cells (EpSC) from equine skin, showing differentiation into keratinocytes and adipocytes [46]. The advantage of developing an eiPSC-KC based construct is that the latter cells have the capacity to regenerate not only the different layers of the epidermis but also accessory adnexal structures of the skin, as shown convincingly in a mouse model [18].

Conclusions

This preclinical, "proof of concept" study reports an efficient method to differentiate equine iPSC into a keratinocyte lineage. Moreover, we describe the morphological and functional characterisation of the differentiated eiPSC-KC, including the migratory ability to cover wounds. The resulting cells, eiPSC-KC, show features that are promising to the development of a stem cell-based skin construct with the potential to fully regenerate lost or damaged skin.

Manufacturers' details:

[a] Invitrogen Corporation, Carlsbad, CA, USA
[b]Sigma Aldrich Corporation, St. Louis, MO, USA
[c]EMD Millipore, Temecula, California, USA
[d]PeproTech, Rocky Hill, NJ, USA
[e]Stemgent, Cambridge, MA, USA
[f]Corning Incorporated, Corning, NY, USA
[g]Fisher Scientific, Ottawa, Ontario, Canada
[h]Jackson Immunologicals, West Grove, PA, USA
[i]ZEISS, Jena, Germany
[j]http://rsb.info.nih.gov/ij/index.html

[k]Qiagen, Valencia, CA, USA
[l] Biometra, Horsham, PA, USA
[m]AbD Serotec, North Carolina, USA
[n]BD Bioscienes, Sparks, MD, USA
[o]SAS Institute Inc., Cary, N.C., USA

Figure Legends:

Fig. 1: (A) Confocal microscopic image of a characteristic eiPSC colony with defined borders and high cell density (left). Images were photographed with an Olympus FV1000 laser-scanning microscope. Scale bar 100µm. Equine iPSC-KC (centre) showing typical 'cobblestone' morphology similar to PEK cultures (right). Centre and right images were photographed with an Axio Observer Z1 microscope. Scale bar 50µm.

(B) Equine iPSC show the endogenous expression of key pluripotency genes *NANOG*, *SOX-2*, *OCT-4* and *KLF-4* that is lost upon differentiation into keratinocytes (eiPSC-KC).

(C) Schematic representation of the protocol used for differentiation of eiPSC into epidermal cells (eiPSC-KC).

Fig. 2: (A) Sequential increase in expression of PANCK in eiPSC after 7, 14 and 30 days of directed differentiation, as detected by immunofluorescence, in comparison to undifferentiated eiPSC (at day 0) and PEK. Columns from left to right show counterstaining with DAPI (staining nuclei blue), PANCK positive cells (staining red), and DAPI+PANCK merge. Scale bar 100µm.

(B) Mean fluorescence intensities (MFI), calculated by Image J, in PANCK+ cells after 7, 14 and 30 days of directed differentiation in comparison to eiPSC before induction and PEK as positive control (n=3). Horizontal bars with asterisks represent group differences (*P<0.0001). There was no statistically significant difference between day 30 eiPSC-KC and PEK however, there were differences between days 0, 7 and 15. Data expressed as mean ± SD.

Fig. 3: Temporal gene expression by eiPSC in response to treatment with ectodermal morphogens. **(A)** *KRT18* **(B)** *KRT14* **(C)** *KRT10* **(D)** *IVL*. Values are normalised to the mean expression values of the housekeeping genes *GAPDH*, *RPL-32* and *SDHA*. Means that share the same letters are not statistically significantly different. Data expressed as mean ± SD.

Fig. 4: Flow cytometric analysis of eiPSC-KC showing keratin 14^+ cells in response to the directed differentiation protocol, in comparison to control PEK and undifferentiated eiPSC. Red line indicates KRT14 staining and black line indicates isotype control.

Fig. 5: (A) *In vitro* scratch wound healing assay showing migration of eiPSC-KC in comparison to control PEK that cover the scratch wound at 96h. Black dotted lines indicate the scratch wound margins.

(B) Graph showing scratch wound coverage by migration of eiPSC-KC and PEK. Images were photographed with an Axio Observer Z1 and percent coverage was calculated using Image J software. Data are presented as mean ± SD of three independent experiments.

(C) Population doubling of eiPSC-KC after formation of epidermal cells in comparison to control PEK cultures. Data are presented as mean ± SD of three independent experiments.

Fig. 6: Equine iPSC-KC and PEK cultured in low calcium medium show cell proliferation by positive immunoreactivity for Ki67. Cell cycle arrest occurs in response to high calcium concentration, as evidenced by loss of immunostaining. (Scale bar 50μm).

Table 1: Primer sequences of analysed genes.

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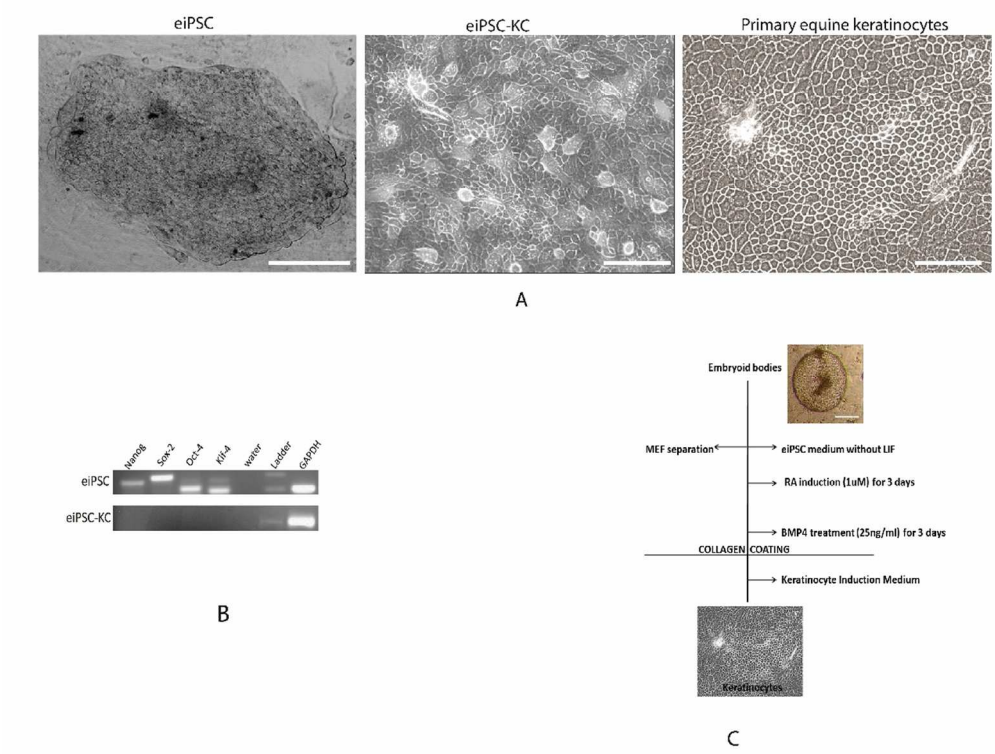

(A) Confocal microscopic image of a characteristic eiPSC colony with defined borders and high cell density (left). Images were photographed with an Olympus FV1000 laser-scanning microscope. Scale bar 100µm. Equine iPSC-KC (centre) showing typical 'cobblestone' morphology similar to PEK cultures (right). Images were photographed with an Axio Observer Z1 microscope. Scale bar 50µm.
(B) Equine iPSC show the endogenous expression of key pluripotency genes NANOG, SOX-2, OCT-4 and KLF-4 that is lost upon differentiation into keratinocytes (eiPSC-KC).
(C) Schematic representation of the protocol used for differentiation of eiPSC into epidermal cells (eiPSC-KC).

