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Safety Assessment of Reprogrammed Cells Prior to Clinical Applications: Potential Approaches to Eliminate Teratoma Formation

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1. Introduction

Human pluripotent stem cells (hPSC) include human embryonic stem cells (hESC) and human induced pluripotent stem cells (hIPSC). Due to their inherent ability to self-renew indefinitely in vitro and to give rise to essentially all cell lineages, both cell types have enormous potential for applications in regenerative medicine, but differ in their origin. HESC are derived from early pre-implantation stage embryos and have the capacity, known as *pluripotency*, to generate any other cell type of the human body. HESC can be differentiated in the laboratory, a procedure aimed at the generation of healthy somatic cells that eventually could be used in a large variety of applications including therapeutic options. However, work with hESC raises ethical concerns regarding the use of human early pre-implantation embryos, as well as concerns regarding the future use of hESC-derived cells in non-autologous cell transplantation therapies due to immune rejection of hESC-derived tissues, given that hESC are non-self. These concerns appeared to be overcome when it was demonstrated that pluripotency could be induced in differentiated somatic (adult) cells of the body by introduction of a cocktail of pluripotency-associated transcription factors, usually *OCT4*, *SOX2*, *KLF4* and *c-MYC* [1]. This process is known as reprogramming, and generates human induced pluripotent stem cells (hIPSC), which show an embryonic-like state similar to hESC (for review see [2]). Human iPSC are considered to have immense potential for regenerative medicine, do not require the use of donated human embryos for their generation and may provide an alternative and suitable resource for autologous cell-based therapies, in which cells obtained from the patient could be used to generate self-hIPSC followed by differentiation to relevant lineages required for therapeutic intervention. However, disturbingly, mouse experiments have shown that autologous mouse iPSC can induce

unexpected T-cell-dependent immune response in syngeneic recipients [3], suggesting that hIPSC-derived cell types should also be evaluated for immunogenicity before any clinical application.

Given that: (i) the generation of human iPSC does not require destruction of embryos, (ii) that many iPSC lines can be established from a single patient, (iii) hIPSC are predicted to lead to patient specific therapies and (iv) that hIPSC could be used as a source of somatic cells for toxicology and drug screening studies, many research programs have shifted their focus from solely hESC-based research to also include work on hIPSC. However, despite the phenotypic similarities with hESC, recent reports described the worrying phenomena of elevated genetic [4-6] and epigenetic abnormalities [7-9] in hIPSC, raising concern about the suitability of hiPSC-derived cell types for future clinical applications. Nevertheless, it appears that these abnormalities are not present in all iPSC cell lines and that at least in mouse studies the current reprogramming methods can produce pluripotent mouse IPSC lines that lack identifiable genomic alterations [10], a result that calls for additional experiments to explain the discrepancies with respect to hIPSC [4-6]. It is becoming increasingly obvious, based on the studies described, that it is extremely important for hIPSC-derived therapies to become a reality in the clinic, that researchers develop diagnostic tools to definitively recognise clinically "safe" and "unsafe" hIPSC lines. This is likely to be a complex and cumbersome task due to the large number of methodological approaches used. To date hIPSC lines have been generated (for review see [2]); using a large number of different vectors to introduce the transgenes, with variations in the combinations of genes used to induce pluripotency, with significant modifications in culture conditions aimed at improving reprogramming efficiency, and from many of the more than 200 cell types in the human body. It will be a challenging undertaking to develop individual safety profiles for the multitude of hIPSC lines developed to date. Additionally, hIPSC-derived cells/tissues intended for clinical applications will need to comply with the following conditions: (i) adequate numbers of cells for transplantation therapy, (ii) hIPSC differentiated progeny need to be tolerated (not immunorejected) by a patient's immune system and (iii) hIPSC-derived cells should not generate teratoma-like tumours at any time after transplantation. In vitro and pre-clinical optimisations for these parameters are essential before hIPSC-derived technologies reach the clinic.

