Immunogenicity and tumorigenicity of pluripotent stem cells

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Part I

Regenerative therapies

Chapter 2

Tracking gene and cell fate

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SUMMARY

The preclinical intersection of molecular imaging and gene- and cell-based therapies will enable more informed and effective clinical translation. We discuss how imaging can monitor cell and gene fate and function *in vivo* and overcome barriers associated with these therapies.

In recent years, impressive progress has been made in the bench-to-bedside translation of gene and cell-based treatments to address a wide range of pathologies in preclinical and clinical settings. Similar advances in bioimaging have provided powerful tools to monitor their *in vivo* fate and function. Here, we provide an overview of relevant gene and cell-based therapies and highlight the applications of molecular imaging technologies to evaluate graft function, regulation and interaction with host tissue. Moreover, we emphasize particular strategies to facilitate the continued, collaborative synergy between molecular imaging technologies and these therapies, which will expedite their assessment and development.

CLINICAL AND PRECLINICAL STUDIES

Significant progress has been made in clinical and preclinical arenas towards the development of gene and cell therapies.

Corrective gene therapy

Gene and stem cell-based therapies have the potential to treat a variety of diseases. Investigators have successfully illustrated the principle of isolating, engineering and re-introducing a 'corrected' graft for a variety of diseases with lineage-restricted phenotypes, including X-linked (Hacein-Bey-Abina et al. 2002) and adenosine deaminase deficient (Aiuti et al. 2009) severe combined immunodeficiency disease, chronic granulomatous disease (Ott et al. 2006), adrenoleukodystrophy (Cartier et al. 2009) and Wiskott–Aldrich syndrome (Boztug et al. 2010). These therapies, largely limited experimentally to retroviral insertion of the corrected gene product in autologously derived hematopoietic stem cells (HSCs), have been met with widely publicized and appropriately directed concerns regarding their safety, despite encouraging demonstrations of phenotype correction. Follow-up reports have shown leukemic (Hacein-Bey-Abina et al. 2003) and pre-leukemic (Stein et al. 2010) induction, clonal T-cell expansion (Howe et al. 2008), and genomic instability (Stein et al. 2010) secondary to retroviral-mediated insertional mutagenesis in or near proto-oncogenes. Such events, as a consequence of untargeted genome editing, served as an impetus for the temporary ban by the Food and Drug Administration on gene therapy in 2002. The subsequent lifting of the ban in 2003 heralded a more skeptical and slow-progressing era that has continued to the present, leaving the field yet to realize its full potential.

Allogeneic stem cell transplantation

Although the aforementioned near pause in gene therapy led to more cautious development in this arena, interest in autologous or allogeneic stem cell-based approaches strengthened significantly. However, despite wide interest in the use of bone-marrow-derived mesenchymal stem cells (MSCs) for a range of regenerative therapies, including those for inflammatory (Griffin et al. 2013), joint (Barry and Murphy 2013) and cardiac diseases (Sheikh et al. 2012), among others, questions regarding the clinical efficacy of various stem cell protocols remain. In addition to marginal improvement observed in several stem cell trials, there is also evidence of detrimental side effects as seen with skeletal myoblast therapy for cardiac repair (Menasche et al. 2008). The discrepancy between the more definitive preclinical success of stem cell therapies and their less promising early clinical results may be partly attributed to a lack of knowledge regarding in vivo graft behaviour.

Promising new therapeutic products are now emerging, in particular those making use of human embryonic stem cell (hESC) and induced pluripotent stem cell (iPSC) derivatives. These include the now defunct Geron trial using allogeneic hESC-derived oligodendrocyte

progenitor cells for spinal cord repair (Strauss 2010), the Advanced Cell Technology trial using hESC-derived retinal pigment epithelium cells (RPEs) for Stargardt's macular dystrophy (Schwartz et al. 2012), and the RIKEN Japan trial using autologous iPSC-derived RPEs for age-related macular degeneration (Cyranoski 2013). As with earlier somatic cell therapies, pluripotent stem cell therapeutics will also need to be extensively tested and evaluated by bioimaging technologies to better understand their fate in vivo.