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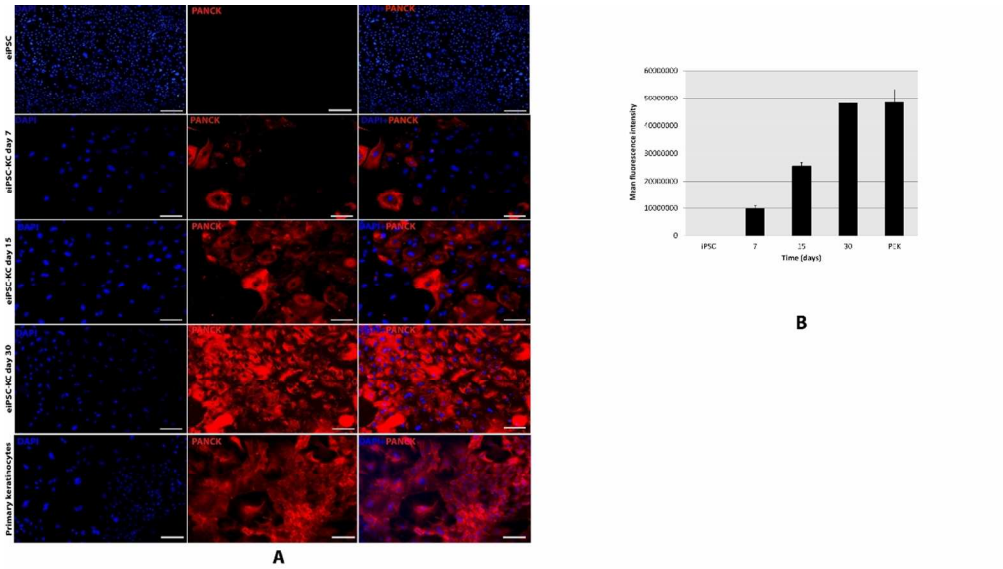

(A) Sequential increase in expression of PANCK in eiPSC after 7, 14 and 30 days of directed differentiation, as detected by immunofluorescence, in comparison to undifferentiated eiPSC (at day 0) and PEK. Columns from left to right show counterstaining with DAPI (staining nuclei blue), PANCK positive cells (staining red), and DAPI+PANCK merge. Scale bar 100µm.
(B) Mean fluorescence intensities (MFI), calculated by Image J, in PANCK+ cells after 7, 14 and 30 days of directed differentiation in comparison to eiPSC before induction and PEK as positive control (n=3). Horizontal bars with asterisks represent group differences (*P<0.0001). There was no statistically significant difference between day 30 eiPSC-KC and PEK however, there were differences between days 0, 7 and 15. Data expressed as mean ± SD.

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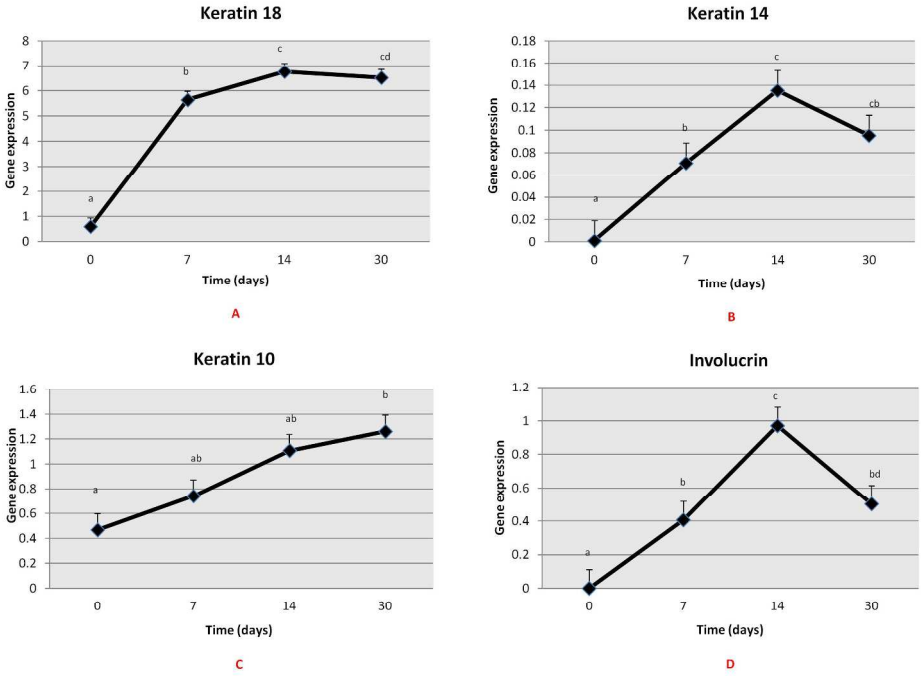

Temporal gene expression by eiPSC in response to treatment with ectodermal morphogens. (A) KRT18 (B) KRT14 (C) KRT10 (D) IVL. Values are normalised to the mean expression values of the housekeeping genes GAPDH, RPL-32 and SDHA. Means that share the same letters are not statistically significantly different.
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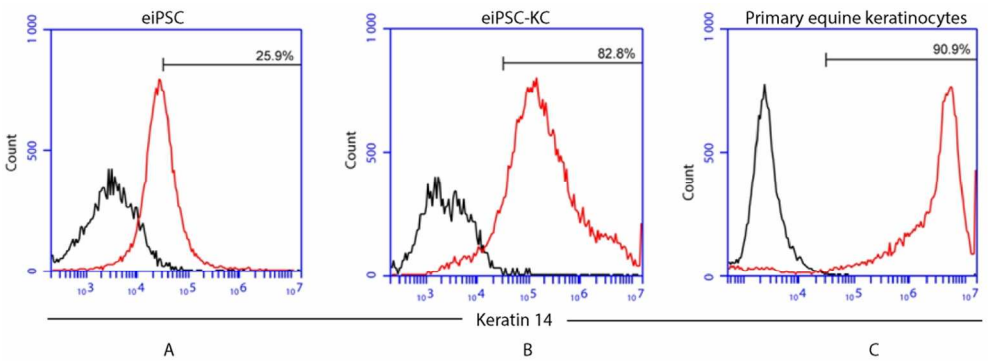
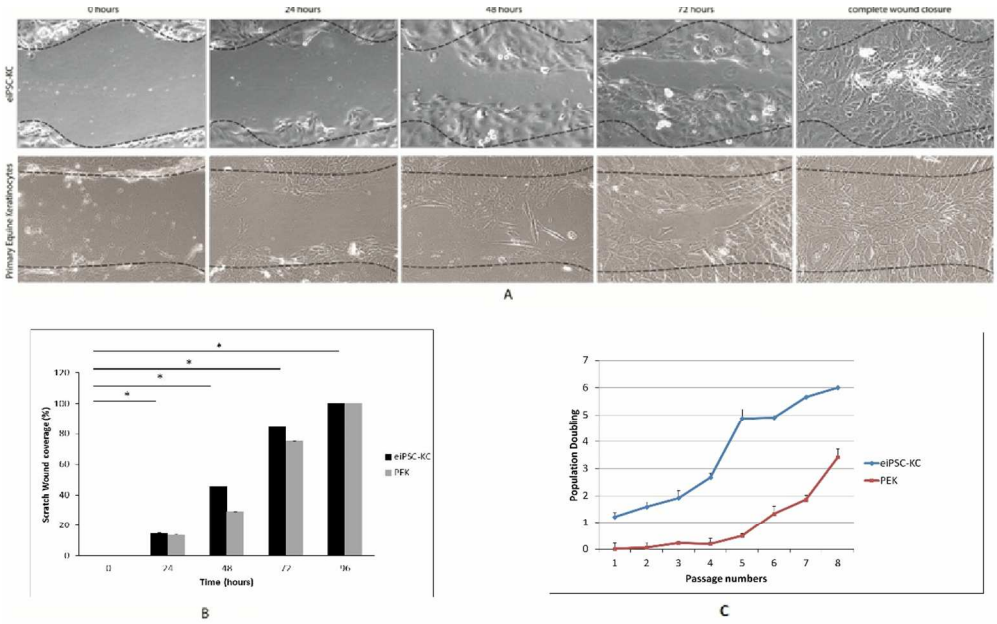
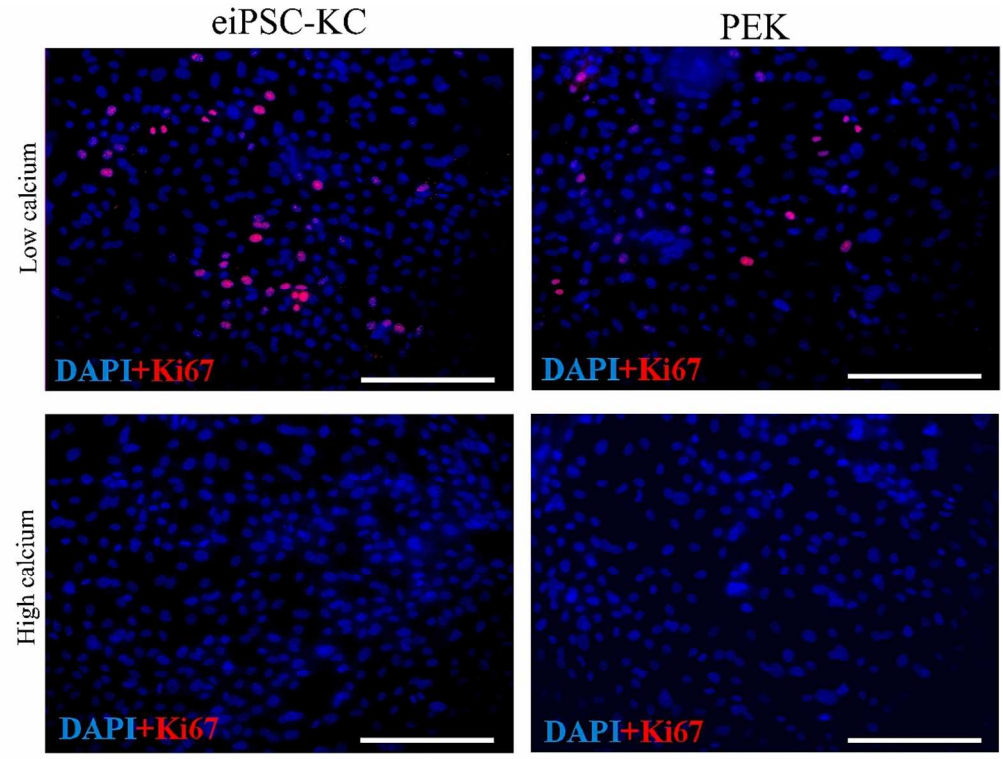