In this Chapter, we discuss the prospects for clinical applications using pluripotent cells, focusing on an evaluation of hIPSC cell potential and on the development of methods for the identification and removal of unwanted residual tumorigenic pluripotent cells from hIPSC-derived cell populations following differentiation.

2. The risk of tumour formation from residual pluripotent cells

In vivo, pluripotent stem cells reside only during a short time in embryonic development. Conversely, in vitro, hESC and hIPSC lines can be propagated indefinitely in the embryonic-like state and remain pluripotent, or with the appropriate cues they can give rise to a range of body cell types. For human cells, the most accepted *in vivo* assay to prove pluripotency is

the generation of *teratomas* in immuno-deficient mice (ie: NOD-SCID and NOD/SCID IL2R$\gamma^{-/-}$ mice), by injection of putative pluripotent hPSC into organs like testis, kidney or muscle. Teratomas are benign solid tumours that contain a mixture of differentiated tissues such as nerve cells, muscle cells or cartilage. If a human cell line generates teratomas, it is considered pluripotent, because teratomas emulate differentiation in the developing embryo, albeit in a disorganised fashion, by generation of tissues resembling different parts of the embryo known as embryonic germ layers (i.e.: Ectoderm, Mesoderm and Endoderm).

In the clinical context, pluripotent stem cells will not be transplanted, rather the progenitors and/or specialised somatic cell types that are derived from hPSC will be used. It is the hope of researchers working in the expanding field of regenerative medicine that hPSC-derived cell populations will integrate into tissues and receive appropriate cues to functionally correct diseased or injured tissue, (i.e.: Parkinson's disease, Huntington's disease, cardiac failure, multiple sclerosis or macular degeneration). Therefore, differentiated somatic cell types are the final product for transplantation and therapeutic applications, and pluripotent stem cells are the stable source to generate those somatic cells or their progenitors (depending upon disease context) in the laboratory. In this context, the presence of even low frequency residual undifferentiated stem cells capable of teratoma formation becomes a highly undesirable feature when considering hPSC-derived somatic cells for transplantation into patients. Differentiated cells will not be deemed safe for use in regenerative medicine if they generate tumours at any time after transplantation. To comply with this requirement, we consider that researchers should aim at the generation of pluripotent stem cell-free samples. Therefore, it will be essential to be able to monitor if any undifferentiated pluripotent cells remain after differentiation protocols, and if so, remove them without damaging the potentially therapeutic differentiated cells. Evidence supporting this statement is that it is known that the numbers of pluripotent cells injected experimentally have a directly proportional effect on how fast the teratomas develop and the size of the tumour [11-13]. It has also been reported that at doses of 1,000 pluripotent cells, teratomas developed with 40% efficiency but with 10,000 cells the efficiency increased to 100% [12]. However, as few as two pluripotent cells have been reported to induce teratoma formation in immuno-deficient mice, although with lower efficiency [11]. Taken together, this might mean that one remaining pluripotent stem cell in a patient bound cell preparation could lead to teratoma formation. There is some limited evidence that potentially refutes the tumorgenic potential of low doses of pluripotent cells. This evidence is demonstrated by experiments showing that two pluripotent cells transplanted into syngeneic immunocompetent mice practically abolished tumour formation [11], most likely because those stem cells were cleared by the immune system. This could be taken to imply that in the clinical context of immuno-competent patients, low contamination with human pluripotent stem cells may be safe, but nevertheless for hPSC-derived cell populations to be approved for use in clinical trials their stringent elimination will be a requirement. Furthermore, the site of transplantation needs to be taken into account as not all places in the body are equally permissive for teratoma growth and development and contaminating hPSC may also migrate to alternative and possibly more permissive sites for teratoma growth post transplantation. For instance, it has been reported that similar number of pluripotent stem cells injected into immuno-deprived mice induced

teratomas with 12.5% efficiency in intramuscular injections, 33% in subcutaneous injections, 60% in intratesticular, and approximately 100% under the kidney capsule [14]. Although many variables can potentially affect teratoma formation, we consider that the most ethical and safest cell population for transplantation into patients should be classified as pluripotent stem cell-free.

