

REVIEW

Tumorigenicity of pluripotent stem cells: biological insights from molecular imaging

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Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the ability (i) to duplicate indefinitely while maintaining pluripotency and (ii) to differentiate into cell types of all three embryonic germ layers. These two properties of ESCs and iPSCs make them potentially suitable for tissue engineering and cell replacement therapy for many different diseases, including Parkinson's disease, diabetes and heart disease. However, one critical obstacle in the clinical application of ESCs or iPSCs is the risk of teratoma formation. The emerging field of molecular imaging is allowing researchers to track transplanted ESCs or iPSCs *in vivo*, enabling early detection of teratomas.

Keywords: stem cells; tumorigenicity; molecular imaging; teratoma; differentiation; regenerative medicine

1. INTRODUCTION

In 1981, mouse embryonic stem cells (ESCs) were first isolated from the mouse blastocyst (Evans & Kaufman 1981; Martin 1981). A decade later, human ESCs (hESCs) were isolated from the human blastocyst (Thomson *et al.* 1998). ESC lines are created from the *in vitro* culture and expansion of the inner cell mass of the blastocyst stage embryo (Evans & Kaufman 1981; Martin 1981; Thomson *et al.* 1998). Subsequent studies found that it was possible to confer totipotency or pluripotency to somatic cells by transferring the nuclear contents of somatic cells into oocytes (Wilmot *et al.* 1997), or by fusion with hESCs (Tada *et al.* 2001; Cowan *et al.* 2005). This led Takahashi and Yamanaka to believe that certain factors, important in the maintenance of ESCs, could induce pluripotency in somatic cells and create mouse induced pluripotent stem cells (iPSCs), a process termed cellular reprogramming (Takahashi & Yamanaka 2006). It was first described using the exogenous expression of four genes, including two pluripotency transcription factors (i.e. *Oct4* and *Sox2*) and two proto-oncogenes (i.e. *c-Myc* and *Klf4*; Takahashi & Yamanaka 2006). The first mouse iPSC line was created in 2006 (Takahashi & Yamanaka 2006), and a year later human iPSC lines

were created independently by James Thomson's and Shinya Yamanaka's groups (Takahashi *et al.* 2007; Yu *et al.* 2007).

The pluripotent state of hESCs and iPSCs is indicated by several cellular characteristics, including the following: (i) expression of transcription factors (e.g. *Nanog*, *Oct4* and *Sox2*), and cell surface molecules (e.g. SSEA3, SES4, Tra-1-60 and Tra-1-81), (ii) high activity of the enzymes alkaline phosphatase and telomerase, and (iii) typical cell morphology (e.g. small cells with a high nucleus to cytoplasm ratio and prominent nucleoli growing in flat colonies; Thomson *et al.* 1998; Amit *et al.* 2000; Takahashi *et al.* 2007). The gold standard for pluripotency is to demonstrate a cell line's ability to form tissue of all three germ layers by creating chimeras (Okita *et al.* 2007) or tetraploid complementation (Zhao *et al.* 2009) with mouse iPSCs, or by forming teratomas with human iPSCs. Teratomas are defined as benign germ cell tumours (GCTs), which consist of mature, well-differentiated tissues (mature teratomas), or embryonic, less-differentiated tissues (immature teratomas) with a normal karyotype (Pierce *et al.* 1960; Oosterhuis & Looijenga 2005). The ability of the first hESC line to form tissue composed of all three germ layers was demonstrated by teratoma assay in immunodeficient SCID mice. Histology showed that every mouse had developed tumours composed of gut epithelium (endoderm); cartilage, bone, smooth muscle and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified

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One contribution to a Theme Supplement 'Translation and commercialization of regenerative medicines'.

squamous epithelium (ectoderm; Thomson *et al.* 1998). This assay was later used to test pluripotency *in vivo* for human iPSCs (Takahashi *et al.* 2007; Yu *et al.* 2007).

Owing to the pluripotency of ESCs and iPSCs, they are considered to possess great potential for regenerative medicine, including possible treatment of Parkinson's disease (Yang *et al.* 2008), diabetes (D'Amour *et al.* 2006) and heart disease (Cao *et al.* 2008). Pre-clinical studies in animal models, however, have reported teratoma formation occurring upon injection of mouse ESC (mESC)-derived beating embryoid bodies (EBs; Cao *et al.* 2006; Xie *et al.* 2007) and ESC-derived neuroprogenitors (Chaudhry *et al.* 2009), highlighting a major hurdle that we have to overcome before clinical translation of pluripotent cells can be realized. The issue being addressed now is how teratoma formation occurs within differentiated ESC- or iPSC-derived cell lines. Although not fully understood, it is believed to be the result of remnant, undifferentiated cells present within the transplanted cell population. This hypothesis is further reinforced by findings that transplantation of a fairly pure hESC-derived cardiomyocyte population ($82.6 \pm 6.6\%$, range 71–95%) into immunosuppressed rats did not result in teratoma formation after four weeks (Laflamme *et al.* 2007), whereas intracardiac injection of undifferentiated mESCs into immunodeficient rats resulted in teratoma formation in all of them after only three to four weeks (Cao *et al.* 2006; Nussbaum *et al.* 2007). Taking pluripotent cells from bench to bedside will therefore partly depend on the ability to purify the cell population and to detect teratoma formation early. The latter can be achieved with the non-invasive tracking of pluripotent cells *in vivo* with molecular imaging as discussed later.

