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PERSPECTIVE

Glypican 4 down-regulation in pluripotent stem cells as a potential strategy to improve differentiation and to impair tumorigenicity of cell transplants

Recent advances in stem cell technologies have opened new avenues for the treatment of a number of diseases still lacking effective therapeutic options. Cell transplantation has emerged as among the most promising clinical intervention for disorders such as injuries, diabetes, liver diseases, neurodegeneration and heart failure (Lee et al., 2013; Forbes and Rosenthal, 2014; Tabar and Studer, 2014). To date much attention is given to the potential application of human pluripotent stem cells (hPSCs) including both embryonic (hESCs) and induced pluripotent stem cells (hiPSCs) for regenerative medicine (Yamanaka, 2012; Lee et al., 2013; Tabar and Studer, 2014). Owing to their remarkable ability to self-renewal indefinitely and to differentiate into all mature cell types hPSCs appear of great advantage to generate an unlimited number of disease-relevant cells. The benefits/pitfalls associated with the use of hPSCs and their derivatives in cell-based transplantation therapies are currently being evaluated in animal models recapitulating human conditions (Hulot et al., 2014; Isacson, 2015). *In vivo* studies have begun to provide proof-of-concept for the efficacy of hPSC-based transplantation therapies to treat pathologies such as Parkinson's, Huntington's and retinal diseases in which symptoms are caused by the selective loss of specific cell types (Tabar and Studer, 2014; Isacson, 2015). Interestingly, cell transplantation therapies utilizing hPSC-derived retinal pigment epithelium cells have reached clinical trials for the treatment of macular degeneration.

While methods for generating hPSC-derived transplantable cells are progressing, important hurdles have emerged that must be overcome before hPSC-based transplantation therapies can enter the clinic in a widespread manner. As potential therapeutics, hPSC-derivatives pose a range of safety concerns. A central issue is the possibility that hPSC-derived transplants might be contaminated with tumor-promoting cells, which would preclude their therapeutic potential by tumorigenic side effects (Lee et al., 2013). Despite current differentiation protocols enable generation of different disease-relevant cell types from hPSCs, in various cases these protocols produce heterogenous cell populations containing contaminating hPSCs/partially differentiated cells that generate teratomas and other forms of embryonal neoplasma after engraftment (Amariglio et al., 2009; Lee et al., 2013).

Cell transplantation studies into immunodeficient hosts have indicated that a threshold of undifferentiated cells ranging from 100 to 245 may be sufficient for tumor development (Hentze et al., 2009; Gropp et al., 2012). Tumor formation is also strongly dependent on the delivery site and reagents employed to improve retention and survival rate of transplanted cells, therefore tumor risks after transplantation might vary according to the delivery site and cell graft preparation (Hentze et al., 2009; Gropp et al., 2012). Yet, the kinetics of tumor development appears proportional to the number of contaminating undifferentiated cells, with tumors arising at a relatively lower frequency and after longer-term follow-up as the percentage of undifferentiated cells within grafts decreases (Gropp et al., 2012).

Strategies such as cytotoxic antibodies/chemical inhibitors and cell sorting methods (FACS and MACS) are currently being developed to eliminate self-renewing hPSCs/progenitors and to obtain nearly homogeneous population of differentiated cells (Lee et al., 2013). Some of these methods are efficient but their broad application still depends on: 1) identifying cellular surface markers specific for distinct cell populations (*e.g.*, PSCs/progenitors *versus* differentiated cell); 2) identifying markers that change drastically during differentiation; 3) minimizing *in vitro* manipulations, shearing forces to preserve viability and functionality of purified cells.

It has also been proposed that tumor risk may be overcome by transplanting hPSCs-derivatives first coaxed into forming more homogeneous cultures of post-mitotic tissue-specific cell types (Tabar and Studer, 2014). To date cell type specific differentiation protocols rely on the use of small-molecules and morphogens known to control embryonic development. Therefore, it is expected that a better understanding of molecular mechanisms underlying cell lineage entry and terminal differentiation should enable controlled differentiation of hPSCs to level of homogeneity suitable for cellular therapy. To become clinically relevant, these differentiation strategies should however be based on methods producing differentiated cells of good manufacturing practice-grade and at cost-effective large scale.