3. How to purge residual tumorigenic pluripotent stem cells from differentiated cell types?

To guarantee that no undifferentiated pluripotent stem cells are present in a hESC or hiPSC-differentiated progeny intended for transplantation into patients, researchers need assays to detect those residual pluripotent cells and efficient methods to purge stem cells from the differentiated cell populations. A good strategy to detect pluripotent cells is using antibodies that detect surface markers on live hPSC that are not present on differentiated cell types. After antibody-mediated detection of stem cells, other technologies could be coupled to the antibodies in order to eliminate residual pluripotent stem cells from the transplantation sample. For instance, Fluorescent or Magnetic Activated Cell Sorting (FACS and MACS) could be used with antibody detection for elimination of the targeted cells.

There are only a few available antibodies that detect cell surface markers on live human pluripotent stem cells (See table 1). Researchers, utilising the available antibodies, have described methods to eliminate residual pluripotent cells from samples of differentiated cell types. For instance the SSEA-4 antibody first demonstrated its utility in purging pluripotent stem cells from simian ESC-derived hematopoietic precursors used for transplantations into monkeys [15]. In this study, researchers used SSEA-4 antibody to detect residual pluripotent cells that persisted despite rigorous and extended differentiation protocols for hematopoietic precursors. SSEA-4 negative cells obtained by fluorescence activated cell sorting (FACS) did not develop teratomas, whereas teratomas were consistently observed in hematopoietic precursors showing presence of SSEA-4 positive cells [15]. The SSEA-4 and Tra-1-60 antibodies have also been compared for their efficiency in detecting and removing residual hPSC, by FACS or magnetic-activated cell sorting MACS [16]. This comparison revealed that MACS technology was not efficient for complete depletion of hESCs, with an average of 82% retention of hESCs, and highlighted that negative selection via FACS may be a preferred approach to eliminate undesirable hESCs from differentiated populations [16]. However, a note of caution against the use of single antibodies to detect hESCs emerged from data showing that 47% of SSEA-4 low-expressing hESCs exhibited a high level of expression for TRA-1-60. Therefore, detection of a single cell-surface marker may not be sufficient to eliminate all pluripotent stem cells, and methods that use multiple antibodies detecting different epitopes expressed by hESCs are more likely to be successful [16].

Antibody	Isotype	Cell-surface antigen	Source/Supplier	Literature reference
GCTM-2	IgM	Keratan sulphate proteogly-can (KSPG)-protein core	Kindly donated by Prof. Martin Pera	Laslett *et al*., 2003 [27]; Pera *et al*.,2003 [28].
mAB 84	IgM	Podocalyxin (PODXL); CD34 family member.	Millipore MAB4414 http://www.millipore.com	Choo *et al*., 2008 [17].
PHM-5	IgG1	Podocalyxin (PODXL); CD34 family member.	Millipore MAB430 http://www.millipore.com	Kerjaschki *et al*., 1986 [29].
SSEA-3	IgM	Globoseries glycolipid	Millipore MAB4303 http://www.millipore.com	Kannagi *et al*., 1983 [30].
SSEA-4	IgG3	Globoseries glycolipid	Millipore MAB4304 http://www.millipore.com	Kannagi *et al*., 1983 [31].
TG30 (CD9)	IgG2a	25kDa tetraspannin protein CD9	Millipore MAB4427 http://www.millipore.com	Laslett *et al*., 2003 [27]; Pera *et al*., 2003 [28].
TG343	IgM	KSPG-protein core (detects the same antigen as the GCTM-2 antibody).	Millipore MAB4346 http://www.millipore.com	Cooper *et al*., 2002 [32].
TRA-1-60	IgM	KSPG-carbohydrate side chain	Millipore MAB4360 http://www.millipore.com	Andrews *et al*., 1984 [33].
TRA-1-81	IgM	KSPG-carbohydrate side chain	Millipore MAB4381 http://www.millipore.com	Andrews *et al*., 1984 [33].