2. THE LINK BETWEEN PLURIPOTENCY AND TUMORIGENICITY

The link between pluripotency and tumorigenicity was discovered as early as the 1960s. At that time, researchers studied teratocarcinomas, the malignant form of teratomas (Pierce *et al.* 1960). The malignancy of teratocarcinomas lies in the presence of embryonic carcinoma (EC) cells, which are capable of self-renewal as well as differentiation into a variety of tissues (Andrews *et al.* 2005). Transplantation of a single EC cell resulted in the formation of a teratocarcinoma, which is composed of many differentiated tissues (Kleinsmith & Pierce 1964; Andrews *et al.* 2005). Subsequent studies showed that teratomas and teratocarcinomas could also be formed by the transplantation of normal mouse embryos or embryonic genital ridges to the adult host, demonstrating that tumour-initiating cells were also present within normal embryos (Stevens & Hummel 1957; Stevens 1964, 1967, 1968). When the first hESCs were isolated in 1998, similarities were observed between the newly isolated ESCs and EC cells, including the expression of the pluripotency markers (e.g. SSEA3, SSEA4, TRA 1–60, TRA 1–81, telomerase and alkaline phosphatase) and the ability to form teratomas in

immunodeficient mice (Thomson *et al.* 1998). Furthermore, Andrews and co-workers hypothesized that EC cells might have a stronger selection for mutations that would increase self-renewal capacities and limit differentiation. Similarly, mutations have been observed when growing hESCs in culture over longer passages (Andrews *et al.* 2005). For example, unbalanced chromosomal translocation involving the long arm of chromosomes 1 and 9, resulting in karyotypic change 46,XX,der(9)t(1;9)(q31;q22), has been reported in H9 cells at passage 120 compared with passage 48 (Xie *et al.* in press). Passaging of three subcultures of hESC line H7 (H7.S0, H7.S6, H7.S9) and the hESC line H14 over a period of several months resulted in a gain of the complete long arm of chromosome 17 (17q; Draper *et al.* 2004), which is also very commonly seen in EC cells derived from teratocarcinomas (Andrews *et al.* 2005). In addition to a gain of chromosome 17, a gain of chromosome 12 has also been observed in a few subpopulations of these cells. The pluripotency marker *Nanog* is sited on this chromosome (Draper *et al.* 2004). The gain of one or more isochromosomes on the short arm of chromosome 12 is associated with testicular GCTs in humans (Mostert *et al.* 1998). Further evidence for the role in chromosome 12 in the tumorigenicity of pluripotent cells was provided by Sperger and co-workers, who compared the gene expression of five hESC lines, 36 GCT cell lines, 14 samples of normal testis and 17 somatic cell lines. Significance analysis of microarrays in this study identified 1760 cDNAs in ESCs, 1299 cDNAs in EC cells and 1518 cDNAs in seminomas that were significantly more highly expressed than the control group of differentiated cells. They found 330 genes that were shared by ESCs, EC cells and seminomas, and among these genes was *POU5F1* (*Oct4*). Furthermore, genes sited on chromosome 12 were highly represented ($p < 0.00001$) among the genes highly overexpressed in EC cells when compared directly with ESCs (Sperger *et al.* 2003).

The molecular basis of the tumorigenicity of pluripotent cells lies in their cancer-resembling properties, namely their ability to self-renew and proliferate, which are promoted by several molecular processes and have been described in detail by Blum & Benvenisty (2008, 2009). These properties include self-renewal, rapid proliferation, lack of contact inhibition and telomerase activity (figure 1). On closer examination of the karyotype of ESCs, polyploidy has been observed in mESCs despite an intact spindle assembly checkpoint (SAC; Malashicheva *et al.* 2002). Mantel and co-workers demonstrated that mESCs and hESCs have an intact SAC, but that mitotic failure-induced polyploidy does not lead to apoptosis of these cells (Mantel *et al.* 2007). Mitotic errors often occur in rapidly proliferating cells and the SAC, typically leading to apoptosis of these cells and the necessity for genome maintenance. Uncoupling of this mechanism will lead to karyotypic abnormalities in ESC culture (figure 1). In addition, when inducing double-strand breaks in the DNA of mouse ESCs and mouse EBs by the DNA-damaging agent etoposide, ESCs were resistant to apoptosis whereas the EBs underwent