Genetically engineered stem cells

With the goal of optimizing clinical impact, the most promising approaches combine gene and cell therapy to deliver a corrected or beneficial gene in a therapeutically relevant cell. As an emerging paradigm, T-cell immunotherapy offers hope for more targeted chemotherapy by genetically instructing T-cell trafficking, direction, or redirection towards tumor cells, with the potential to engineer bispecific T cells with engineered proliferation and antitumor specificities (Kershaw et al. 2002). A noteworthy, single- patient-case example of using positron emission tomography (PET) imaging to track the fate of genetically labelled and therapeutically manipulated cytolytic T lymphocytes (CTLs) has demonstrated a valuable platform for integrating gene and cell therapy with molecular imaging (Yaghoubi et al. 2009). In this report, CTLs labelled with the PET reporter gene herpes simplex virus thymidine kinase (HSV-tk) were infused intracranially after resection of a glioblastoma multiforme. PET imaging after administration of the PET reporter probe 9-(4-[18F]-fluoro-3-hydroxymethylbutyl)- guanine ([18F]FHBG) revealed not only accumulation of the engineered CTLs in the patient's primary tumor but also homing to another metastatic site.

In addition to studying the in vivo fate of transplanted engineered cells, ex vivo edited cells also offer a valuable investigative platform. For instance, the ability to reprogram patient-specific adult somatic cells to iPSCs by overexpression of pluripotent transcription factors (Takahashi et al. 2007) has been used for ex vivo disease modelling. Notable examples of recapitulating disease phenotypes in a dish include amyotrophic lateral sclerosis (Dimos et al. 2008), spinal muscular atrophy (Ebert et al. 2009), long QT syndrome (Moretti et al. 2010) and inherited cardiomyopathies (Sun et al. 2012, Lan et al. 2013), among others. Beyond disease modelling, this platform has also expedited development of high-throughput drug screening (Mordwinkin et al. 2013) as well as gene correction in monogenic diseases (Simara et al. 2013).

BIOIMAGING

Molecular imaging technologies can importantly further the progress of gene- and cell-based therapies.

Disease modelling and monitoring

Gene- and cell-based approaches have suffered from a lack of knowledge and control over *in vivo* graft behavior. Requiring years of preclinical testing, their combined progression will need to overcome the obstacles that have impeded these approaches independently and should benefit significantly from insights gained from bioimaging of gene and cell fate. Historically, lineage mapping by physical or genetic labelling has contributed extensively to our understanding of developmental processes and stem cell behavior and assisted in the isolation of important stem cell populations. To better understand why gene and cell therapies have fallen short of their potential so far, an approach similar to that taken by developmental biologists should be more fully adopted by molecular imaging specialists and translational researchers. The coupling of therapeutic cells or vectors to reporter cassettes to permit live, longitudinal imaging of cellular processes may provide key insights that will help elucidate and harness their full regenerative and corrective capacities, while simultaneously addressing safety and regulatory concerns (Wang et al. 2012, Joung and Sander 2013).

LABELLING THE THERAPEUTIC POPULATION

A diverse range of cell labelling and tracking modalities are available to investigators.

