Biochemical and Biophysical Research Communications 422 (2012) 75–79

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# *In vivo* tissue engineering chamber supports human induced pluripotent stem cell survival and rapid differentiation

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ARTICLE INFO

*Article history:*
Received 17 April 2012
Available online 27 April 2012

*Keywords:*
Induced pluripotent stem cells
Teratoma
Tissue engineering

ABSTRACT

Pluripotent stem cells are a potential source of autologous cells for cell and tissue regenerative therapies. They have the ability to renew indefinitely while retaining the capacity to differentiate into all cell types in the body. With developments in cell therapy and tissue engineering these cells may provide an option for treating tissue loss in organs which do not repair themselves. Limitations to clinical translation of pluripotent stem cells include poor cell survival and low cell engraftment *in vivo* and the risk of teratoma formation when the cells do survive through implantation. In this study, implantation of human induced-pluripotent stem (hiPS) cells, suspended in Matrigel, into an *in vivo* vascularized tissue engineering chamber in nude rats resulted in substantial engraftment of the cells into the highly vascularized rat tissues formed within the chamber. Differentiation of cells in the chamber environment was shown by teratoma formation, with all three germ lineages evident within 4 weeks. The rate of teratoma formation was higher with partially differentiated hiPS cells (as embryoid bodies) compared to undifferentiated hiPS cells (100% versus 60%). In conclusion, the *in vivo* vascularized tissue engineering chamber supports the survival through implantation of human iPS cells and their differentiated progeny, as well as a novel platform for rapid teratoma assay screening for pluripotency.



## 1. Introduction

Autologous cell sources are ideal for cell-based therapy and tissue engineering because of the lack of immunological issues such as rejection of the transplants. However, autologous cells impose substantial limitations where the cell types required cannot be harvested in substantial numbers or cannot be sufficiently expanded *in vitro*, such as for cardiomyocytes and neurons. With the advent of induced pluripotent stem (iPS) cells [1], the potential to generate large numbers of autologous precursors or differentiated cells may provide a solution to this limitation. iPS cells are characterized by the capability of indefinite self-renewal and pluripotent differentiation into all three germ layers, with potential to generate all tissue types of the body without many of the ethical issues associated with embryonic stem cells [2,3]. While recent studies suggest that iPS cells may not have complete immunoprotection [4,5], with careful and detailed evaluation iPS cells may ultimately become an unlimited source of donor cardiomyocytes (and other cell types) for patient-specific cell therapy and tissue engineering.

A defining shortfall of iPS cell application is the associated tumourigenesis, especially the formation of teratoma in the implanted region [6]. These serious adverse effects have delayed the translation to clinical trials using pluripotent stem cells for therapy. Screening for teratoma formation is a routine procedure for demonstrating pluripotency, but looking forward towards clinical applications these assays may also become essential safety screening for cell therapies which use populations differentiated from iPS cells. The assays will need to offer highly supportive environments for implanted cells, allow robust differentiation and be sufficiently

*Abbreviations:* AVL, arteriovenous loop; bFGF, fibroblast growth factor-2; EBs, embryoid bodies; FA, Friedreich Ataxia; GFP, green fluorescence protein; H&E, hematoxylin and eosin; hiPS cells, human induced pluripotent stem cells.

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http://dx.doi.org/10.1016/j.bbrc.2012.04.108

rapid to test cell populations quickly. Current teratoma assay protocols use nude mice as hosts, and cells are delivered into a vascularized environment, such as testes, kidney capsule or subcutaneous tissue space [6,7]. In order to allow cells to grow and differentiate, the time course for these assays extends into several months and efficiency is variable [7,8]. We have developed a supportive tissue engineering environment which allows for implantation of human adult stem cells and subsequent multipotent differentiation, but without teratoma formation [9]. We propose here that this *in vivo* vascularized chamber may provide a similarly supportive environment for testing survival through implantation of human iPS (hiPS) cells and their differentiated progeny, as well as a novel platform for rapid screening teratoma assays.