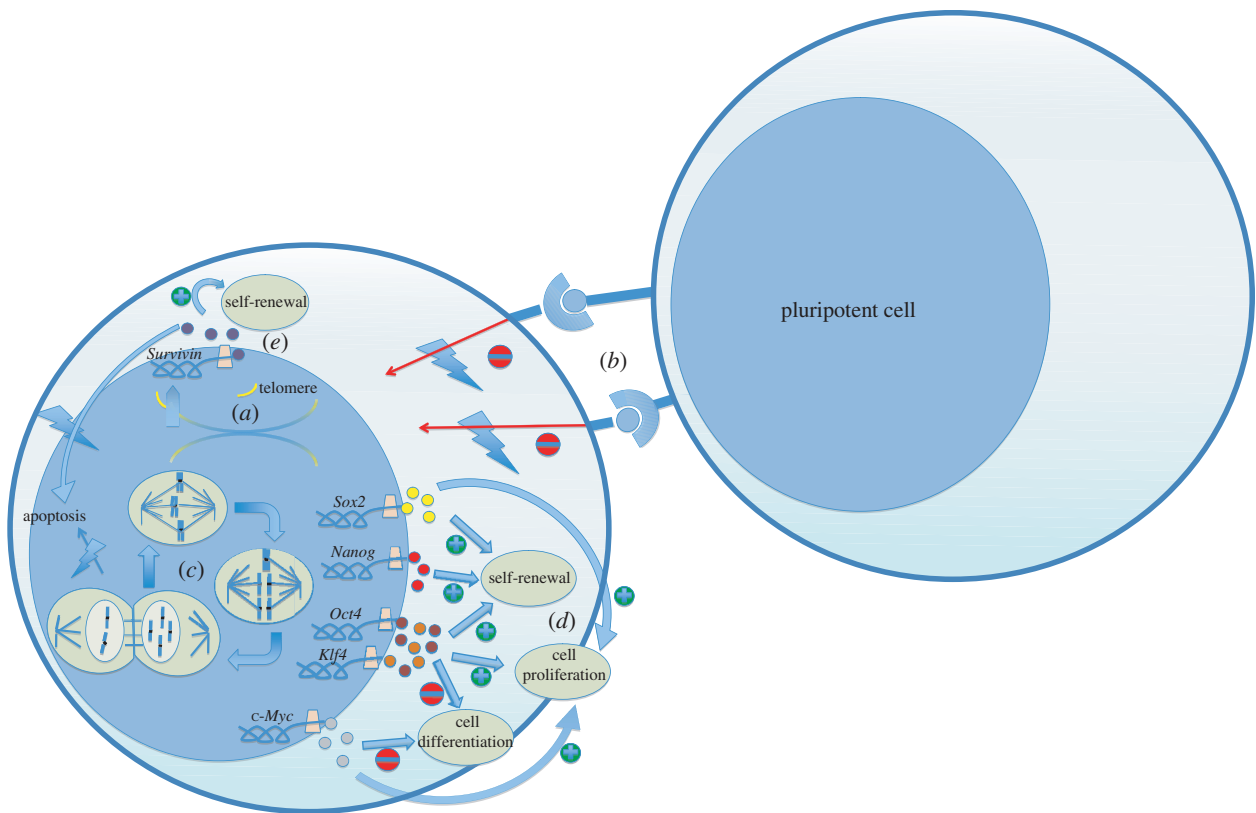


Figure 1. The molecular basis of the tumorigenicity of pluripotent cells. (a) Increased telomerase activity results in extended lifespan of the pluripotent cells by adding telomere ends to the chromosomes. (b) Lack of contact inhibition allows for proliferation and cell growth, even if pluripotent cells are in contact with neighbouring pluripotent cells. (c) Spindle assembly checkpoint (SAC) uncoupling results in failure to induce apoptosis of ESCs with karyotypic abnormalities such as mitotic failure-induced polyploidy. (d, e) Overexpression of the pluripotency markers leads to a highly proliferative, undifferentiated and self-renewal state. *Survivin* (*BIRC5*), in addition to the self-renewal properties, functions as an anti-apoptotic gene.

caspase-3-dependent apoptosis. This suggests that polyploidy tolerance in ESCs will give way to apoptosis upon lineage-specific differentiation. This hypothesis has been confirmed for mouse as well as human mononuclear polyploidy/aneuploid cells, indicating that checkpoint–apoptosis uncoupling is an intrinsic behaviour of mESCs and hESCs (Mantel *et al.* 2007).

The examination of gene expression in several human tumours has shown that the pluripotency markers used in reprogramming somatic cells to iPSCs are directly oncogenic (e.g. *c-Myc* and *Klf4*) or are involved in tumorigenesis (e.g. *Sox2*, *Nanog*, Knoepfler (2009); and *Oct3/4*, Palma *et al.* (2008)) Targets of *Nanog*, *Sox2* and *Oct4* that encode for transcription regulators, are overexpressed and prove especially active in high-grade breast tumours (Ben-Porath *et al.* 2008). Upregulation of *Nanog* and *Oct4* is also associated with poor outcomes in patients with oral cancer (Chiou *et al.* 2008). The pluripotent genes *Oct3/4*, *Sox2*, *Nanog*, *c-Myc* and *Klf4* were also present in prostate tumour cell lines, as well as in primary prostate tumour tissue. Furthermore, injection of these tumour cells containing the pluripotent genes created strong tumorigenicity in immunodeficient mice (Bae *et al.* 2010).

Using a mouse model in which *Oct4* could be activated by doxycycline administration, Hochedlinger and co-workers evaluated the effects of *Oct4* overexpression in somatic differentiation and tumorigenesis. *Oct4*-induced dysplasia of the epithelial tissues could

be seen as early as 5 days after initiating doxycycline. Dysplasia was only seen in the epithelial progenitor and/or stem cells, and *Oct4* overexpression had no effect on the cellular phenotype of differentiated cells. Withdrawal of doxycycline caused a complete reversal of the tumour phenotype, again showing the association of *Oct4* with tumorigenesis (Hochedlinger *et al.* 2005).

The biological link between pluripotency and tumorigenicity can perhaps best be described by *c-Myc*. Although not required for the induction of pluripotency in iPSCs, *c-Myc* augments the reprogramming ability of *Oct4*, *Sox2* and *Klf4* (Takahashi *et al.* 2007), inhibits differentiation and promotes proliferation (figure 1). Myc proteins have been shown by Eilers & Eisenman (2008) to influence several thousand genes. The majority of these are upregulated and involved in cell growth; the few genes that are downregulated are involved in cell cycle arrest, cell adhesion and cell–cell communication. The influence of *Myc* on these nuclear processes is oncogenic as shown by the many tumours described in this review (Eilers & Eisenman 2008). However, not all members of the *Myc* family possess the same properties. *L-Myc* for instance has a decreased transformation activity and only few human cancers are associated with an aberrant expression of *L-Myc*. A recent study reported the creation of iPSCs with *L-Myc* instead of *c-Myc* and chimeric mice derived from these *L-Myc* iPSCs showed reduced tumorigenicity (Nakagawa *et al.* 2010).

Recently, 21 genes were found to be highly expressed in hESCs as well as in teratomas (Blum *et al.* 2009). These genes were then credited as known oncogenes, if they were involved in cell-cycle progression, inhibition of apoptosis, signal transduction, transcription or translation. Of these 21 genes, *Survivin* (*BIRC5*) was found to be the strongest candidate gene. *Survivin* is an anti-apoptotic gene that is also involved in mitotic regulation and is expressed in the majority of cancers (Ambrosini *et al.* 1997; Li *et al.* 1998; figure 1). It was highly expressed in hESCs and teratomas and downregulated in mature EBs (Blum *et al.* 2009). From these insights into the gene expression of hESCs and iPSCs, it can be concluded that tumour formation is not dependent on the presence of EC-like cells, but is more an intrinsic property of pluripotent cells. Furthermore, this tumorigenicity of pluripotent cells is reduced upon differentiation. The dilemma now facing investigators is that in reducing the tumorigenicity of stem cells, the very essence of stem cells that makes them useful also has to be reduced, namely self-renewal and pluripotency.