Imaging modalities

For regenerative medicine, several cell types are of interest because of their multipotent (for example, MSCs) or pluripotent (for example, hESCs and iPSCs) nature (Fig. 1a). Therapeutic applications of some of these cells have been explored through clinical trials, but unresolved concerns surrounding safety and efficacy are limiting their full clinical implementation (de Almeida et al. 2013). The principal barriers to full regulatory acceptance of pluripotent stem cell-based therapeutics are their immunogenic and tumorigenic potential; addressing these will require *in vivo* tracking of transplanted grafts (Kooreman and Wu 2010). *In vivo* tracking of cell fate involves either 'direct' physical labelling of cells by incubating them with a contrast agent, or 'indirect' genetic labelling of cells by transfecting them with reporter gene construct(s) (Fig. 1b). The position of and signal from these labels can then be tracked using a CCD (charged coupled device) camera for bioluminescence imaging (BLI) or fluorescence imaging (FLI), single photon emission computed tomography (SPECT), PET and magnetic resonance imaging (MRI), among other modalities. Selection of the optimal labelling technique and imaging modality depends on the cellular processes that need to be studied as well as the readouts that are most desirable, with each labelling and imaging strategy having distinct advantages and disadvantages (Youn and Chung 2013). Until now, most clinical studies have relied on direct labelling strategies to track homing and migration of multipotent stem cells or engineered cells (see Table 1). These studies have answered critical

questions in regenerative medicine, such as the importance of early stem cell engraftment on predicting late functional improvement (Vrtovec et al. 2013) and the optimal route of cell delivery (comparing transendocardial versus intracoronary routes) into the heart (Vrtovec et al. 2013). Furthermore, these cardiac regenerative studies highlight the importance of combining bioimaging of organ function with that of cell homing to assess which part of a diseased organ might benefit most from cell therapy (Musialek et al. 2013). For immunotherapy using modified CTLs or tumor-specific dendritic cells (DCs), bioimaging has provided clinicians with important insight into the kinetics of antitumor cell infiltration into tumor tissue (de Vries et al. 2005, Yaghoubi et al. 2009). By using fluorinated (^{19}F) contrast agents to label human DCs for MRI, preclinical studies have further demonstrated