Table 1. Antibodies that are reactive with cell surface markers expressed on human pluripotent stem cells

The studies described above point to FACS technology coupled to antibody detection of surface markers as a good strategy to eliminate residual undifferentiated pluripotent cells and recover differentiated live cells for further applications such as re-culture or transplantation.

However, as the viability of hPSC-derived lineage progenitors or more mature cell types can be compromised post-FACS, caused by shearing forces, laser damage or osmotic stress, other technologies such as MACS may be better suited in these instances. Although MACS does not completely remove all hESCs in a single pass [16], this technology exhibits higher cell viability than FACS and it is possible that subsequent positive selections by MACS using multiple antibodies for different hESC cell surface markers could completely remove all hESCs. An alternative approach to MACS could be to use cytotoxic antibodies directed against hESC surface antigens or chemicals that could selectively eliminate hESCs without affecting their derivatives. An example of a cytotoxic antibody that detects and removes hESCs is the monoclonal antibody mAB-84 [17], which binds to PODXL (Podocalyxin-like protein 1) on hESCs and initiates a sequence of events that leads to hESC-membrane damage by formation of leaking pores [18]. It has been proposed that using the monoclonal antibody mAB-84 in a two-step cell-cell separation approach can eliminate teratoma-forming hESC from differentiated cell types [19]. In this strategy, an initial depletion of hESCs was achieved via MACS using a panel of commonly used hESC cell-surface markers, which was followed by selective elimination of residual undifferentiated stem cells post-MACS using the cytotoxic antibody mAB-84, an approach that appears to increase the safety of cell transplantation [19].

Selective elimination of residual human pluripotent stem cells after differentiation can also be achieved by targeting apoptosis-meditating receptors that are differentially expressed in undifferentiated stem cells and absent in hESC derivatives. Therefore, stimulation of these specific hESC apoptotic receptors induce programmed cell death only in the residual stem cells without affecting their differentiated progeny. One example of this kind of receptor is the prostate apoptosis response-4 (PAR-4), which mediates ceramide or ceramide-analogue-induced apoptosis in proliferating stem cells [20]. The apoptotic response appears to be specific for PAR-4(+) stem cells, and given that ESC-differentiated progenies such as neuro-progenitors express very low levels of PAR-4, they are less sensitive to ceramide induced apoptosis [20]. Using this approach, ceramide treatment appears to prevent teratoma formation when transplanting neural progenitors derived from ES cells [20] although it is likely that regulatory assays will require a more stringent method. Although PAR-4 induced apoptosis by ceramides appears an effective way to eliminate residual pluripotent stem cells following differentiation, this approach has not been broadly tested.

4. Antibodies against cell surface markers of human stem cells

The scarcity of antibodies directed against cell surface markers that recognize live human pluripotent stem cells (See table 1) is compounded by the fact that most of these antibodies lack identification of their encoding gene. Indeed, some cell surface antibodies do not recognize proteins, but complex carbohydrate and lipid moieties for which the corresponding gene is not yet identified. Despite this, these complex moieties are strong

antigens that elicit highly sensitive antibodies that recognize human pluripotent stem cells. Furthermore, a caveat is that stem-cell antibodies could also be immunoreactive with some embryonic tissues, or some mature cell types, becoming problematic with some hESC differentiation protocols. Therefore, depending on the phenotype of the target somatic cells, selected antibodies used to detect human pluripotent cells should be selected that do not react with the differentiated cells intended for transplantation. For instance, if working with hESC-derived renal tissues for treatment of kidney disorders, PODXL antibodies should not be used alone to detect stem cells because Podocalyxin protein is also expressed in glomerular podocytes.