Flow cytometric analysis of eiPSC-KC showing keratin 14+ cells in response to the directed differentiation protocol, in comparison to control PEK and undifferentiated eiPSC. Red line indicates KRT14 staining and black line indicates isotype control.
108x38mm (300 x 300 DPI)



(A) In vitro scratch wound healing assay showing migration of eiPSC-KC in comparison to control PEK that cover the scratch wound at 96h. Black dotted lines indicate the scratch wound margins.
(B) Graph showing scratch wound coverage by migration of eiPSC-KC and PEK. Images were photographed with an Axio Observer Z1 and percent coverage was calculated using Image J software. Data are presented as mean ± SD of three independent experiments.
(C) Population Doubling of eiPSC-KC after formation of epidermal cells in comparison to control PEK cultures. Data are presented as mean ± SD of three independent experiments.

338x209mm (96 x 96 DPI)



Equine iPSC-KC and PEK cultured in low calcium medium show cell proliferation by positive immunoreactivity for Ki67. Cell cycle arrest occurs in response to high calcium concentration, as evidenced by loss of immunostaining. (Scale bar 50μm).

108x82mm (300 x 300 DPI)

Gene	Forward	Reverse
GAPDH	GAGATCCCGCCAACATCAAA	AAGTGAGCCCCAGCCTTCTC
RPL 32	GAAGCACATGCTGCCCAGT	CTTTGCGGTTCTTGGAGGAG
SDHA	GCACCTACTTCAGCTGCACG	AACTCCAAGTCCTGGCAGGG
Keratin 18	ACATCCGAGCCCAGTATGAAG	GTGGCCTCAGCAGTGCCTATC
Keratin 14	TCTCCTCCAGCCGCTACTCCT	TTCTGCATGGTCACCTTCTCA
Keratin 10	GGTTCAGTGGTGGCTCTTTTA	CCTAAGCTGCTGCTTCCATAG
Involucrin	ACATCACAAAGCGGAAAACC	GAGCTTCTTCTCCTGTTCCA
Keratin 5	ACCGATTTGGAGCCGTTGCTG	CCTAAGCTGCTGCTTCCATAG
Nanog	CGGGGCTCTATTCCTACCACC	GAGCACCAGGTCTGACTGTT
Sox 2	CTTGGCTCCATGGGTTCG	TGGTAGTGCTGGGACATGTGA
Klf 4	GTGCCCCAAGATCAAGCAG	TGCTGAGAGGGGGTCCAGT
Oct 4	GAGAGGCAACCTGGAGAACAT	ACTCGTACCACGTCCTTCTCG

Primer sequences of analysed genes.
254x190mm (300 x 300 DPI)

V. General Discussion

A. Undifferentiated eiPSC weakly express molecules of the MHC

The high polymorphism of the MHC complex represents a considerable barrier in tissue transplantation since incompatibility between donor and recipient may lead to immune rejection. Data showed that reprogrammed parental fibroblasts significantly downregulate MHC expression. One of the eiPSC lines analyzed for MHC expression (MHC-I = 4% and MHC-II = 1%) was injected into the horse from which it was derived, thereby serving as an autologous eiPSC control. While this line did not elicit acute graft rejection, it did cause the formation of inflammatory foci within the dermis, similarly to the allogeneic eiPSC line (MHC-I = 1% and MHC-II = 12%), presumably due to cell line differentiation or transient culture conditions that upregulate MHC-II expression [179]. Analysis of an eESC line that served as an *in vitro* cell control showed that MHC-I expression was low (2.5%), however MHC-II expression was far greater (27%) than observed in other species [180] (data in appendix- **Fig. A1)**. Equine eESC are rather an ES-like trophoblast cell line contaminated by other cell types [80]. Moreover, they have been shown to differentiate very rapidly and spontaneously into various cell types (a significant drawback with most veterinary ESC lines). Nevertheless, no other *in vitro* studies have measured MHC expression in eESC and there may be differences among cell lines. Because they are allogeneic (and embryonic) in origin, eESC might have been an appropriate (positive) cell control with which to compare the immunogenicity of eiPSC *in vivo*. However, due to the difficulty in keeping them in an undifferentiated state [103] and

their high MHC-II expression, that could further be upregulated upon differentiation *in vivo*, eES-like cells may not be suitable for clinical applications.

Since the aim of my first study was to determine the immunogenicity of allogeneic eiPSC, ELA typing was carried out to confirm the allogeneic nature of the eiPSC line to be transplanted intradermally. Genomic DNA from skin samples obtained from the experimental horses was used for microsatellite PCR amplification and fragment analysis, carried out by Dr. Antczak at the Baker Institute for Animal Health (Cornell University) – see supplementary data of article 1.

An advantage of the eiPSC used in my studies is their expression of GFP, a reporter gene enabling the identification of iPSC colonies successfully transfected with the exogenous pluripotency factors. An incidental finding was that eiPSC were heterogeneous with respect to GFP expression and cell size, as observed in other species [181]. In any case, pluripotency is unrelated to cell size and to GFP expression. Further, GFP is an unstable protein and spontaneous silencing of transgenes can occur in ESC and iPSC, a common manifestation in transgenic animals [182]. Nevertheless, the GFP protein may trigger an inflammatory response in the host, potentially contributing to graft rejection [183]. Thus, stem cell differentiated derivatives should ideally not be marked with GFP in a clinical setting.

B. Undifferentiated eiPSC elicit a chronic inflammatory response in immunocompetent hosts

In vitro studies have mostly shown that autologous, undifferentiated murine and human iPSC are lowly or non immunogenic [158], though a study by Zhao *et al.* (2011) found that autologous, undifferentiated iPSC elicit an immune response in syngeneic mice

[136]. Interestingly, my histologic data reveal no statistically significant difference in the inflammatory response elicited by autologous or allogeneic undifferentiated eiPSC (data in appendix- **Fig. A2)**. Both autologous and allogeneic eiPSC-injected sites initially formed small inflammatory foci that grew larger over 30 days, with inflammatory exudate extending throughout the dermis. A recent study in mice demonstrated that undifferentiated, allogeneic iPSC survive and proliferate in immunocompetent recipients early post-transplantation, accompanied by a mild immune cell infiltration [184]. My study extends these findings to a large animal model. The GFP^+ eiPSC were detectable up to one week post-transplantation in tissue sections of injected sites. Infiltration of $CD4^+$ and $CD8^+$ T-cells around the transplantation sites suggests activation of the immune system. These data imply the development of a chronic and progressive inflammatory response to eiPSC in the MHC-mismatched hosts, with persistence of $CD4^+$ and $CD8^+$ T-cells that increasingly infiltrated the transplanted site over 30 days, in comparison to saline-injected controls. The absence of GFP^+ eiPSC at the site of transplantation after seven days may be due to migration of the cells away from the site or phagocytosis and lysis of the cells by T-cell mediated effects. The undifferentiated eiPSC, being doxycycline dependent, require other cytokines such as LIF, bFGF etc. to sustain their undifferentiated state, possibly even in *in vivo* conditions [87]. The absence of these stimuli in the dermis may have caused sluggish activity or death of the transplanted eiPSC.