3. MOLECULAR IMAGING FOR TRACKING CELL FATE

In the last few decades, the field of medical imaging has advanced enormously, giving rise to different *in vivo* imaging technologies such as magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), single photon emission computed tomography (SPECT), ultrasound and the charge-coupled device (CCD) camera (Blasberg & Tjuvavej 2003). These modalities have been used in the field of nuclear medicine, in which radiolabelled imaging probes are injected into living subjects to provide information about structure and functionality of different organ systems. Although the field of nuclear medicine gave physicians non-invasive insights into the anatomy of organs, a better understanding of changes in biological processes at the cellular and molecular levels in disease was needed. This need gave rise to the field of molecular imaging, in which the interaction of an imaging probe with a molecular target (DNA, RNA or protein) can be detected using the appropriate imaging system.

In addition to cardiovascular, neuronal and oncological applications, molecular imaging is also used in tracking the survival and proliferation of stem cells *in vivo*. For the latter purpose, two approaches can be used to label the stem cells before transplantation. The first approach is directly labelling the cells by incubation with a contrast agent such as nanoparticles, magnetic particles or radionuclides. The second approach is by a reporter-gene construct transferred into the cells by a vector-delivery system. Transcription and translation of the reporter gene will result in a reporter protein that can either be an intracellular enzyme, a cell surface receptor, a transmembrane protein or an intracellular storage protein.

3.1. Nanoparticle imaging

Semiconductor quantum dots (QDs) can be manipulated to emit different wavelengths of light and are

very photostable, making them interesting options for *in vivo* tracking of stem cells. In addition, QDs provide brighter and more stable signals for molecular and cellular imaging (Dubertret *et al.* 2002; Jaiswal *et al.* 2003; Lin *et al.* 2007). However, QDs tend to aggregate in the cytosol, are difficult to deliver to the cells and have non-specific binding to multiple molecules.

3.2. Iron particle imaging

The monitoring of stem cells with MRI is mostly based on endocytosis of a contrast agent such as superparamagnetic iron oxide particles (SPIOs) or ultrasmall superparamagnetic iron oxide particles (USPIOs) that can elicit changes in T_2 relaxivity, which allows their detection *in vivo*. Usage of MRI for the tracking of mesenchymal stem cells labelled by furomoxides has been described by several groups (Kraitchman *et al.* 2003; Amado *et al.* 2005; Arai *et al.* 2006). MRI signals in these studies could be detected from three weeks to two months. However, one concern is that the MRI signals can still be present even when the transplanted cells are no longer alive. This is because the iron particles can be engulfed by nearby cells, especially macrophages (figure 2; Li *et al.* 2008).

3.3. Radionuclide imaging

Stem cell imaging by directly labelling cells with radionuclides is the only direct labelling approach that has been used in human studies, using either bone marrow-derived stem cells (Hofmann *et al.* 2005) or circulating progenitor cells (Kang *et al.* 2006; Schachinger *et al.* 2008). Tracking these cells using SPECT, PET and gamma camera imaging is realized by a labelling agent that is introduced into the cells of interest before transplantation. The major advantage of radionuclide imaging over MRI is its higher imaging sensitivity. However, these cells can only be tracked for several hours to days, depending on their individual decay half-lives (e.g. ^{99m}Tc : 6 h; ^{111}In : 2.8 days; ^{18}F : 109 min). Another problem is that radionuclides can leak into other non-target cells and hence give rise to false-positive signals.

3.4. Reporter gene imaging

Reporter gene imaging is based on a reporter gene linked to a promoter/enhancer. These DNA sequences are transcribed into mRNAs and will lead to the production of reporter proteins that will interact with exogenously administered reporter probes. This reaction will lead to a detectable signal, which can be visualized by a CCD camera, SPECT, PET or MRI. As mentioned earlier, this can be (i) an intracellular enzyme that interacts with an exogenous reporter probe to give rise to a detectable signal (Cao *et al.* 2006), (ii) a cell surface receptor that binds to an exogenously delivered probe (e.g. dopamine 2-like receptor; MacLaren 1999), (iii) a transmembrane protein that mediates in the intracellular uptake of radioisotopes (e.g. sodium/iodide symporter; Miyagawa *et al.* 2005), or (iv) an intracellular storage protein that actively concentrates