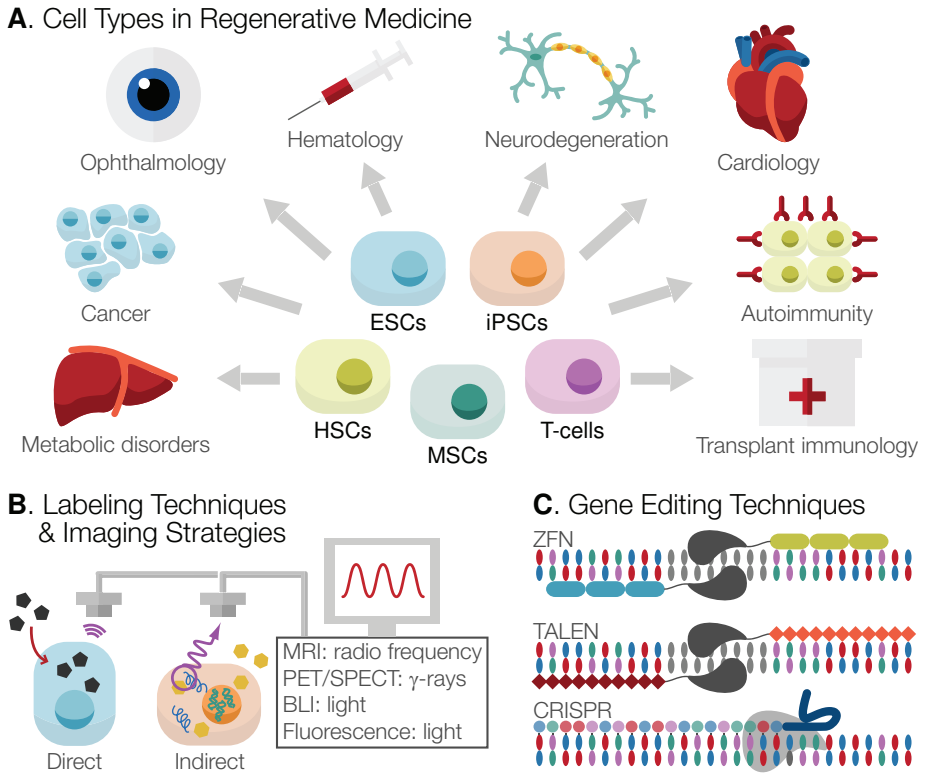

Figure 1. a, A variety of cell types, including somatic cells (T cells), adult stem cells (HSCs, MSCs) and pluripotent stem cell (ESCs, iPSCs) derivatives, are available to researchers to address a wide range of pathologies across fields. b, To enable *in vivo* monitoring of transplanted cells by protein, enzyme and receptor-based platforms, cells can be labelled either 'directly' (with a physical compound such as iron particles or radiotracers) or 'indirectly' (by genetic integration of reporter gene(s)). c, Targeted genome editing can be achieved by several techniques, including ZFN, TALEN and CRISPR approaches. Dual editing of cells to integrate corrected gene products with reporter cassettes will facilitate informed assessment of their safety and efficacy by bioimaging. Image courtesy of Ryan Tucker and Christina Sicoli (Manuscript by Design).

DC migration to draining lymph nodes, with superior assessment of cell quantity compared with that obtained by other MRI labels (Helfer et al. 2010, Bonetto et al. 2011).

With the current focus on moving pluripotent stem cell derivatives to the clinic (Garber 2013), *in vivo* tracking of these cells is critical in assessing their homing and proliferative potential over time, as well as the exclusion of teratoma formation (Lee et al. 2013). In preclinical studies, more emphasis is placed on indirect genetic labelling over direct physical labelling because the former technique is not subject to signal dilution on cell division nor discordance between signal intensity and cell viability on graft loss (Li et al. 2008, Higuchi et al. 2009). Of note, label uptake by inflammatory cells may produce a false positive readout of graft persistence, as a consequence of uncoupling of the label from its original host cell (Amsalem et al. 2007). Preclinical genetic labelling of stem cells with fluorescent proteins or bioluminescent enzymes has provided researchers with important information regarding graft behavior in small animal models, offering both fast readouts of longitudinal cell survival and low costs per imaging study. However, the penetrance of these signals is too low for detection in humans, hence largely limiting their application to small animal models (Contag and Bachmann 2002). By contrast, PET and SPECT provide higher sensitivity than optical techniques, making them better suited for monitoring biological processes in large animal and human studies (Kang et al. 2006, Schachinger et al. 2008, Yaghoubi et al. 2009, Vrtovec et al. 2013, Vrtovec et al. 2013), while also sensitively permitting visualization of as low as 1×10^5 engrafted cells (Templin et al. 2012). Although spatial resolution remains a limitation with nuclear medicine imaging, it could be overcome by combining with computed tomography (CT) (Doyle et al. 2007) or with MRI. However, the combined PET–CT or SPECT–CT approaches may not be ideal for repetitive assessment of gene or cell fate because of the high exposure to ionizing radiation. Hence, the combined PET–MRI approach may be an attractive alternative because it offers decreased radiation and high spatial resolution qualities (Wehrl et al. 2009).

Cell labelling strategies

Labelling cells to enable a combined PET–MRI approach will be of great clinical value for gene and cell therapies. Numerous PET reporter systems have been previously described, including those using dopamine receptor D2 and somatostatin receptors. These systems, however, suffer from low sensitivity, as endogenous receptor expression leads to high background signal (MacLaren et al. 1999, Liang et al. 2002, Kwekkeboom et al. 2010). The sodium iodide symporter (NIS) has been proposed as an alternative reporter gene as a result of its wide substrate availability, labelling stability, and well-understood metabolism and substrate clearance (Chung 2002). However, the presence of NIS in other tissues such as the thyroid, stomach and urinary tract reduces its reporter specificity. For PET imaging in gene- and cell-based therapies, the most widely used label so far has been the *HSV-tk* reporter gene (Black et al. 1996, Wu et al. 2003, Cao et al. 2006, Yaghoubi et al. 2009). This

Table 1.
Human clinical trials or case reports using direct and indirect labelling strategies for *in vivo* bioimaging to monitor homing and migration of transplanted cells. ^{18}F-FHBG, fluorine 18-9-[4-fluoro-3-(hydroxymethyl)butyl]guanine; ^{18}F-FDG, fluorine 18-fluorodeoxyglucose; SPIO, superparamagnetic iron oxide; ^{111}In-oxine, indium oxine; ^{99m}Tc-HMPAO, ^{99m}Tc-hexamethylpropyleneamine oxime; *HSV-tk*, herpes simplex virus thymidine kinase; LVEF, left ventricular ejection fraction; PBMC, peripheral blood mononuclear cells.