The information in the previous section demonstrates that FACS and MACS technologies are potential methods for the elimination of residual pluripotent cells following *in vitro* differentiation (Figure 1). Both methodological approaches use cell surface antibodies for the labelling and detection of undifferentiated live hPSC. The advantage of live cell detection using either FACS or MACS is the ability to retrieve live hESC or hIPSC-derivatives that could be used for *in vitro* re-culture and expansion, or, ultimately, transplantation. However, FACS and MACS studies have also revealed the immunological complexity of *in-vitro* hESC cultures. HESC cultures contain a continuum of different subpopulations, where some hESC subpopulations express low levels of one surface marker and at the same time high levels of another [16, 21-23]. These findings imply strongly that a single cell-surface marker is not sufficient to eliminate all pluripotent stem cells [16, 21-23]. Therefore, any attempt to eliminate all hESC pluripotent subpopulations should rely on methods that use multiple antibodies detecting different epitopes expressed by hESCs. For instance, SSEA-4-coupled MACS showed an average 82% retention of hESCs [16], but when a panel of cell surface antibodies directed to different epitopes was used with MACS, the removal of undifferentiated hESCs raised to 98% on average [19].

In our laboratory, we have been working on the development of monoclonal antibody panels against extracellular markers that allow efficient human pluripotent cell separation from mixed populations of cultured cells, an essential requirement for safe hESC or hIPSC-based therapeutics [21-24]. Towards this end, we have reported a FACS-based immuno-transcriptional profiling system based on the detection of two pluripotency-associated cell surface antigens TG30 (CD9) and GCTM-2, [25-26]. This method is useful to characterise multiple human pluripotent stem cell lines, and to identify the subpopulations that are found in hESC *in-vitro* continuous culture [21-22]. Ongoing unpublished observations indicate that this double staining of human stem cells using two cell-surface markers is a better way to eliminate residual and persistent undifferentiated pluripotent cells using FACS in both hESC and hIPSC lines. Nevertheless, we are aware that there will be differentiation contexts in which TG30 (CD9) and GCTM-2 might not be appropriate or sufficient to purge pluripotent cells from particular differentiated hPSC-derivatives. Therefore there is a real need for new monoclonal antibodies that detect cell surface proteins on live hPSC.
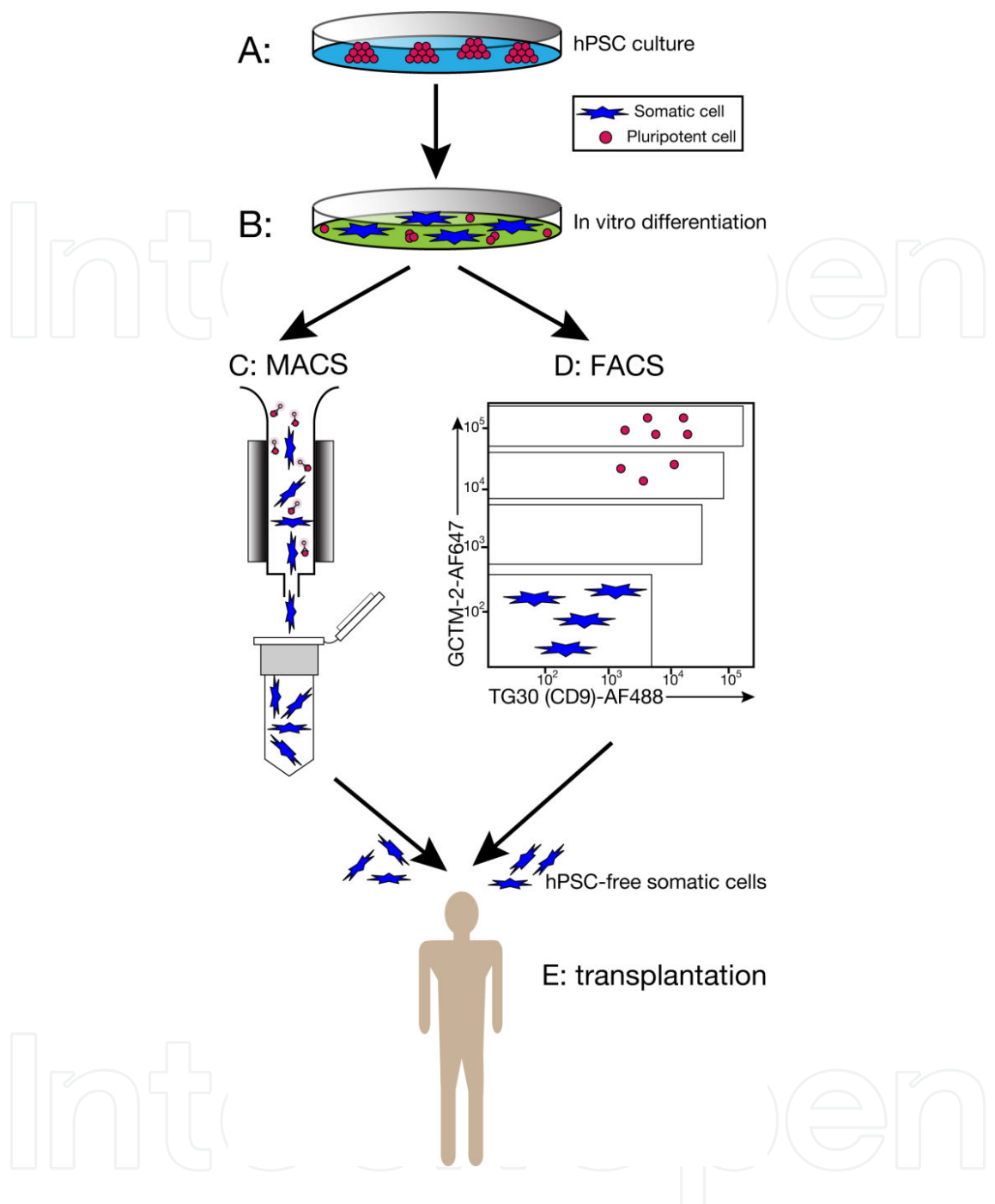
Figure 1. Potential approaches to eliminate residual pluripotent stem cells after in vitro differentiation. Shown are two potential methods that could be used to purge residual tumorigenic pluripotent stem cells from differentiated cell types. (A): Human pluripotent stem cells (hPSC) are able to self-renew indefinitely *in vitro*. (B): These pluripotent cells can be induced to differentiate *in vitro* to generate healthy progenitors and/or specialised somatic cell types that could potentially be used for transplantation and therapeutic applications. However, it is essential to monitor if any residual undifferentiated pluripotent cells remain after differentiation protocols. If undifferentiated stem cells remain, these cells should be removed without damaging the potentially therapeutic differentiated cells. Two good strategies for elimination of residual pluripotent cells are Magnetic Activated Cell Sorting (C: MACS) and Fluorescence Activated Cell Sorting (D: FACS). Both technologies are coupled to antibody detection of cell surface markers and allow retrieval of live hPSC-derivatives that could be used for further *in vitro* re-culture and expansion, or in due course transplantation (E).