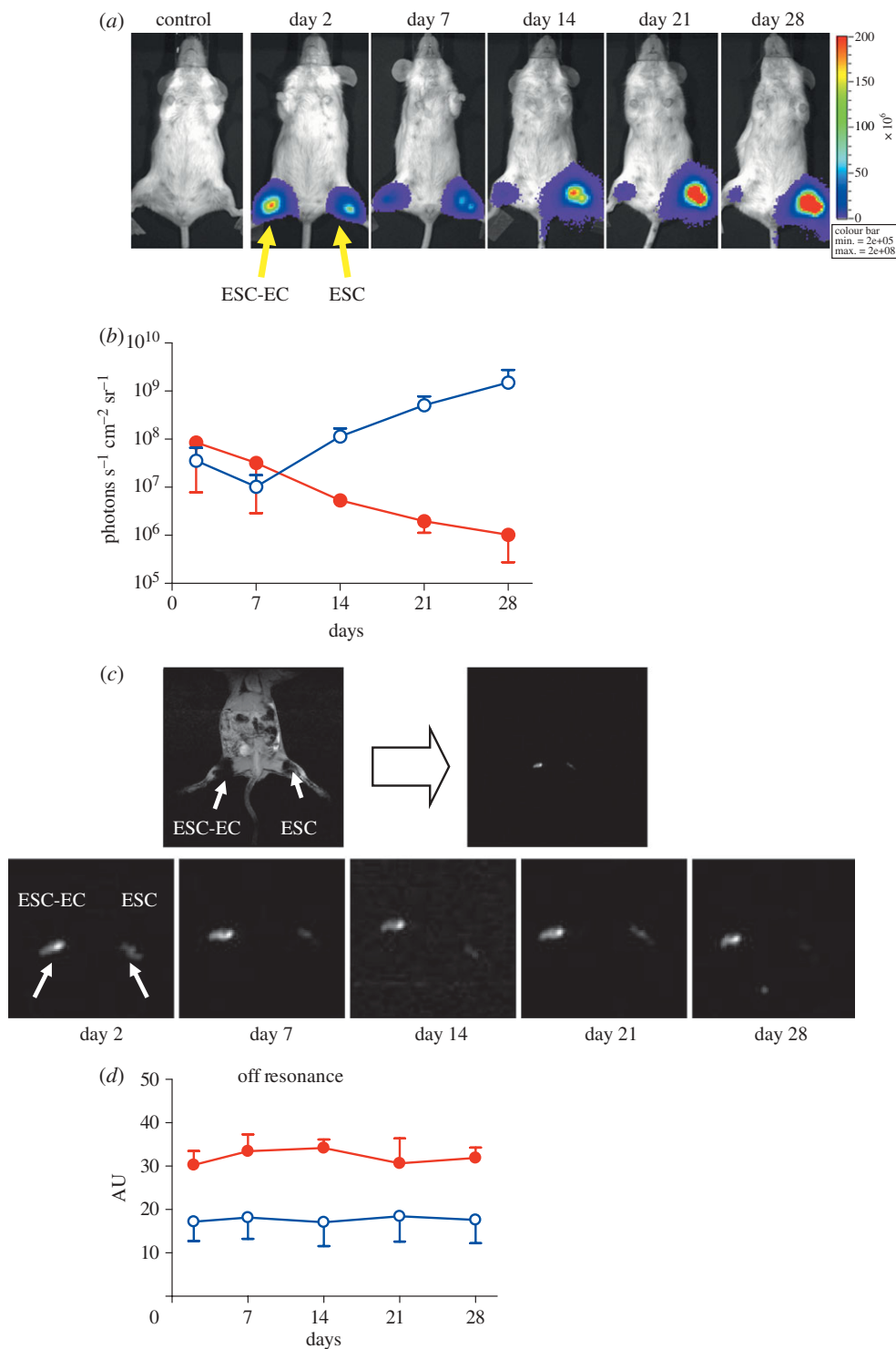


Figure 2. Comparison of physical labelling versus reporter gene labelling for tracking cell fate. Human ESCs were stably transduced with Fluc-eGFP double-fusion reporter gene as well as incubated with superparamagnetic iron oxide (SPIO). Immunodeficient SCID mice were injected with either undifferentiated ESCs or ESCs differentiated into endothelial cells (ESC-EC). (a) Representative animal injected with 1×10^6 ESC-ECs (right hind limb) shows significant bioluminescence activity at day 2, which decreases progressively over the next four weeks owing to acute donor cell death. In contrast, undifferentiated ESCs (left hind limb) proliferated dramatically over four weeks owing to teratoma formation. (b) Detailed quantitative analysis of bioluminescence signals from all animals transplanted with hESCs (blue open circles) versus hESC-ECs (red filled circles). Signal activity is expressed as photons $s^{-1} cm^{-2} sr^{-1}$. Note that the *y*-axis is shown as a logarithmic scale. (c) Representative *in vivo* gradient-recalled echo (GRE) imaging of the animals as shown in (a) and (b). MR signals showed no significant difference between undifferentiated ESCs and differentiated human ESC-ECs from day 2 to day 28. MR image by GRE at day 28 shows bulking expansion of the left hind limb injected with undifferentiated ESCs owing to teratoma formation (arrow head). (d) Detailed quantitative analysis of GRE signals from all animals transplanted with hESCs (blue open circles) and hESC-ECs (red filled circles) (signal activity is expressed as authority unit (AU)). The persistent MRI signal is owing to macrophages engulfing SPIO particles from transplanted cells that have died. Overall, unlike reporter gene imaging, MRI was *unable* to distinguish cell viability from cell proliferation as its signals remained relatively constant over the four-week time span. Reprinted with permission from Li *et al* (2008).