The eiPSC used in my study require the continuous expression of doxycycline controlled reprogramming factors to maintain pluripotency [87]. Because previous studies show an association between the reprogramming factor *c-MYC* and the formation of

malignant neoplasms [185], the eiPSC were cultured in doxycycline-free media prior to transplantation, in order to eliminate potential tumorigenicity associated with *c-MYC*. In doing so, loss of reprogramming transgenes occurred after 48h (data in appendix- **Fig. A3**). Predictably, there was a concurrent significant downregulation of pluripotency genes *NANOG, c-MYC* and *KLF-4*, suggesting the initiation of differentiation of eiPSC (data in appendix- **Fig. A4**). Nevertheless, the eiPSC continued to express pluripotency genes (albeit at a lower level), compared to eiPSC cultured in doxycycline containing media (data in appendix- **Fig. A5**). Further, there was no remarkable change in MHC-I or II expression by eiPSC after 48h in doxycycline-free media (data in appendix- **Fig. A6**). These preliminary data are promising since culturing in doxycycline-free media reduces potential tumorigenic risk of the transgenes, especially *c-MYC*, while early differentiation of eiPSC does not seem to increase their immunogenic potential. The absence of teratocarcinomas in this study is possibly attributed to factors such as the site of transplantation, the use of immunocompetent animals and the type of non-genomic integrating eiPSC.

Because iPSC are unlikely to spontaneously differentiate into a desired cell lineage *in vivo*, due to the absence of external morphogens or stimulants to direct their differentiation [128], the next step of my project aimed to develop an efficient method to drive the differentiation of eiPSC into a keratinocyte lineage, of interest for coverage of wounds in horses.

C. Standardized protocol to drive eiPSC to commit to a keratinocyte lineage

In this study, a specific combination of signalling molecules induced iPSC differentiation by recapitulating the conditions occurring during embryogenesis [186].

Thus, sequential application of ectodermal morphogens RA and BMP-4, directs the differentiation of iPSC towards ectoderm and finally keratinocytes of the epidermis. Various concentration (0.5, 1, 1.5, 2μM) of RA and BMP-4 (25ng/ml) were applied to eiPSC, either together or sequentially (data not shown), and it was found that concentrations of 1μM RA for three days, followed by 25ng/ml of BMP-4 for three days were most effective for inducing ectodermal differentiation in the equine iPSC model, reflecting previous studies carried out in man and mice [124; 128]. Controls for this directed differentiation protocol were undifferentiated eiPSC cultures, separated from feeder layers but not treated with ectodermal morphogens.

In response to precise induction with ectodermal morphogens, undifferentiated eiPSC were driven to eiPSC-KC that express keratinocyte markers at both gene and protein levels. The eiPSC-KC expressed *KRT18*, *KRT14*, *KRT10* and *IVL* as shown in prior iPSC differentiation studies [128; 131]. The antibody clone used to measure PANCK expression in response to the differentiation protocol is a cocktail of high molecular weight (MW) KRT 1, 2, 3, 4, 5, 6, 10, 14, 15 and 16, and low MW KRT 7, 8 and 19 (verified by WB, data in appendix- **Fig. A7)**. Thus, immunostaining with this antibody implies expression of several of these keratins specific to the epidermis suggesting successful differentiation. Expression of genes of suprabasal layers of the epidermis (*KRT 10, IVL*) indicates keratinization, a characteristic absent in other stratified squamous epithelia such as esophagus, oral mucosa, cornea etc.

In addition, flow cytometry confirmed that a highly pure population of cells of the basal epidermal subtype were obtained following the differentiation protocol: 82.5% of the

differentiated eiPSC expressed KRT14, in comparison to 90.9% of the PEK (positive control). However, the concentration of molecules on the surface of eiPSC-KC varied from that on PEK, evidenced by differences in fluorescence intensities measured by cytometry. However, the concentration of molecules on the surface of eiPSC-KC varied from that on PEK, evidenced by differences in fluorescence intensities measured by cytometry suggesting differences in distribution of surface molecules between eiPSC-KC and their primary counterparts. Nevertheless, the purity achieved in my study is comparable to that of a recent study in man (75% KRT 14^{+} cells and 86% KRT 14^{+} cells) using the same ectodermal inducers [128; 131].

Normal mouse and human keratinocytes proliferate when cultured in low calcium media and can be induced to terminally differentiate and stratify when exposed to higher levels [36]. This was shown by prior studies that investigated the effects of a 'calcium switch', beginning with low calcium media (0.02mM) and increasing through gradients of 0.5, 1, 2, 4, 6 and 8mM [187]. Both eiPSC-KC and PEK suffered cellular detachment and apoptosis when culture in media with concentrations >5mM calcium. The two cell types (eiPSC-KC and PEK) were therefore treated with 1.8mM calcium (based on the results of a titration assay) for up to three days. Analysis of keratin genes and *IVL* did not show discernible changes in response to calcium for either cell type (in response to calcium treatment, basal keratinocytes markers *KRT 5* and *KRT 14* are expected to decrease, with an increase in suprabasal markers *IVL* and *KRT 10*). Nevertheless, the calcium switch did induce morphological changes and growth arrest in both eiPSC-KC and PEK suggesting calcium had artificially induced terminal differentiation as seen by the squames floating in

the culture media. These results are in accordance with those observed in previous differentiation studies [186].

D. Primary equine keratinocytes (PEK) cell culture

Primary equine keratinocytes isolated from horse skin have not been widely characterized, although there have been reports of PEK isolated from equine hoof and lip epithelium [188; 189]. Recently, PEK monolayers were obtained from skin explants in horses [60] however this approach may be plagued by variations in cellular composition leading to susceptibility to fibroblast overgrowth, poor subculturability and low growth rate [190]. I developed a modified culture system with low serum medium of 2% based on techniques used for keratinocyte isolation in mice and man, and a single report in horses using 10% serum containing keratinocyte medium [189]. The PEK obtained by this method were negative for vimentin, a marker of fibroblasts, confirming that fibroblast-free cultures of PEK were obtained **(**data in appendix- **Fig. A8)** contrary to what is usually achievable using explant cultures [190].

The recent development of equine skin equivalents (ESE), seeded with PEK and dermal fibroblasts, with the ability to form suprabasal skin layers in 3D models is exciting [60] [191]. However, a major caveat of these latest studies is the absence of a comprehensive molecular characterization necessary to confirm their identity as PEK, and absence of data on proliferative and migratory capacities. Another recent addition to equine

regenerative medicine is the isolation, from equine skin, of EpSC [192] capable of self-renewal and with a bipotent differentiation capacity (into keratinocytes and adipocytes). These cells displayed regenerative capabilities by increasing vascularization, elastin content and follicle-like structures when implanted into experimental skin wounds in horses. Equine iPSC-KC should be tested in this *in vivo* wound model to the respective abilities of these two cells types to engraft and regenerate skin and its appendages.

E. Phenotype and function of eiPSC-KC and PEK

The eiPSC subjected to the directed differentiation protocol described herein rapidly undergo morphologic changes, assuming epithelial cell morphology; the cells take on a typical cobblestone appearance, characteristic of keratinocytes of the epidermis [193] and undergo terminal differentiation in response to the calcium switch (low to high). These keratin-expressing cells attach to collagen-coated dishes, a characteristic of KSC, which concurs with the behavior of mouse and human iPSC transformed into a multipotent keratinocyte lineage [128]. Based on these attributes, differentiated eiPSC were found to have a similar phenotype to PEK.