Therefore, the present study aims to evaluate the supportive environment of *in vivo* vascularized tissue engineering chambers for implanted hiPS cells, using undifferentiated cultures and spontaneously differentiated embryoid bodies, to demonstrate their survival and their potential for differentiation in the chamber.

## 2. Materials and methods

### 2.1. Culture of hiPS cells and embryoid body formation

The hiPS cell line (FA-hiPS) utilized in the current study was generated by retroviral transduction with OCT-4, SOX2, KLF4 and c-MYC of human skin fibroblasts isolated from Friedreich's Ataxia patients [10]. GFP labeled cells (GFP-FA3-hiPS) were generated by transfecting FA3 iPS cells with the piggyBac transposon vector (Wellcome Trust Sanger Institute) modified to contain a GFP expression cassette, driven by the human elongation factor 1 alpha promoter. Positive GFP-expressing cells were enriched by mechanical dissection of transfected FA3 iPS cells, using a Leica MZFIII Fluorescence Stereomicroscope. The undifferentiated GFP-FA3-hiPS cell colonies were maintained on a feeder layer of mitotically inactivated human foreskin fibroblasts (HFF:D551) in cultured media containing DMEM/F-12, GlutaMAX media (Invitrogen, CA, USA) supplemented with 20% Knockout serum replacement (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), penicillin/streptomycin (Invitrogen) and 20 ng/mL recombinant human fibroblast growth factor-2 (bFGF; Millipore, CA, USA) (Fig. 1A). To induce differentiation, areas of GFP-FA3-hiPS cell colonies were dissected into approximately 0.1 $mm^3$ pieces (Fig. 1B) and transferred to low attachment plates in culture media without bFGF for 7 days, where they aggregated to form embryoid bodies (EBs, Fig. 1C).

### 2.2. Animals

Experimental procedures were approved by the Animal and Human Ethics Committees of St. Vincent's Hospital (Melbourne, Australia) and were conducted in accordance with the Australian National Health and Medical Research Council guidelines for the care and maintenance of animals. Nude rats (CBH-rnu) were purchased from Animal Resources Centre (Perth, Western Australia) and maintained with a 12 h dark/light cycle and given water and chow ad libitum.

### 2.3. In vivo vascularized tissue engineering chamber and experimental groups

An arteriovenous loop (AVL) was constructed in the groin region of adult male nude rats weighing between 200 and 250 g as previously described [9,11]. Briefly, rats were anesthetized with isoflurane inhalation (4% induction and 2% maintenance). A vein graft was dissected from the left femoral vein and interposed between the right femoral artery and right femoral vein with