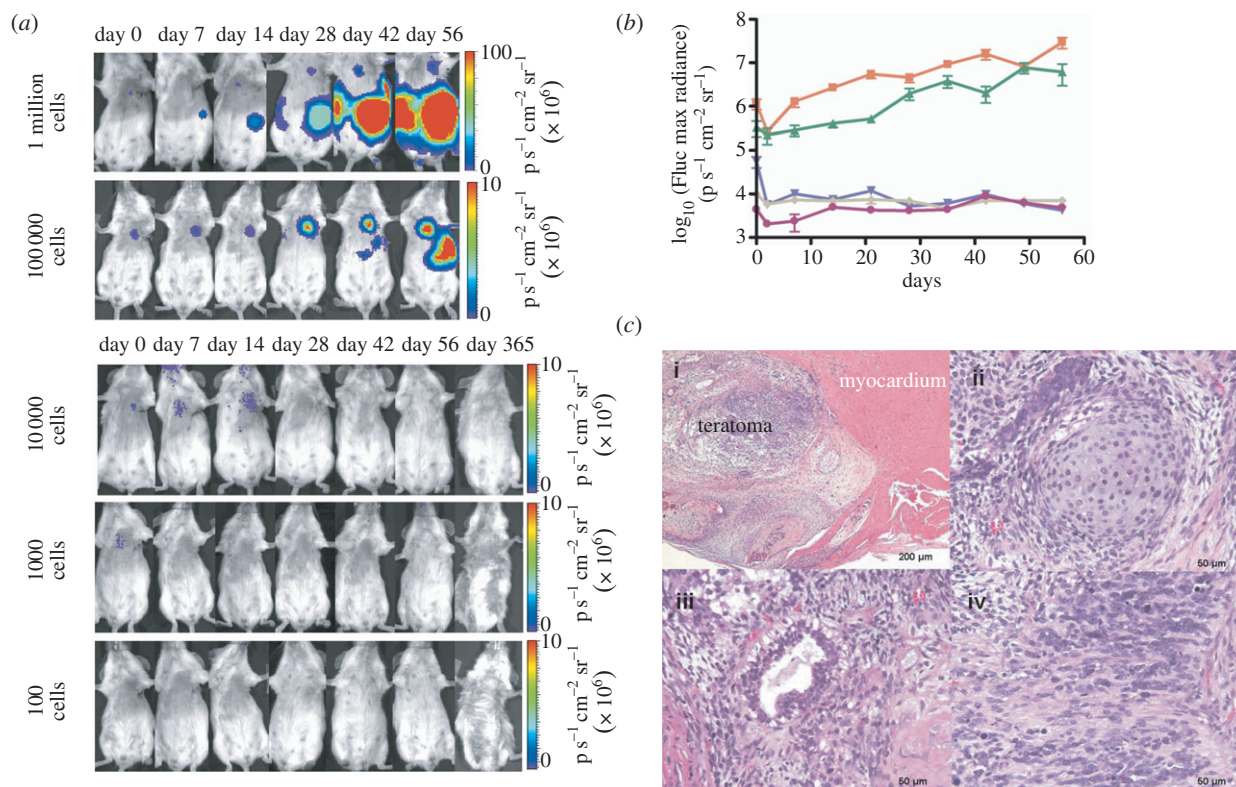


Figure 3. Potential for varying numbers of hESCs to form teratomas following intramyocardial injection. (a) Different numbers of undifferentiated Fluc-eGFP-positive hESCs were injected intramyocardially into SCID mice. BLI reveals tumour development in animals transplanted with 1×10^5 or 1×10^6 cells but not in animals receiving 1×10^4 cells or less. (b) Quantitative analysis of the kinetics of teratoma development using BLI. Orange squares, 1 million; green triangles, 100 000; violet inverted triangles, 10 000; grey diamonds, 1000; pink circles, 100. (c) Representative hematoxylin and eosin staining of intramyocardial teratoma: (i) low-power view of intramyocardial teratoma, (ii) cartilage (mesoderm), (iii) mucinous glandular epithelium (endoderm), and (iv) neural tissue (ectoderm) confirm the presence of cellular derivatives from all three germ layers. Reprinted with permission from Lee *et al.* (2009).

endogenous contrast elements (e.g. intracellular iron in ferritin-bound form; Liu *et al.* 2009a).

For small animal imaging, several reporter gene constructs have been developed. A double-fusion construct containing the firefly luciferase (*Fluc*) and enhanced green fluorescence protein (*eGFP*) reporter gene has been used in several studies (van der Bogt *et al.* 2006; Cao *et al.* 2007; Lee *et al.* 2009). In these studies, the Fluc enzyme interacts with the reporter probe D-luciferin to generate low-energy photons (2–3 eV) that can be detected by an ultrasensitive CCD camera. In addition, the eGFP allows confirmation of *in vivo* imaging signals with traditional *ex vivo* post-mortem histology. A triple-fusion construct for monitoring the *in vivo* survival, proliferation and migration of mESCs after intramyocardial transplantation was reported by Cao *et al.* (2006). This triple-fusion construct used Fluc for high-throughput bioluminescence imaging (BLI), monomeric red fluorescent protein (mRFP) for fluorescence-activated cell sorting and fluorescence microscopy, and herpes simplex virus truncated thymidine kinase (HSVtk) for deep tissue PET imaging (Cao *et al.* 2006). For PET reporter gene imaging, the HSVtk phosphorylates its radioactive substrate, 9-(4-[^{18}F]-fluoro-3-[hydroxymethyl]butyl)guanine ([^{18}F]-FHBG), intracellularly. The [^{18}F] generates two 511 keV photons that can be detected by PET.

Compared with BLI, PET imaging provides higher anatomical details and allows imaging in large animals and humans (Yaghoubi *et al.* 2009).

Finally, it should be mentioned that stable integration of the various reporter genes (*Fluc*, *eGFP*, *mRFP* and *HSVtk*) into cellular chromosomes allows the mother cell to pass on the reporter genes to daughter cells. Hence, this genetic approach can truly evaluate survival and proliferation, unlike physical approaches such as iron particle, QD or radionuclide labelling. However, the main disadvantage here is the risk of altering cellular behaviours from gene insertion. Although previous genomic (Wu *et al.* 2006a) and proteomic (Wu *et al.* 2006b) studies have not shown any significant adverse effects of mouse ESCs expressing the triple-fusion reporter genes, further optimization using safer site-specific integration approaches is probably needed in the future (Keravala *et al.* 2009).

3.5. Imaging for tumorigenicity

Several imaging modalities have been described here to track the survival and proliferation of pluripotent cells. Owing to the apparent risk of teratoma formation after transplantation of pluripotent cells, it is imperative that molecular imaging be used to detect these tumours at an early stage. In this pre-clinical setting of