5. Conclusions

Human pluripotent stem cells, namely hESC and hIPSC lines, may be the future main-stay of medicine, providing a plethora of medical applications and transplantation thera-pies aimed at the correction of an important number of pathological disorders. However, reaching clinical applications based on hPSC-therapies has not been as fast as expected. The ability to generate hIPSC lines from a variety of tissue sources has brought hIPSC re-search clearly into the spotlight, but reports on their epigenetic instability and genetic variability suggest that these cells are not yet clinic-ready. In addition, the concern of tu-morigenesis or teratoma formation is an unsolved problem for both hESC and hIPSC re-search. If differentiation protocols are not 100% efficient and yield a mixture of differentiated and undifferentiated cells, this presents a significant risk of teratoma for-mation after transplantation. It is clear that adequate safety assays for hESC or hIPSC-de-rived technologies are of the utmost importance to aid in the safe translation from the bench to the clinic. This includes the essential monitoring of any residual undifferentiated pluripotent cells after differentiation protocols, an unavoidable methodological step in any sample to be used in the clinic. A variety of approaches have been discussed in this chapter to help to eliminate the undesirable residual pluripotent stem cells from samples intended for transplantation. However, there is an ongoing need to improve these separa-tion methods in order to achieve hPSC free samples in a rapid, easy, safe, cost effective, scalable and clinically applicable way. We expect that novel cell-surface antibodies recog-nizing live pluripotent stem cells will strongly contribute to this ongoing search.

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