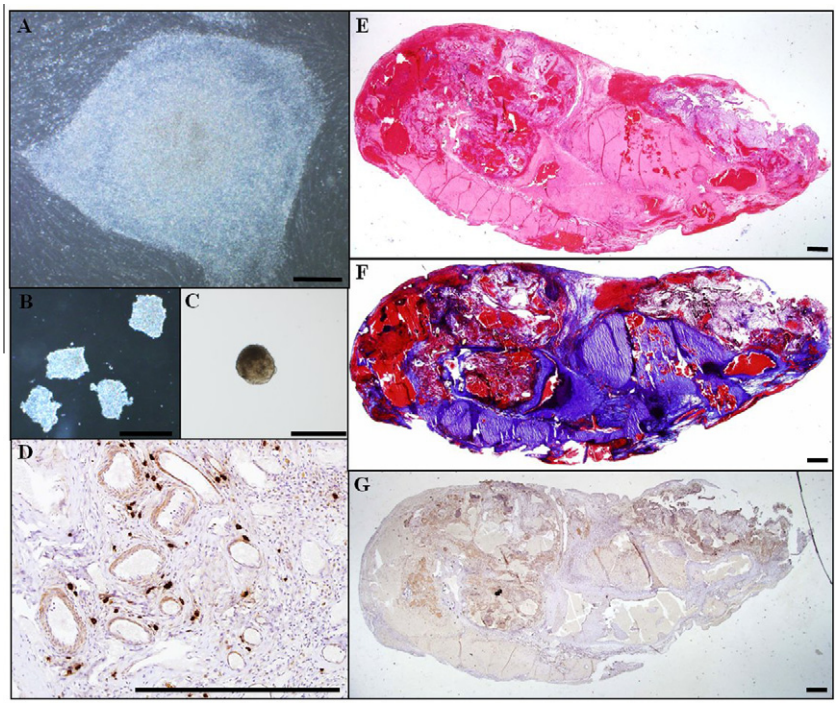


**Fig. 1.** Preparation of GFP-FA3-hiPS cells for *in vivo* vascularized tissue engineering chambers. (A) Representative bright field micrograph of an undifferentiated GFP-FA3-hiPS cells colony cultured on human fibroblast feeder cell layer. (B) Pieces of undifferentiated GFP-FA3-hiPS cells following manual dissection. (C) Differentiated EBs derived from GFP-FA3-hiPS cells. (D) Representative micrograph of OCT3/4-stained section demonstrating pluripotent cells engrafted in the tissue construct and in close proximity to blood vessels. Representative tissue construct generated at 4 weeks post-implantation with GFP-FA3-hiPS cells stained with H&E (E), Masson's trichrome (F) and GFP immunohistochemistry (G). Scale bar = 500 μm.

microanastomoses. The AVL was laid onto the base of a polycarbonate chamber (0.5 ml internal volume, 1.3 cm internal diameter and 0.5 cm height; Department of Chemical Engineering, The University of Melbourne, Melbourne, Australia), which was sutured into the underlying muscle fascia. 250 μL of Matrigel (BD Biosciences Pharmingen) containing GFP-iPS cells was placed into the well of the chamber and allowed to solidify before the lid of the chamber was attached. Skin wounds were then closed in two layers and the animals were allowed to recover. Animals were randomized to receive chambers containing undifferentiated GFP-FA3-hiPS cells (approximately 60 pieces containing 1,200,000 cells, $n = 5$) or differentiated GFP-FA3-hiPS cells (approximately 30 intact EBs containing 700,000 cells, $n = 4$).

### 2.4. Harvest of chamber tissue and histological analysis

At 4 weeks postoperatively, rats were anesthetized and the tissue constructs formed in the chambers were harvested. The tissue constructs were fixed in 4% paraformaldehyde for 24 h, then divided into serial 2 mm thick transverse slices and all slices from each construct embedded into the same block for histology and immunohistochemistry. Paraffin-embedded 5 μm-thick sections were stained for (1) hematoxylin and eosin (H&E), (2), Masson's trichrome, (3) toluidine blue, (4) OCT3/4 (1:200, mouse monoclonal IgG, sc-5279; Santa Cruz, CA, USA), (5) desmin (1:100, mouse anti-human monoclonal IgG, M0760; Dako, Denmark), (6) GFP (1:400, rabbit polyclonal IgG, A11122; Invitrogen), (7) α-fetoprotein (1:500, rabbit anti-human polyclonal IgG, A0008; Dako), (8) nestin (1:100, rabbit monoclonal IgG, ab105389; Abcam, MA, USA), and (9) troponin T (1:500, mouse monoclonal IgG, ab8295; Abcam). For immunohistochemistry, sections were then incubated with the appropriate secondary antibodies: rabbit anti-mouse (1:200, E0464; Dako) or goat anti-rabbit (1:200, BA-1000; Vector, CA, USA). Peroxidase activity was visualized with diaminobenzidine (Thermo Scientific, Rockford, IL, USA) and sections were counterstained with hematoxylin before mounting. The appropriate IgG antibodies (rabbit or mouse) were used as negative controls. The incidence of teratoma in the tissue constructs was evaluated by a pathologist (JS) in a blinded fashion.