Although highly differentiated hPSCs could be instrumental to overcome tumor risks, it is still not clear whether they will become the best therapeutics for most human disorders. It has been hypothesized that: 1) treatment of diseases such as heart failure might benefit from transplants involving lineage committed rather than differentiated cells (Hulot et al., 2014); 2) less differentiated cells (*e.g.*, committed/progenitor cells) may be more robust and able to survive in the hostile graft environment (Hulot et al., 2014); 3) the host tissues might provide the best environment for their proper maturation, survival and integration (Forbes and Rosenthal, 2014).

The issues above discussed show that developing an efficient procedure to eliminate tumor-forming cells is a significant hurdle that requires careful study of PSC biology both *in vitro* and after transplantation so that we can develop the necessary stringent technologies essential for good manufacturing practices.

We study signaling mechanisms regulating the balance between SC self-renewal and differentiation. We have recently explored whether interfering with genes that are essential for self-renewal, but dispensable for lineage entry, would provide an alternative strategy to interfere with tumor formation by promoting a more efficient depletion of self-renewing cells (Fico et al., 2012). We found that one these genes encodes the heparan sulfate proteoglycan (HSPG) Glypican 4 (Gpc4). In embryos, Glypicans, such as Gpc4, act as gatekeepers of extracellularextra-cellular signal such as Fgfs, Bmps and Wnts to modulate their interaction with targeted cells (Fico et al., 2011). This property allows them to impact on mechanisms such as receptor-ligand interactions, temporal and quantitative supply of active signals to cells. Other and we have shown that Gpc4 is a cell surface marker of different SC types (Fico et al., 2011; Fico et al., 2012). Our loss-of-function studies revealed that Gpc4 is required to maintain self-renewal in mouse ESCs and neural SCs as Gpc4 down-regulation orients these SCs towards an accelerated and efficient differentiation (Fico et al., 2012). We demonstrated also that Gpc4 down-regulation in mouse ESCs disrupts the intrinsic potential for teratoma development, but does not interfere with pluripotent differentiation potential (Fico et al., 2012). In conclusion, our work has shown that modulation of Gpc4 activity can be considered a new molecular strategy to regulate SC numbers and fate in order to minimize tumor risks.

An obvious question that comes up when impairing the expression of genes regulating self-renewal in hPSCs is whether this approach would compromise the generation of large numbers of therapeutically relevant cells as a consequence of premature depletion of progenitor pools. In our latest report, we tested this hypothesis by performing cell transplantation experiments with Gpc4-mutant PSCs using Parkinson's disease (PD) as a disease model of cell-based therapy. The basic principle of cell-transplantation therapy for PD is to implant midbrain dopaminergic (DA) neurons in the targeted striatum as a mean to restore dopamine levels and neurotransmission (Lindvall and Bjorklund, 2004). According to previous work in Isacson's lab, PSCs kept as embryoid bodies for 4 days and then injected in the striatum of rat models of PD (6-OHDA-lesioned rats) at low doses (1,000–2,000 single cells) develop into a cell population containing DA neurons (Bjorklund et al., 2002). Although these DA neurons improve amphetamine-induced rotational asymmetry in a fraction of Parkinsonian rats, these cell grafts are at high risk for tumorigenicity. Using this challenging experimental