To further demonstrate that differentiated eiPSC are functionally similar to PEK and therefore warrant the appellation 'eiPSC-KC', a wound healing assay was performed to predict their ability to heal an *in vitro* wound created in a monolayer of cells. To my knowledge, no previous study reports the use of such an *in vitro* wound healing assay for PEK, however my data parallel those of mouse and human models [194]. Results suggest that eiPSC-KC and PEK would be equally valuable for application on *in vivo* wound healing models. Importantly, eiPSC-KC showed greater proliferative capacity compared to

PEK that had a limited life span. Thus, eiPSC-KC may be superior alternatives in clinical settings since they could be used to generate a large number of cells required to populate the graft *in vivo*. Equine iPSC-KC consist of KRT14 basal epidermal cells that have the capacity to form the suprabasal layers of the epidermis. An advantage of my study design is the feeder free system used to generate eiPSC-KC, making these cells ideal for future transplantation in horses since free of xenogeneic components that would necessarily increase immunogenicity. It is expected that eiPSC-KC would have the capacity to differentiate into the adnexal structures of the epidermis, as shown in previous *in vivo* mouse models [128], essentially regenerating the skin by restoring pigmentation, nerve and vascular plexuses as well as immune capabilities.

VI. Study Limitations

A. Immune response to undifferentiated eiPSC

Prior to transplanting cells into the experimental subjects, the MHC expression of undifferentiated eiPSC was measured in an effort to predict the eventual *in vivo* immune response. While MHC expression by the two available eiPSC lines was low, analysis of additional lines would enable selection of the least immunogenic lines. Although the *in vivo* portion of my study has provided valuable information on the horse's immune response to transplantation of eiPSC, the data must be interpreted with caution since limited to two horses. Moreover, while the dose of eiPSC ($1x10^6$) was based on that currently used in mouse models [136], due to technical difficulties and time constraints it was unfortunately impossible to generate sufficient numbers of cells to inject all the sites in both horses, such that a single allogeneic line provided duplicate data sets.

Skin samples were obtained at days 2, 3, 7 and 30 following intradermal transplantation of eiPSC. While these times were based on a previous study model, chronic inflammation may have persisted and evolved beyond the study duration, causing tissue fibrosis and scarring [195] which, in this study, went unobserved. A considerable technical difficulty with flow cytometry was that the overlap of GFP expression by eiPSC and secondary antibody staining (R-Phycoerythrin) hindered fluorescence compensation of GFP. This might be overcome by using a GFP^- eiPSC line; as this was unavailable we used the parental fibroblast cell line as a negative control to enable determination of immunolabelling with the secondary antibody. This situation could be avoided in the future

by staining with fluorochromes further ahead in the fluorescent spectrum such as APC or Alexafluors (Alexa647) with wavelengths >650nm.

Because the eiPSC used in this study were doxycycline dependent, the withdrawal from doxycycline may have lead to the death of these cells *in vivo*. While supplementation of doxycycline in the feed might have sustained the survival of eiPSC *in vivo*, this is not easily feasible in horses due to cost and toxicity concerns. No doubt the most significant limitation of the first study is that undifferentiated eiPSC were studied, which does not reflect the scenario likely to be used clinically. Therefore, this experiment should be repeated using differentiated derivatives of potential clinical utility, such as the eiPSC-KC subsequently developed.

B. Differentiation of eiPSC into keratinocytes

The next objective of my PhD research was to differentiate eiPSC to a keratinocyte lineage given the therapeutic potential of this cell type in wound management. A major caveat of this second study is that the protocol I developed was fully successful in a single eiPSC line. While reproducible in the same eiPSC line, other available eiPSC lines did not respond similarly, even with varying concentrations of the ectodermal morphogens RA and BMP-4, possibly due to dissimilar differentiation capacities [196].

Another drawback was that the available eES-like lines and undifferentiated eiPSC lines had inherent keratin expression suggesting epigenetic memory or incompletely reprogrammed keratinocytes in these lines as also observed in human iPSC lines [197]. The H2A line used in this study showed baseline expression of KRT14 prior to being subjected to the directed differentiation protocol, which was not the case in other species' iPSC subjected to the same protocol [131]. This baseline expression was also seen in WB by

PANCK staining of undifferentiated eiPSC lysates (data in appendix- **Fig. A7)**. Differentiated eiPSC-KC expressed high MW keratins (similar to PEK) as well as low MW keratins, confirming the expected keratin upregulation in response to the differentiation protocol. While undifferentiated eiPSC also expressed (baseline) high MW keratins, this may be attributed to contaminating MEF in addition to inherent keratin expression by the H2A line. This baseline keratin expression of the H2A line is attributable to either epigenetic memory or contamination with keratinocytes that were unsuccessfully or incompletely reprogrammed since fetal tissue (skin with underlying cutaneous muscle) was used for reprogramming [197]. Moreover, studies have documented that iPSC show preferential lineage-specific differentiation such that the phenotype may be influenced by the cell of origin [198], a characteristic that might well be exploited therapeutically [199].

Next, the absence of commercial, equine-specific antibodies against protein markers of the KSC limited my ability to fully compare the obtained eiPSC-KC population to the 'true' keratinocytes (PEK). In this regard, it has been reported that only 4% of human antibodies react with the equivalent equine epitopes [200]. Finding suitable antibodies is, therefore, a challenge in equine studies.

An important clinical consideration is the time required to generate autologous iPSC-KC grafts for wound management. Reprogramming of iPSC from patient fibroblasts is followed by characterization of the iPSC line, done by karyotyping, flow cytometry analysis of pluripotency genes, qRT-PCR analysis, differentiation analysis *in vitro* and *in vivo* for each germ layer. All of these assays require >5 weeks to derive an established autologous iPSC line. Subsequently, the protocol to differentiate iPSC into keratinocytes requires an additional 4-5 weeks of waiting time. Thus, allogeneic iPSC-KC might be

superior for clinical use since they could be prepared in advance and banked. Alternatively, autologous PEK-derived grafts, engineered in approximately three weeks, may be suitable temporary dressings to be used while awaiting the preparation of autologous eiPSC-KC grafts, should further studies reveal that immunogenicity of allogeneic products is excessive.

VII. Research Perspectives

A. Immunogenicity of eiPSC

A consensus as to the effect of the reprogramming method on the immunogenicity of iPSC has yet to be reached. Consequently, reports on the immunogenicity of iPSC derivatives are contradictory, although the most recent suggests acceptance of skin grafts engineered from chimeric iPSC [118]. Thus, studies comparing immunogenicity of non-virally reprogrammed iPSC and other integration-free lines must be conducted on animal models in an effort to determine iPSC lines that are clinically viable. Nevertheless, since undifferentiated iPSC are not likely to be useful clinically, most of the following suggestions apply to iPSC derivatives, in particular the eiPSC-KC described in the second article of this thesis.

In addition to striving for a better MHC match between host and recipient prior to grafting, the immune response might be predicted by performing *in vitro* Mixed Lymphocyte Reaction (MLR) testing [157]. This test aims to estimate histocompatibility between the donor and the host. In addition to the MHC antigens, costimulatory molecules such as CD40, CD80, CD86 and mHC also play integral roles in activation of the immune system by presenting foreign antigens to the TCR. Therefore, to further characterize the immunogenicity of eiPSC and better predict the *in vivo* response to grafting, a comprehensive study of surface molecules involved in the immune response must be conducted. Of course, analysis of a large number of eiPSC lines is necessary to select, for eventual banking purposes, those that are lowly immunogenic on account of their MHC and costimulatory molecule expression profile. Analysis of costimulatory molecules on iPSC

would additionally permit identification of the activated cells and their pathways, thus permitting their blockade, thereby facilitating graft acceptance [201].

The logical next step would be to determine the ability of iPSC to survive amidst *in vivo* inflammatory conditions, given that the cells are ultimately to be used therapeutically for disease conditions involving inflammation. This might be done by challenging eiPSC with IFN-ϒ *in vitro*, in such a manner as to simulate inflammatory *in vivo* conditions. Interestingly, a previous study has shown that undifferentiated iPSC exert paracrine effects by homing to the site of acute lung injury in mice and causing downregulation of the inflammatory response [202]. Analysis of the MHC molecules in response to simulated inflammatory environments (e.g. wounds) will determine the ability of these cells to survive amidst the inflammatory mediators and potentially modulate the host environment.