## 3. Results

All 9 rats implanted with GFP-FA3-hiPS cells remained healthy throughout the 4 weeks experimental duration. GFP-FA3-hiPS cells implanted in the *in vivo* vascularized chamber survived through 4 weeks post-implantation and resulted in teratoma formation in 7 of 9 chambers. The tissue constructs formed all had patent vasculature, including the original implanted arteriovenous loop and the microcirculation which forms off it and fills the chamber within the first week. Substantial tissue volumes were formed (Fig. 1E and F) by cells from the host rat and integrated within them were cells of human origin, indicated by constitutive GFP expression (Fig. 1G). Although tumour formation was observed after implanting either undifferentiated (as freshly dissected pieces, Fig. 1B) or differentiated (as intact EBs, Fig. 1C) GFP-FA3-hiPS cells, chambers with differentiated GFP-FA3-hiPS cells had a higher incidence of teratoma formation (4 out of 4 chambers) compared to undifferentiated GFP-FA3-hiPS cells (3 out of 5 chambers).

Detailed immunohistochemical analysis of the teratoma tissue constructs revealed human cells representing all three germ layers, including mesoderm (Fig. 2A–F), endoderm (Fig. 2G–K) and ectoderm (Fig. 2L), as well as residual undifferentiated hiPS cells expressing pluripotency markers, OCT3/4, in close proximity to blood vessels (Fig. 1D). These pluripotent cells were retained through implantation and incorporated into tissues in the chamber by 4 weeks. Interestingly, OCT3/4 positive cells were detected in all chamber constructs including the two constructs with no evidence of teratoma tissues.

Mesoderm-derived cells included skeletal muscle (Fig. 2A), GFP-positive blood vessels (Fig. 2B), GFP-positive adipose tissues (Fig. 2C), as well as chondrocytes within developed cartilage (Fig. 2D–F). Some of the cartilaginous areas were undergoing calcification and ossification (Fig. 2D) and toluidine blue staining indicated that there was proteoglycan-containing extracellular matrix in the tumour tissues (Fig. 2F). Staining with cardiac specific marker troponin T did not detect any spontaneous cardiomyocyte differentiation in the tissue construct (data not shown). H&E staining also revealed many glandular elements lined with columnar epithelial cells (Fig. 2G–J) throughout the teratoma tissues indicating a supportive environment for endoderm differentiation of hiPS cells in the *in vivo* vascularized chambers. Some teratomas also contained extra-embryonic endodermal tissue where the glandular structures were arranged in a reticular pattern within the edematous stroma (Fig. 2I) and others contained epithelial cells with vacuolated areas within the cytoplasm (Fig 2J). The endoderm lineage was further shown by positive staining with α-fetoprotein (Fig. 2K). Ectoderm lineage of implanted GFP-FA3-hiPS cells was shown by nestin positive structures resembling neuronal cells, with clear filamentous extensions (Fig. 2L).

## 4. Discussion

In the present study, we showed for the first time that a vascularized tissue engineering chamber supports survival and differentiation of implanted GFP-FA3-hiPS cells *in vivo*. Implanting GFP-FA3-hiPS cells into the chamber has a high success rate in producing teratomas with all three germ cell lineages and multiple tissue types within 4 weeks. Furthermore, all animals in this study survived and remained healthy throughout the experimental period, indicating that the *in vivo* vascularized chamber offers a tumor-prone environment for pluripotency and safety evaluation without aggravating the welfare of host animals.

Teratoma formation in immunodeficient animals has been regarded as the gold standard assay to evaluate pluripotentiality of human pluripotent stem cells and may be further developed to assess their safety prior to clinical translation into human therapies [12]. The standard teratoma assay may involve *in vivo* administration of pluripotent cells as a single cell suspension or small pieces. Dissociating GFP-FA3-hiPS cells into single cell suspension significantly reduces cell viability and they may not survive the implantation process. Therefore, in the present study, GFP-FA3-hiPS cells were implanted as either pieces of 2D culture or EBs to maintain their cell-cell contact to prevent anoikis and thus reduce cell death. Additionally, GFP-FA3-hiPS cells were also implanted with Matrigel to further enhance cell survival and growth [7]. Interestingly, implantation of partially differentiated GFP-FA3-hiPS cells in the form of EBs resulted in a higher incidence of teratoma formation in the chamber compared to undifferentiated GFP-FA3-hiPS cells in the form of pieces. This observation seems unlikely to be explained by the pluripotency of implanted cells because previous findings demonstrated that teratoma formation from pluripotent stem cells was positively correlated with the residual presence of undifferentiated cells [13]. Another possible explanation would be survival through implantation of higher cell numbers, as suggested by previous studies reporting that efficiency of teratoma formation in the heart was dependent on implanted cell number [8,14] and the site of delivery [8]. Refinements to the present model, such as the development of a modified chamber which removes the technical requirements for microanastomoses and identification of the minimum number of cells required for a successful teratoma assay in this model are the subject of ongoing studies.