Labeling:	Imaging Modality	Cell type	Treatment	Procedure	Outcome	Source
^{18}F–FHBG	PET/MRI	Cytolytic T-cells (CTL) *($1x10^9$ cells)*	Immunotherapy for glioblastoma multiforme	-Genetically modified CTLs with *HSV-tk* to target glioblastoma -Administration of CTLs (after tumor resection) with Rickham reservoir	-Tracking of CTLs up to 2 hours after infusion -Possibility to induce programmed cell death of CTLs after ganciclovir administration	Yaghoubi, Jensen et al. 2009
^{18}F-FDG	PET/CT	Hematopoietic stem cells (HSC) *($4.5x10^8$ cells)*	Cell therapy after myocardial infarction	-HSC isolation via spectra apheresis -40 min incubation with ^{18}F-FDG -Administration via balloon catheter	-High resolution assessment of retention in myocardium (1.5% of cells) -Short half-life of ^{18}F-FDG limits usefulness after 20 hours	Kang, et al. 2006
SPIO + ^{111}In-oxine	MRI and scintigraphy	Dendritic cells (DC) *($15x10^6$ cells)*	Immunotherapy for melanoma	-DC isolation from PBMC -15 min incubation with ^{111}In-oxine -SPIO added to culture media -Injection into draining lymph node of resected area	-Similar sensitivity between scintigraphy and MRI -Scintigraphy allows quantification of cells -MRI superior in assessing location and migration of labeled cells	de Vries, Lesterhuis et al. 2005
SPIO	MRI	Neural stem cells (NSC)	Cell therapy for brain damage	-NSC isolation from exposed neural tissue -60 min incubation with SPIO -Stereotactic implantation in region of brain damage	-Non-invasive tracking of engraftment and migration of labeled cells up to 7 weeks	Zhu et al. 2006
^{111}In-oxine	PET	Circulating progenitor cells (CPC) *(~10^6 cells)*	Cell therapy after myocardial infarction (MI)	-CPC isolation from PBMC -60 min incubation with ^{111}In-oxine -Administration via balloon catheter	-Reduced homing of cells in chronic MI (> 1 year) versus acute MI (<14 days)	Schachinger, Aicher et al. 2008
^{99m}Tc-HMPAO	SPECT	Bone marrow mononuclear cells (BMMNC) *(~100×10^6)*	Cell therapy for non-ischemic dilated cardiomyopathy	-BMMNC isolation via apheresis -Labeling of $CD34^+$ cells with ^{99m}Tc-HMPAO before injection -Intracoronary or transendocardial injection in affected myocardium	-Better homing of cells leads to increase in LVEF at 3 and 12 months after procedure -Superior engraftment with transendocardial injection	Vrtovec, Poglajen et al. 2013; Vrtovec, Poglajen et al. 2013

construct offers several benefits, including quantitative and anatomic evaluation of reporter gene expression (Gambhir et al. 2000). It also has the ability to function as a suicide gene on administration of exogenous acycloguanosine substrates in pharmacological amounts, making it particularly ideal for ablating unwanted tumorigenic findings (Cao et al. 2006).