Because the fate of the transplanted eiPSC is uncertain in my study (no detection of the GFP marker beyond seven days of grafting), strategies to improve both *in vivo* cell tracking and graft survival must be developed. Several fluorophores and probes can be introduced into stem cells for *in vitro* and *in vivo* dynamic tracking, with bioluminescent imaging (BLI) modalities such as magnetic resonance imaging and optical or nuclear imaging being some of the most common [67]. Moreover, alternative fluorophores, less immunogenic than GFP, should be considered for *in vivo* tracking studies.

Detachment of cells in culture from ECM/feeders/attachment to other cells initiates the apoptotic pathway (called anoikis) eventually leads to cell death due to the loss of adhesion-related survival signals. The concurrent use of biomaterials mimicking the ECM has been explored as a means to provide a niche environment rich in pro-survival factors in order to increase retention of cells at the site of transplantation [203]. Alternatively, co-

transplantation of iPSC with other cell types such as MSC or with PRP are interesting measures since the latter may ensure immunomodulation and cytokine release at the site of transplantation, thereby enhancing graft 'take'.

B. Differentiation of eiPSC into keratinocytes

The second article in my thesis describes the generation of eiPSC-KC that are phenotypically similar to their primary counterparts (PEK). While the eiPSC-KC do express key keratinocyte genes and proteins, differences in the levels of gene transcripts are apparent. For example, *KRT18* was highly expressed in eiPSC-KC suggesting these cells have yet to reach a state of complete differentiation [204]. Further studies are therefore warranted to establish the exact differentiation status of these cells. Analysis of other markers of basal keratinocytes (e.g $\alpha_6\beta_4$ integrins, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins of the lateral surfaces of epidermal basal cells) is warranted. Enrichment of these cells by double labelling with antibodies against integrins and key markers such as KRT5, KRT6, KRT15 and KRT14, is required. Precise *in vivo* localization of these putative stem cells, to determine the niche from which they originate, must be explored to tap into these sources for future clinical use.

In order to confirm the clinical value of the eiPSC-KC generated via the directed differentiation protocol I have developed, the ability of these cells to fully integrate and develop into the various strata that comprise equine skin must be studied, first *in vitro* then *in vivo*. Seeding of eiPSC-KC in 3D constructs should help demonstrate this capacity by 1- characterizing the construct, using specific markers to determine whether iPSC-KC have differentiated into adnexa and 2- raising the 3D construct to the air-liquid interface to verify the ability of eiPSC-KC to stratify and form corneocytes. Then, using an *in vivo*

experimental model such as that recently described by Broeckx *et al*. (2014) [192], the contribution of these eiPSC-KC to wound healing in horses could be measured and possibly compared to that of equine EpSC.

While eiPSC-KC have shown promising indications (proliferation potential) of being superior to PEK for grafting onto wounds to enhance healing, several questions as to their applicability and safety must be answered before they can be considered clinically. In the interim, PEK-based grafts might present an interesting but unexplored option for equine regenerative medicine. However, KSC specific markers must first be developed to permit immunophenotypical characterization of PEK. Efforts to further define basal cells as well as determine the source and niche of these cells (e.g.: interfollicular epidermis, hair follicle bulge) are required. Enrichment of these respective populations must then be tested in *in vivo* models to confirm the multipotency of these cells. Moreover, improvement of culture conditions of PEK is required to prolong their lifespan *in vitro*; inspiration might come from studies in mice and man wherein primary keratinocytes are maintained for >20 passages [205]. Alternatively, strategies to immortalize PEK lines and/or the development of transformed cell lines might produce an important tool for research, similar to the classic HaCaT line that serves to study wound repair, cancer, and as platform for gene delivery, drug testing [206].

VIII. Conclusion

The overall objective of my research program was to develop a tissue engineering approach, using stem cells, to improve the management of chronic, non-healing wounds in horses. In order to achieve this I first investigated the immune potential of eiPSC in an effort to characterize this possible barrier to therapeutic use of eiPSC. Initial enthusiasm for PSC derivatives was based on the assumption that they may escape immune surveillance on account of low MHC expression. I used an intradermal transplantation model along with GFP-marked eiPSC to effectively study, *in vivo*, the immune response to iPSC in horses. My results show that the weak expression of MHC molecules is insufficient to ensure engraftment of autologous and allogeneic eiPSC; thus a plethora of other factors must also contribute to the immunogenicity of iPSC and thus the success of engraftment of iPSC-derived grafts. Ultimately, screening and banking of hypoimmune allogeneic eiPSC lines should allow selection of those most suited for directed differentiation into lineages of clinical interest.

In equine wound healing, the keratinocyte is of critical importance to ensure proper epithelialization and control the development of EGT; unfortunately, this cell population is often deficient, particular in the limb wounds of horses. This was the impetus for the standardization of a protocol to induce the differentiation of eiPSC into cells of a keratinocyte lineage that might ultimately be used to provide effective coverage in the form of a graft. Results of my study show that eiPSC-KC express markers characteristic of the basal cells of the epidermis and are phenotypically as well as functionally similar to primary keratinocytes. Moreover, the demonstrable migratory ability of eiPSC-KC along

with their superior proliferative capacity, suggest their value as a cell source for grafting onto *in vivo* models requiring large numbers of highly proliferative cells.

Taken together, my studies mark a step forward for equine regenerative medicine. Nevertheless, before clinical application may be envisaged, several barriers are to be overcome. High quality cell products must be developed in good manufacturing practice conditions and standardized non-genomic integration derivation methods must be developed. Further studies are necessary to ensure consistency and genomic stability of PSC derivatives since heterogeneity and tumorigenicity are justifiable concerns. Flow-based methods to enrich for pure, differentiated populations must be perfected prior to clinical use. Finally, the ability of stem cell derivatives to engraft and function in the host over the long term is prerequisite to their therapeutic success. The constant evolution of regenerative medicine with safe and effective means of using iPSC should help to make iPSC-derived grafts the new 'gold standard' for future therapeutics.

IX. Bibliography

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X. Appendix: Supplementary Results

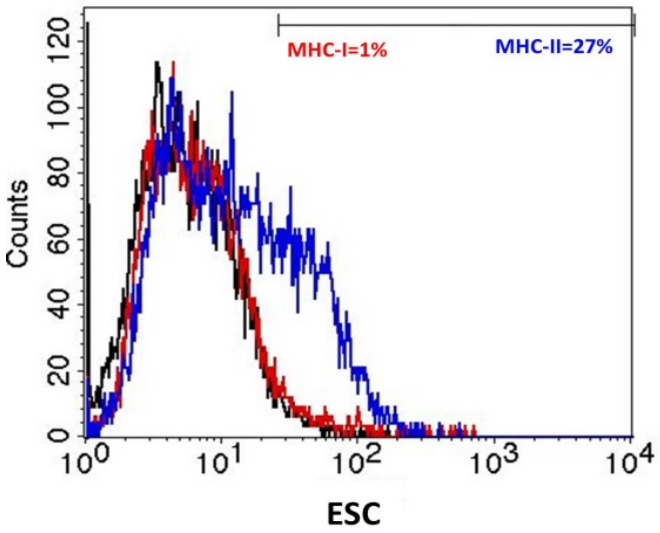


Fig. A1: Flow cytometric analysis of MHC expression by equine ES-like cells: Flow cytometric analysis of equine ES-like cells shows low MHC-I expression (1%) and high MHC-II expression (27%).