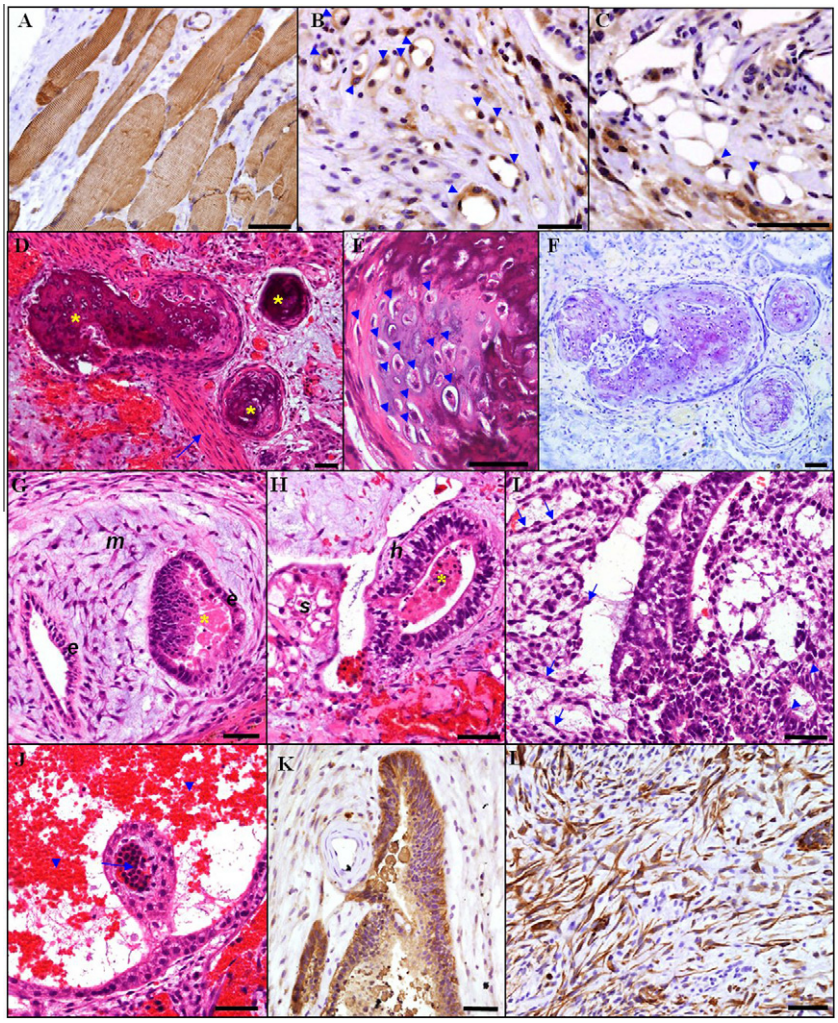


**Fig. 2.** GFP-FA3-hiPS cells differentiate into mesoderm (A–E), endoderm (G–J) and ectoderm (K) lineages. Mesoderm: (A) Representative micrograph of desmin-stained section demonstrating skeletal muscle with sarcomeric striation pattern. GFP-stained sections showing GFP-FA3-hiPS cells derived blood vessels (arrow heads in B) and adipose tissue (arrow heads in C). Representative micrographs of H&E (D and E) and toluidine blue (F) stained sections demonstrating chondrogenic and osteogenic differentiation of GFP-FA3-hiPS cells in the chambers. Some cartilaginous areas were undergoing calcification and ossification (∗ in D), connected by a bundle of smooth muscle cells (arrow in D) and containing chondrocytes (arrow heads in E). Endoderm: Representative micrograph of H&E (G–I) and α-fetoprotein (J) stained sections. (G) Glands in myxoid stroma (*m*) with epithelial structures (*e*) that contained secretory products and cellular debris within the lumen (∗). (H) Columnar epithelium resembling hair follicles (*h*) and with sebaceous glands (*s*) in close proximity. (I) Gland structures with epithelial cells arranged in a columnar pattern (arrowheads) or in a reticular pattern (arrows) within oedematous stroma. (J) Glomeruloid structures with polypoid projections into the lumen that contained mature erythrocytes (arrow heads) and immature nucleated red blood cells (arrows). The gland structure was lined with epithelial cells with vacuolated areas within the cytoplasm. (K) Gland structures were stained positively for α-fetoprotein. Ectoderm: (L) Representative micrographs of nestin-stained sections demonstrating positive cells with filamentous extensions. Scale bar = 50 μm.

The teratoma formed in the *in vivo* vascularized chamber consisted of all three germ lineages. Moreover, extraembryonic endodermal tissue, an important feature during embryogenesis, can also be detected in the teratoma, indicating yolk sac differentiation of GFP-FA3-hiPS cells in the chamber further proving the pluripotency of GFP-FA3-hiPS cells. The continued expression of cells with the pluripotency marker (OCT3/4) in close proximity to blood vessels indicated that pluripotent cells can survive *in vivo* implantation and found supportive niches in the chamber, which were able to maintain pluripotency for the duration of the assay. Furthermore, implanted GFP-FA3-hiPS cells can contribute to angiogenesis of the teratoma tissues as evidenced by GFP positive blood vessels. We did not however observe any human cardiomyocyte development in teratomas, despite this being one of the spontaneous differentiation outcomes observed with hiPS cells *in vitro*.