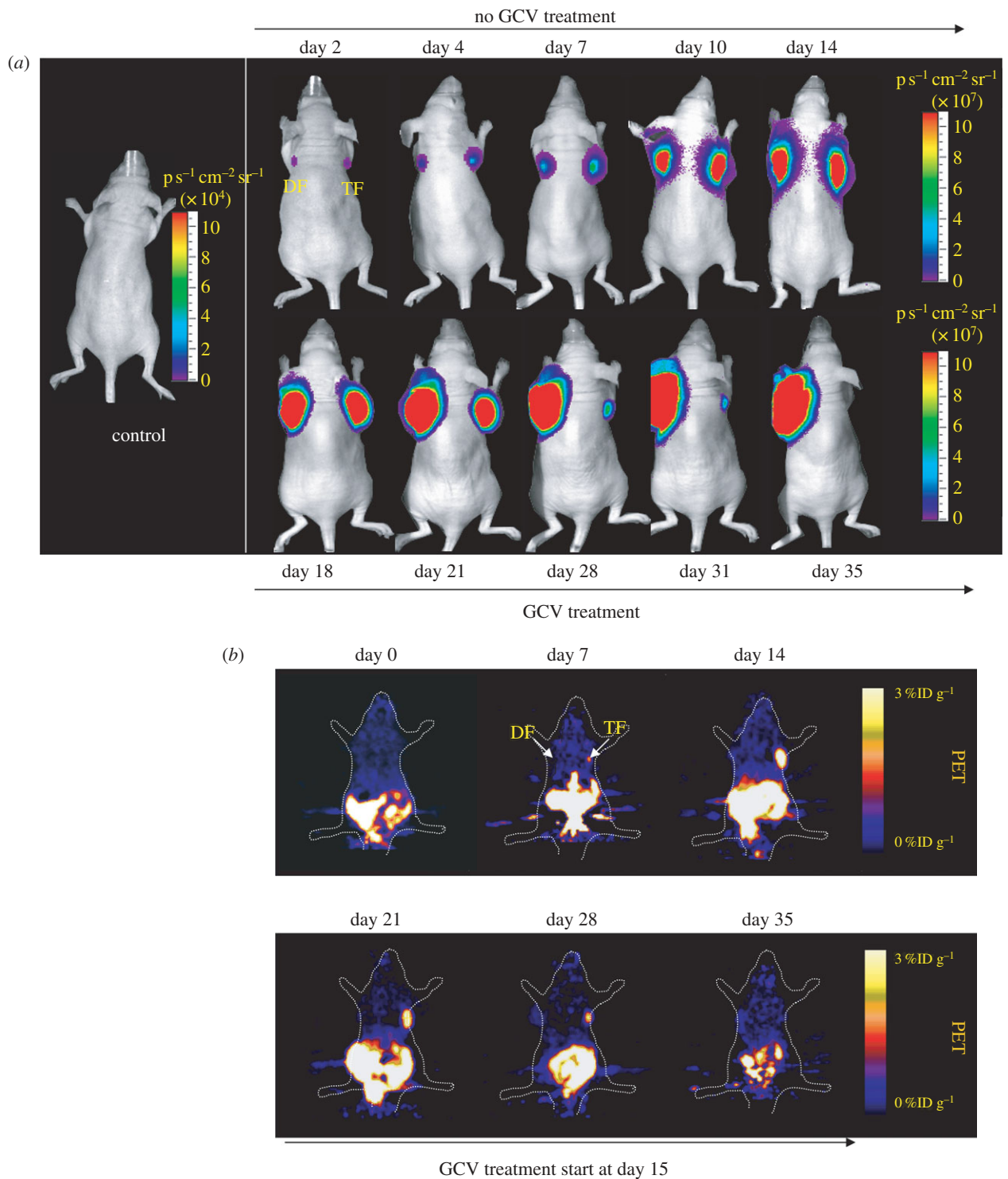


Figure 4. Ablation of teratoma formation using *HSVtk* reporter and suicide gene. mESCs were stably transduced with a triple-fusion (ESC-TF) reporter gene that consisted of monomeric red fluorescence protein (*mRFP*), *Fluc* and *HSVtk* or a double-fusion (ESC-DF) reporter gene that consisted of enhanced *GFP* and *Fluc*. Stably transduced ESC-TF and ESC-DF cells (1×10^6 each group) were transplanted into right and left shoulders of adult nude mice, respectively. (a) BLI and (b) PET imaging were performed at various time points using D-luciferin and ^{18}F -FHBG reporter probes, respectively. The marked difference in PET activities is consistent with the expression of *HSVtk* reporter gene in ESC-TF cells but not ESC-DF cells. Ganciclovir (GCV) treatment was started at week 2 and continued until week 5. After three weeks, ESC-TF cells (right shoulder) were completely ablated by GCV treatment, whereas ESC-DF cells were not affected as they lack *HSVtk*. Reprinted with permission from Cao *et al.* (2007).

transplanting pluripotent cells, several groups have looked at the different imaging modalities for the detection of tumour formation in small animals. BLI of mice and rats using *Fluc* is most commonly used for tumour

detection, because it is easy to use and very sensitive (figure 3). Teratoma formation can be detected by a strong BLI signal, even before a palpable tumour is formed (van der Bogt *et al.* 2006; Lee *et al.* 2009).