For MRI, chemical exchange saturation transfer (CEST) has been used to improve detection sensitivity. It is based on the principle that mobile protons resonate at a frequency distinct from those in bulk fluid. A proton signal specific to a molecule or CEST substrate is selectively saturated with signal. These protons' movement towards, exchange with, and subsequent signal transfer to bulk water results in the exchange transfer of signal loss (Ward et al. 2000). Whereas all ^{1}H-based magnetic resonance reporter genes rely on (super) paramagnetic substances and water relaxation for contrast (Louie et al. 2000, Moore et al. 2001, Genove et al. 2005), the contrast produced by CEST agents can additionally be switched on and off by frequency selection. Using a lysine-rich protein (LRP) reporter, Gilad *et al.* demonstrated this principle by creating a contrast material that is detectable in the micromolar range, biodegradable and capable of distinguishing live from dead cells, thus enabling the constant monitoring of endogenous expression levels in daughter cells (Gilad et al. 2007). Newer CEST contrast agents, such as human protamine-1, address immunogenic concerns regarding the use of animal reporter proteins, and are being investigated for *in vivo* imaging applications (Bar-Shir et al. 2013). Another advantage is that the separation of signals from different CEST contrast agents enable multiple, simultaneous measurements possible from distinct target populations (McMahon et al. 2008, Liu et al. 2012). In contrast to fusion reporter genes, the use of a single reporter gene for multimodal imaging with photoacoustics, MRI and PET is also being explored (Qin et al. 2013).

Targeted genome editing

Significant advances have been made with respect to genome editing technologies, but these advances have not yet been extensively integrated with bioimaging technologies (Fig. 1c). The majority of genetic engineering to a targeted locus so far has been accomplished by use of zinc- finger nucleases (ZFNs). More recently, transcription activator-like effector nucleases (TALENs) have offered a similarly promising tool, coupling a generic *Fok*I nuclease domain with a specific DNA- binding domain. For ZFNs and TALENs, DNA-binding modules are engineered to match a target DNA sequence. There, they direct double strand breaks and facilitate potential DNA alterations and repair under non-homologous end joining or homology-directed repair. A third genome editing system also offers strong potential for improving targeted gene therapies: the clustered regularly interspaced short palindromic repeats (CRISPR) system uses RNA-guided Cas9 DNAse activity to generate sequence-specific target cleavage (Horii et al. 2013).

So far, these three genome editing approaches have been used for correction or modelling of α1-antitrypsin disease (Yusa et al. 2011), sickle cell anemia (Sebastiano et al. 2011)

and Parkinson's disease (Soldner et al. 2011). Recently, Wang *et al.* demonstrated how ZFN technology can introduce a triple fusion reporter cassette into the safe-harbor AAVS1 locus of hESCs and iPSCs with readout capacity by three independent systems: BLI (firefly luciferase), PET (*HSV-tk*), and FLI (monomeric red fluorescent protein) (Wang et al. 2012). This work provides a platform for future introduction of a dual reporter gene and corrected cassette under the control of the target site promoter, providing important insights into temporal and spatial activity of cell fate. Collectively, these three approaches (ZFN, TALEN and CRISPR) make potent tools for genomic targeting. Investigators now have the ability to guide genomic integration of reporter genes and corrected genes, and to monitor the behavior of edited grafts with bioimaging platforms.

CONCLUSIONS

Gene and stem cell therapies, individually or integrated into one therapeutic product, have yet to realize their full potential. Two significant hurdles are the lack of regulatory confidence in the safety and specificity of genomic manipulations in gene correction or in cell differentiation, and a lack of understanding into the long-term survival kinetics and behavior of transplanted cells or integrated gene products. We propose here that bioimaging will play a critical role in overcoming these barriers by providing more quantitative and longitudinal readouts of graft and vector behavior, and lead to more informed and comprehensive patient care. The use of bioimaging in an integrated, collaborative approach will offer valuable insight into the delivery, engraftment, survival and host tissue interactions of vectors and cells, as well as early knowledge of off-target behavior and oncogenic events. By providing powerful tools for guiding clinical practice and scientific development, bioimaging is assured of a bright future as a research field.

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