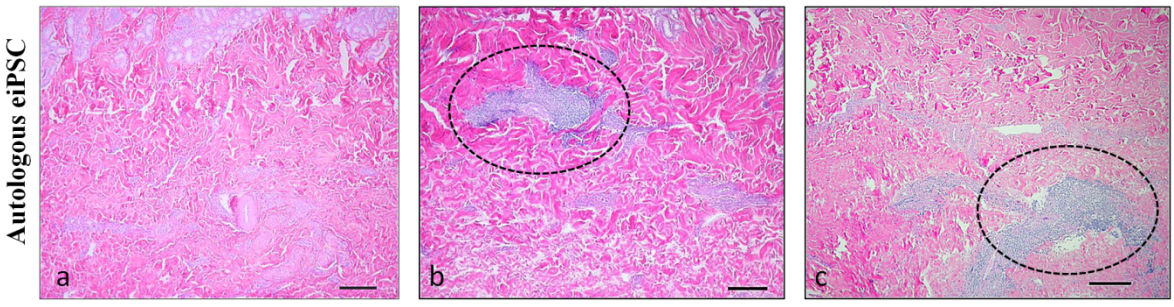
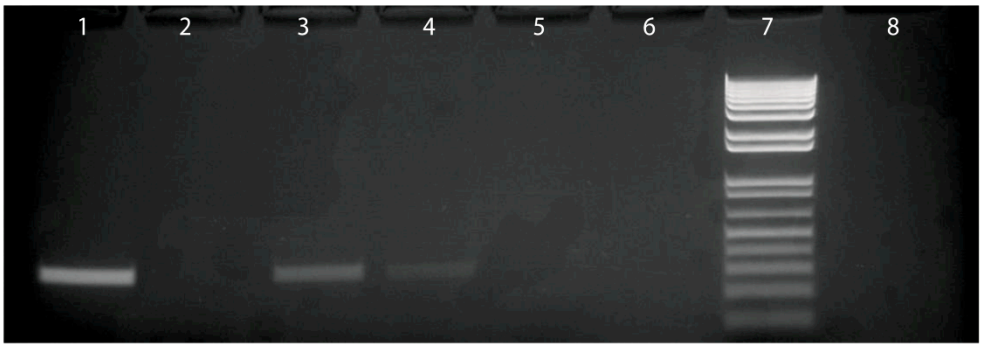


Fig. A2: H&E section of skin injected with undifferentiated autologous eiPSC intradermally in the lateral neck of experimental horses showing moderate immune response at days 7(b) and 30(c), and no response at 2 days (a). (Black dotted circles show the area of inflammatory response)



1- iPSC Line 1 in complete iPSC media showing expression of the mouse exogene
2 - iPSC line 1 in inhibitor free media showing loss of the mouse exogenes after 48 hours
3-iPSC line 2 in complete iPSC media showing expression of the mouse exogene
4 –iPSC line 2 in inhibitor free media showing negligible expression of the mouse exogene
5- RT negative
6-water
7-1Kb ladder
8-water negative control

Fig. A3: Loss of murine transgenes after 48h in doxycycline free media: PCR products of line 1 eiPSC confirm presence of the mouse plasmid, that disappears after doxycycline withdrawal. Similarly, line 2 eiPSC lose expression of the mouse plasmid in the absence of doxycycline.

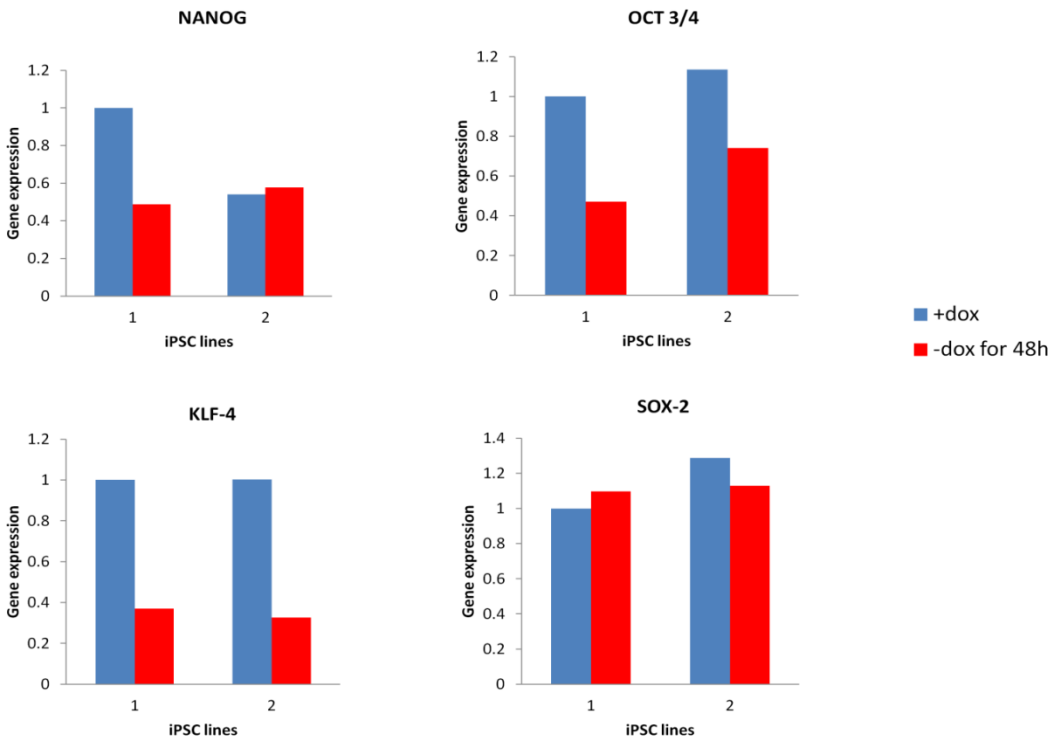
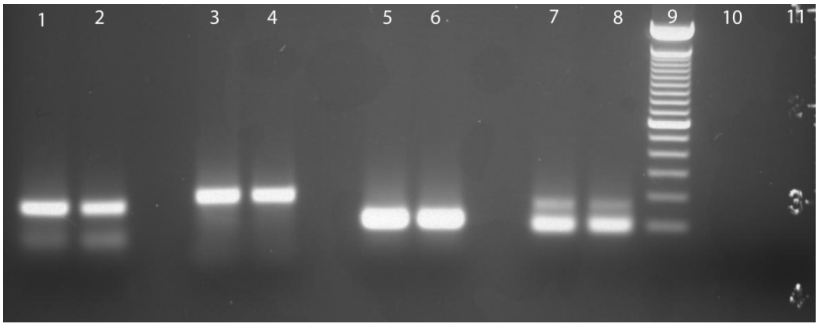


Fig. A4: **Reduction in pluripotency genes in the absence of inhibitors:** Expression of pluripotency genes of eiPSC cultured in complete doxycycline medium (blue bars) and gene expression after doxycycline withdrawal for 48h (red bars) shows reduction in levels of *NANOG, OCT 3/4, KLF-4* suggesting initiation of differentiation in eiPSC lines 1 and 2.



1- Line 1 eiPSC without doxycycline for 48 hours showing expression of Nanog
2- Line 2 eiPSC without doxycycline for 48 hours showing expression of Nanog
3- Line 1 eiPSC without doxycycline for 48 hours showing expression of Sox - 2
4- Line 2 eiPSC without doxycycline for 48 hours showing expression of Sox - 2
5- Line 1 eiPSC without doxycycline for 48 hours showing expression of Oct - 4
6- Line 2 eiPSC without doxycycline for 48 hours showing expression of Oct - 4
7- Line 1 eiPSC without doxycycline for 48 hours showing expression of Klf - 4
8- Line 2 eiPSC without doxycycline for 48 hours showing expression of Klf - 4
9- Ladder
10- RT negative
11- water

Fig. A5: Persistent pluripotency gene expression after 48h: PCR products of eiPSC lines 1 and 2 after doxycycline withdrawal for 48h show expression of pluripotency genes *NANOG, SOX-2, OCT-4* and *KLF-4.*