In the present study, GFP-FA3-hiPS cells were implanted in the groin region of nude rats but did not appear to preferentially differentiate into any specific lineage. Whether the site of construct formation will affect the type of tissue that pluripotent stem cells develop remains unclear. Studies injecting embryonic stem cells into the heart reported directed cardiomyocyte differentiation [15,16]. Similarly, the growth and composition of teratoma generated by hiPS cells has been shown to be influenced by the site of delivery [17]. In contrast, Nussbaum and colleagues did not observe that tissue environment guided differentiation in the heart [14]. Others have also reported that there were no site-specific differences in the teratoma composition with human pluripotent stem cells, suggesting a lack of microenvironment influence on their differentiation [6,7]. This tissue engineering chamber may provide the opportunity for co-implanting inductive cells, scaffolds or other instructive cues required for guided differentiation, as we

previously showed for cardiac differentiation of adult mesenchymal stem cells from adipose tissue co-implanted with donor cardiomyocytes [9].

In conclusion, implantation of GFP-FA3-hiPS cells in the *in vivo* vascularized chamber represents an effective model for teratoma formation in immunodeficient rats with turnaround time of only 4 weeks. Moreover, the teratomas were well contained and separated from the surrounding host tissues and therefore could be easily excised from the animal for analysis. Future studies to verify the consistency of this *in vivo* vascularized chamber in supporting teratoma formation with other pluripotent stem cell lines are warranted. The present approach may prove useful for testing efficiency of purification protocols, to select *in vivo* differentiated cells of interest and to remove residual pluripotent cells, in order to achieve tumor-free tissue engineering with a pluripotent donor source.

## Acknowledgments

The authors would like to acknowledge grant support from the National Health and Medical Research Council (509274, 400303), The National Heart Foundation and Friedriechs Ataxia Research Alliance. The O'Brien Institute acknowledges the Victorian State Government's Department of Innovation, Industry and Regional Development's Operational Infrastructure Support Program.

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