paradigm, we demonstrated that down-regulation of Gpc4 in mouse ESCs increases safety and functional potential properties of PSC derived cell grafts. We found that engraftment of Gpc4-mutant cells strongly enhances the survival rate of transplanted rats compared to that of rats receiving wild-type cells (Fico et al., 2014). Post-mortem analysis of rat brains at 6 weeks post transplantation showed that these findings correlated with impaired teratoma development of Gpc4-mutant cell grafts and with reduced numbers of self-renewing cells such as nestin-positive precursors compared to wild-type grafts (Fico et al., 2014). Together these findings reinforce the concept that down-regulating Gpc4 in PSCs is a new strategy to reduce tumor side effects. Besides enhancing safety, we found that Gpc4-mutant cells in the host striatum generate grafts enriched in DA neurons when compared to transplanted wild-type cells at 6 weeks post transplantation (Fico et al., 2014). It is likely that Gpc4 down-regulation in neuronal precursors promotes distinct cell fates. This possibility is supported by our differentiation studies showing that: 1) *In vitro*, the proportion of TH-positive presumptive DA neurons within the entire neuronal population was doubled in comparison to that of wild-type cells; 2) *in vivo*, DA neurons were predominant over GABAergic and serotonergic neurons and they co-express markers of ventral mesencephalic DAs (*e.g.*, $TH^+/FOXA2^+$) (Fico et al., 2014). Compared to wild-type cells, Gpc4-mutant ventral mesencephalic DA neurons had remarkable functional properties. These cells improved the rotational asymmetry of unilateral 6-OHDA lesioned rats in the amphetamine-induced rotation-test and rescued akinesia-like deficits as assessed by the non-drug induced cylinder test, which is considered as among the most stringent assays for monitoring motor recovery effects in PD rat models (Fico et al., 2014).

Overall, the above results highlight that targeting PSC self-renewal through genes such as Gpc4 permits an efficient depletion of tumor-promoting cells and does not prevent the generation of DA neurons to a level sufficient for behavioral recovery. The next essential steps include assessing the long-term outcomes in transplanted PD animal models and identifying the molecular/signaling pathways underlying efficient differentiation of Gpc4-mutant PSCs into ventral mesencephalic DA neurons.

Different transplantation studies have established that DA neurons characterized by a ventral mesencephalic phenotype are the most appropriate cell types for PD therapies. Nowadays, highly enriched population of $FOXA2^+/TH^+$ ventral mesencephalic DA neurons are obtained by using protocols that combine prolonged *in vitro* hPSC differentiation with temporal administration of distinct patterning cues in order to recapitulate the progressive developmental events triggering ventral mesencephalic fate determination in embryos (Tabar and Studer, 2014; Isacson, 2015). In non-human primates around 10–50 millions of transplantable cells have been engrafted to reach the minimal number of 13,000 surviving ventral mesencephalic DA neurons required to restore motor deficits at a significant level (Tabar and Studer, 2014; Isacson, 2015). From a clinical perspective, the dosage of grafted cells will need to increase substantially as at least 100,000 surviving fetal DA neurons are required to provide symptomatic relief in PD patients (Lindvall and Bjorklund, 2004). Therefore, the next challenge is to improve these experimental protocols in the perspective of clinical use. In the light of the intrinsic properties of Gpc4-mutant PSCs, we think that this cellular system may help to achieve safe, scalable and cost-effective differentiation methods for generating ventral mesencephalic DA neurons.

Although we have performed studies on Parkinson's disease, safety and efficacy of Gpc4 mutant pluripotent stem cells should be exploited in animal models of other human diseases. Reduced Gpc4 activity in PSCs does not interfere with the pluripotent differentiation potential and mutant PSCs also efficiently differentiate into cardiomyocytes and endothelial cells (Fico et al., 2012). Therefore studies of cell transplantation into ischemic hearts could be fundamental to understand whether the modulation of Gpc4 levels in PSCs provides more general insights into the development of hPSC-based replacement therapies. As Gpc4 is a cell membrane protein, it is an attractive target to design agents such as antibodies to prime hPSCs into a "safe state" without genetic engineering.

Cell replacement is not a new concept as transplantation of hematopoietic SCs is the oldest and most widely used therapy for blood system. From a clinical perspective, it is likely that hPSC-derivatives rather than the "bonafide" hPSCs will be transplanted. Although the risk of having tumor-promoting cells in grafts might be relatively low when transplanting small cell numbers (*e.g.*, in retinal regeneration), the greatest challenge occurs when treating diseases requiring large quantities of hPSC-derived cells. As knowledge on transplantation-based therapies will advance, new variables such as batch-to-batch differences in hPSC differentiation efficacy might arise. A variety of approaches are being developed to ensure that no self-renewing cells remain in the grafts. However, there is an ongoing need to improve them in order to achieve realistic, cost effective and clinically applicable strategies. It will be important to evaluate whether the safest procedure will arise from combining different technologies.

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