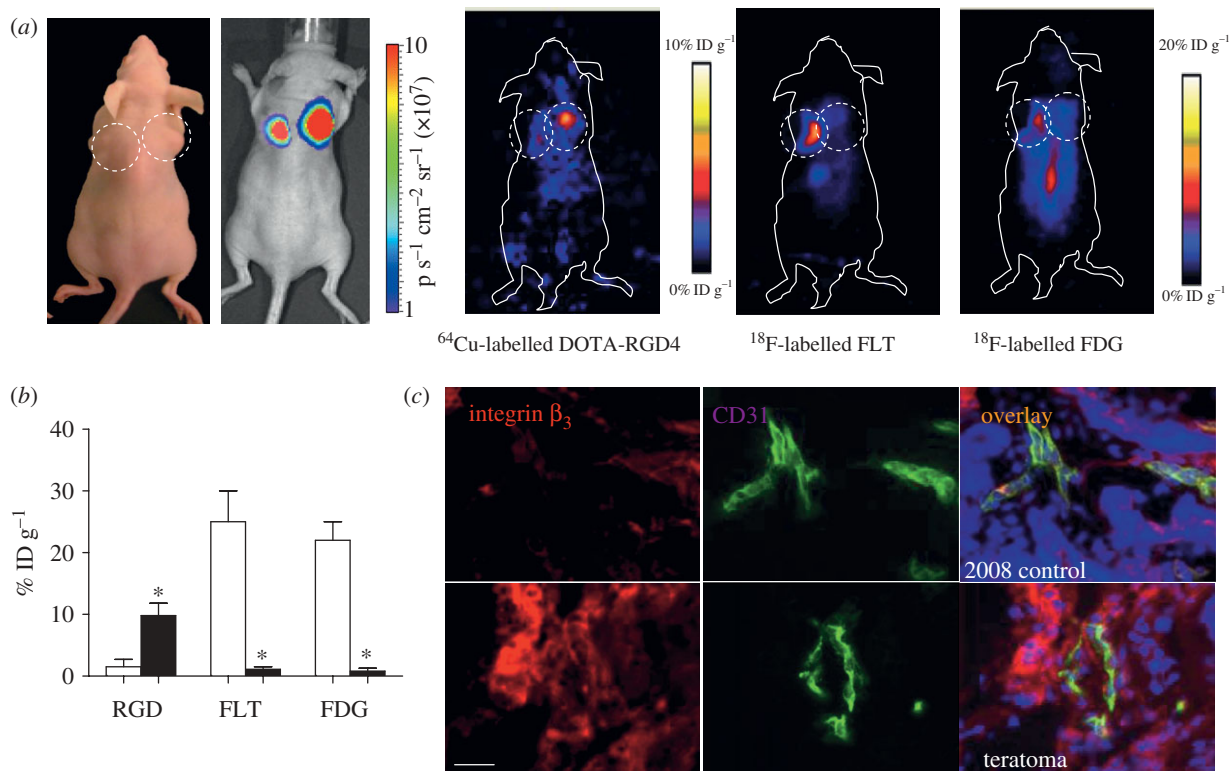


Figure 5. Non-invasive de novo imaging of hESC-derived teratoma formation. MicroPET imaging of 2-deoxy-2- ^{18}F fluoro-D-glucose (^{18}F -FDG), 3'-deoxy-3'- ^{18}F -fluorothymidine (^{18}F -FLT) and ^{64}Cu -DOTA-RGD4 on ESC-derived teratoma. (a) Static photograph and bioluminescence imaging show teratoma formation by H9 hESC-DF cells at the right shoulder and tumour formation by the control 2008 cell line at the left shoulder, which were confirmed by bioluminescence imaging ($7.3 \pm 2.5 \times 10^7$ versus $4.5 \pm 1.6 \times 10^7$ photons $\text{s}^{-1} \text{cm}^{-2} \text{sr}^{-1}$, respectively, $p = \text{n.s.}$). Specific and prominent uptake of ^{64}Cu -DOTA-RGD4 was found in vascularized teratoma, but not in control human ovarian carcinoma cell line (2008) tumour with low integrin expression ($p < 0.01$). By contrast, hESC-derived teratomas had low uptake of ^{18}F -FDG and ^{18}F -FLT, whereas the 2008 ovarian cancer xenografts had both high ^{18}F -FDG and ^{18}F -FLT uptake. (b) Quantification of different microPET imaging patterns between ESC-derived teratoma (filled bars) and control human ovarian carcinoma (unfilled bars). * $p < 0.01$. (c) Higher expression of β_3 integrin (red) was observed in ESC-derived teratoma (bottom) than that in control 2008 tumour (upper), which can be partly co-localized with CD31 staining (green; blue: DAPI; scale bar = 50 μm). Reprinted with permission from Cao *et al.* (2009).

Using BLI, Lee *et al.* have found that they could inject a maximum of 1×10^3 undifferentiated ESCs in the skeletal muscle and 1×10^4 undifferentiated hESCs in the cardiac muscle of immunodeficient mice without teratoma formation (Lee *et al.* 2009). These findings provided important pre-clinical insights into the effects of hESC numbers and local niches on teratoma development as well as the kinetics of teratoma formation. In addition, BLI together with PET imaging have provided insights into teratoma ablation using a reporter–suicide gene construct (figure 4; Cao *et al.* 2007). However, *Fluc* is not suited for clinical application because of the limited penetration of the signal through tissue in larger animals. Furthermore, BLI only gives a two-dimensional image and reveals little spatial information. In contrast, MRI and PET provide a higher spatial resolution and can be used in clinical imaging (Kraitchman *et al.* 2003; Hinds *et al.* 2003; Yaghoubi *et al.* 2009), but with significantly lower detection threshold. Taking into account that every imaging modality has its advantages and drawbacks, combining two or several imaging modalities may provide a better solution. This multimodality approach together with the continuing technological advances will help

scientists take tissue engineering and cell-replacement therapies into the clinical phase in the near future.

4. DISCUSSION

The field of regenerative medicine using pluripotent stem cells holds great promise if clinical hurdles can be overcome. One significant problem is the tumorigenic property of pluripotent cells. This is highlighted by a recent case report involving a child that received foetal neural stem cells as a treatment for the neurodegenerative disease ataxia telangiectasia but later developed multifocal glioneural tumour from these transplanted neural stem cells (Amariglio *et al.* 2009). As gene expression of pluripotent cells becomes widely investigated, more and more correlations in gene expression between pluripotent cells and cancer cells are being found. Not only are some of the pluripotent markers used in the creation of iPSCs directly oncogenic, other pluripotent genes are found to be highly associated with tumour differentiation as well.

Because of the tumorigenicity of pluripotent cells, tracking them *in vivo* is of high importance for future

clinical application, and several imaging modalities can be used for this purpose. To make regenerative medicine therapies safe, one option is to use a reporter–suicide gene mechanism that would allow for the *in vivo* tracking of the transplanted pluripotent cells and would target these cells for elimination in the case of tumour formation (figure 4; Cao *et al.* 2007). Although this study showed ablation of teratomas formed from ESCs, the construct is still based on genetically modifying the pluripotent cells by viral transduction, which has tumorigenic potential as well. Future approaches therefore should focus on site-specific genomic integration approaches such as zinc finger nuclease (Hockemeyer *et al.* 2009) or phiC31 integrase (Liu *et al.* 2009b) to minimize potential adverse effects to the cells. Alternatively, one could bypass the reporter gene technique by designing molecular probes that target cell surface receptors of teratoma, as was recently demonstrated using ⁶⁴Cu-labelled RGD tetramer that targets $\alpha\beta 3$ integrin receptors on hESC-derived teratomas (figure 5; Cao *et al.* 2009).

Ultimately, clear insights into the gene expression of pluripotent cells will allow researchers to select a pluripotent cell population with a reduced tumorigenic signature. In addition, the advances in the field of molecular imaging will allow for (i) labelling of these cells without adverse effects to cellular function, (ii) tracking them *in vivo* with high sensitivity, spatial resolution, and over long periods of time without loss of signal (Thomson *et al.* 1998), and (iii) targeting these cells upon tumour formation. These exciting novel developments will pave the way for safe regenerative medicine.

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