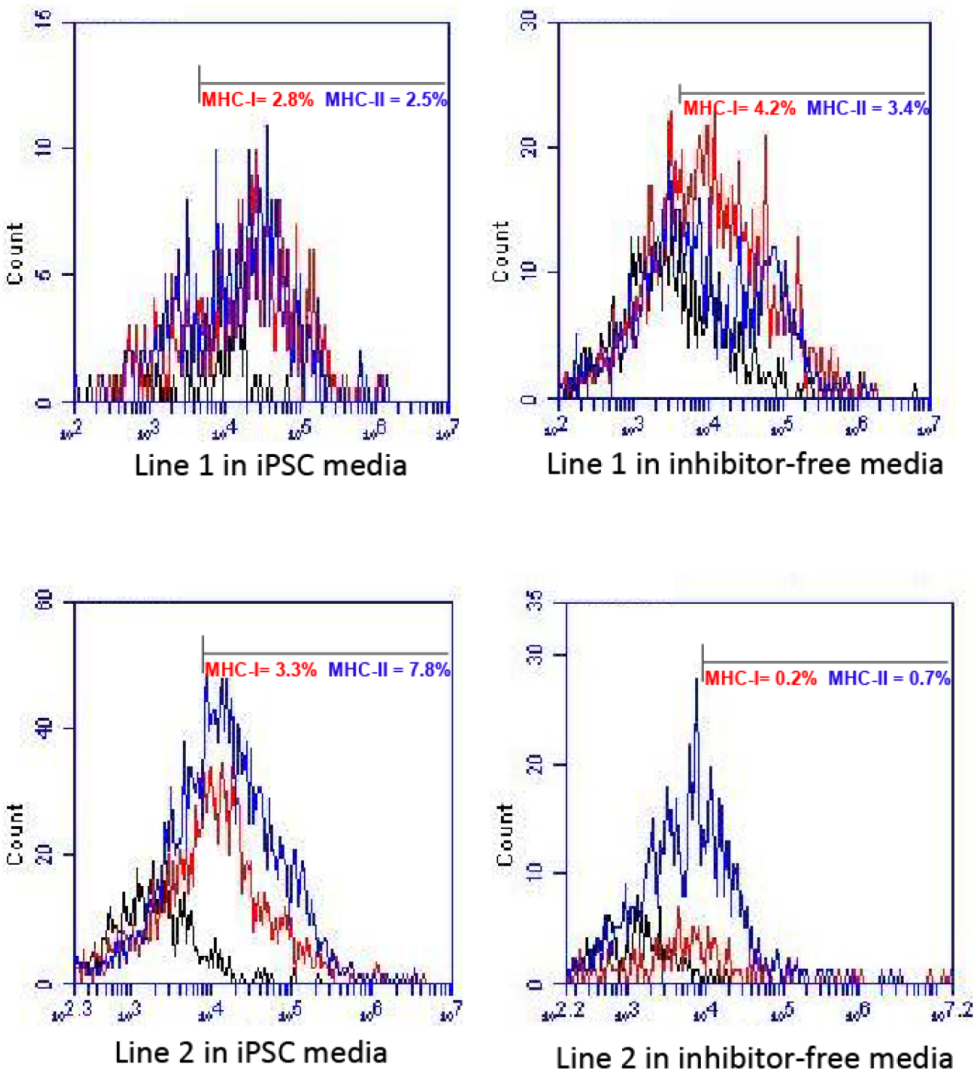


Fig. A6: Flow cytometric analysis of MHC expression by undifferentiated eiPSC: MHC expression by lines 1 and 2 in complete iPSC media and in inhibitor free media (doxycycline and LIF free media) for 48h. Equine iPSC line 1 had MHC-I expression of 2.8% and MHC-II expression of 2.5% in eiPSC media. After 48h of inhibitor withdrawal, MHC-I expression was of 4.2% and MHC-II expression was of 3.4%. Similarly, eiPSC line 2 showed MHC-I expression of 3.3% and MHC-II expression of 7.8%. After 48h of inhibitor withdrawal, MHC-I expression was of 0.2% and MHC-II expression was of 0.7%. These data suggest that doxycycline removal does not increase immunogenicity by MHC expression.

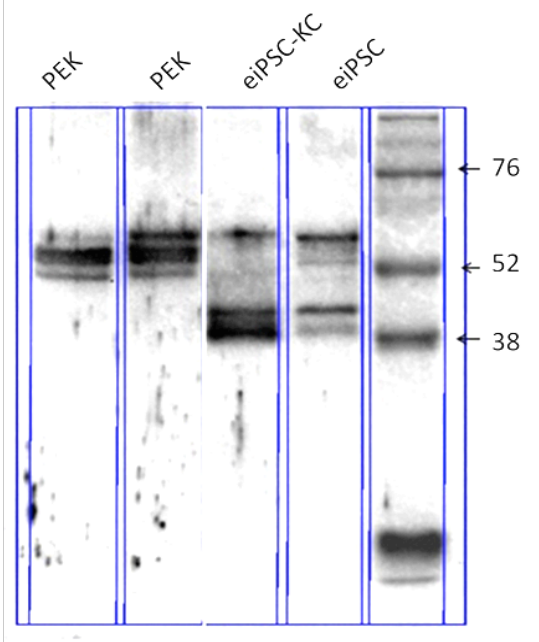


Fig. A7: Immunoblot of Pancytokeratin: PANCK has MW ranging from 40-67kDa. PEK show higher MW keratins (50-60 kDa), whereas eiPSC-KC show low MW keratins (40-60 kDa). Undifferentiated eiPSC also express low and high MW keratins (40-60 kDa).

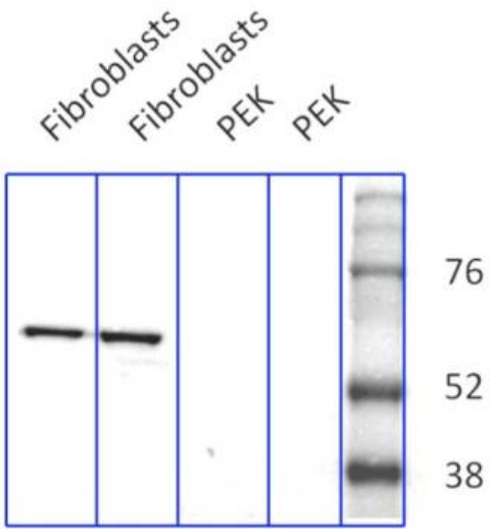


Fig. A8: Immunoblot of Vimentin: Cell lysates of fibroblasts have a molecular weight (MW) of 60 kDa whereas PEK are negative for Vimentin.

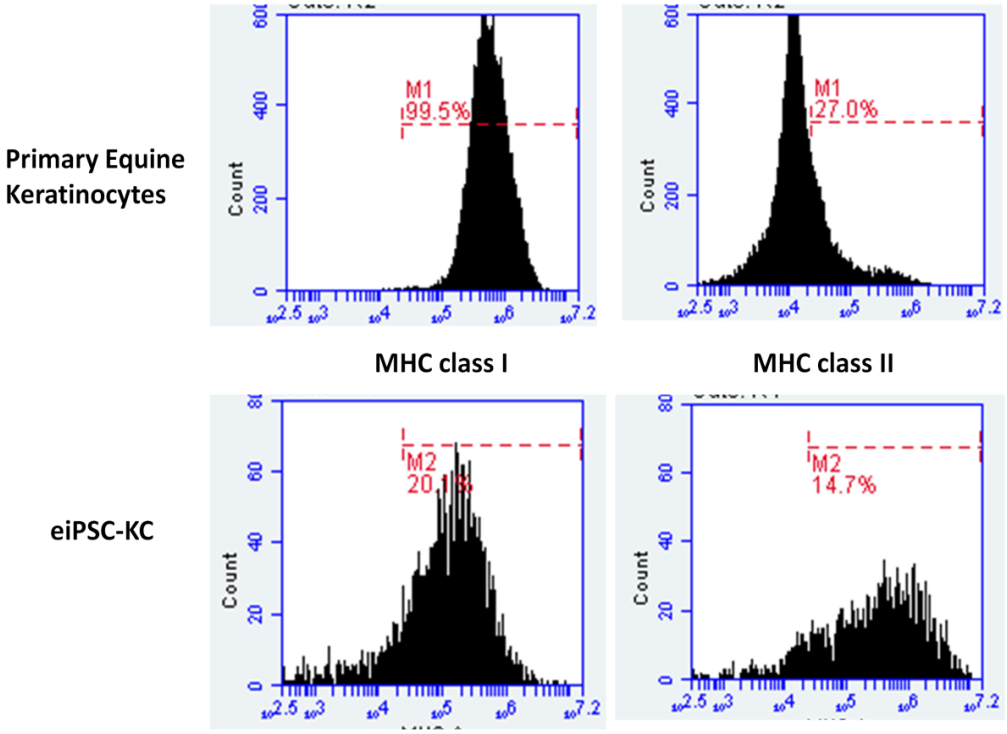


Fig. A9: Flow cytometric analysis of MHC expression by PEK and eiPSC-KC: MHC-I expression of PEK was 99.5% and that of eiPSC-KC was 20.18%. MHC-II was of PEK was 27% and that of eiPSC-KC was14.7%.

Equine Veterinary Journal

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August 19, 2014

Re: EVJ-GA-14-203.R3, Immune potential of allogeneic equine induced pluripotent stem cells, Aguiar et al.
Acceptedfor publication by Equine Veterinary Journal, 13 August 2014.

Dear Dr Aguiar

I confirm that you have my permission use this version of the manuscript as part of your PhD thesis.

Celia M Marr,
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