Cell

CellNet—Where Your Cells Are Standing

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The manufacturing of clinically relevant cells is a widely used strategy in regenerative medicine. Cahan et al. develop a network biology platform named CellNet to accurately assess the fidelity of such cells and spot aberrant regulatory networks, and Morris et al. apply this platform to improve cell manufacturing.

Proper lineage commitment and cell fate determination are crucial to ensuring appropriate development. Specifically, gene regulatory networks (GRNs) control the dynamic spatial and temporal patterns of regulatory gene expression during development (Davidson, 2010). Cells in the organism receive all the signals required to attain the highest level of functionality. Despite many years of research, the underlying basis for full cell functionality in vivo remains elusive. In essence, we fail to understand why a given cell—regardless of whether it is derived by directed differentiation of stem cells or by direct conversion from somatic cells—does not faithfully recapitulate the in vivo cell properties when manufactured in vitro (Jopling et al., 2011). This drawback can be overcome only by garnering a better understanding of cell similarity and unraveling the pathways for improving the sameness and, consequently, functionality of the manufactured cells. In the current issue of *Cell*, two studies by the groups of James Collins and George Daley (Cahan et al., 2014; Morris et al., 2014) introduce a novel network biology platform, named CellNet. This platform compares the GRNs of cells manufactured in vitro with those of their in vivo counterparts and attributes a score to them.

Cahan et al. (2014) report that cells differentiated from embryonic stem cells (ESCs) have higher scores than directly converted cells, although the differentiated cells differ from their in vivo counterparts (Figure 1A). One reason for this difference is that the desired tissue- and cell-type GRNs are incompletely or improperly established. Promoter inaccessibility is likely the major barrier. Developing strategies that augment the opening of closed chromatin may enable complete establishment of the desired tissue- and cell-type GRN status. The difference could also be attributed to the presence of residual ESC GRNs. The residual GRNs in ESC-differentiated cells are closely associated with ESC cell-cycle regulation, which may engender the resultant cells with tumorigenic potential. This is particularly problematic, as the resultant cells could be of clinical relevance. Thus, meticulous inspection is required to ensure the differentiated cells are free of ESC GRNs. CellNet can serve as a rigorous inspector in this regard. Finally, unexpected generation of different cell-type-specific GRNs during differentiation is yet another reason.

Cells directly converted from fibroblasts have lower classification scores, as the desired tissue- and cell-type GRNs are incompletely established (Figure 1B). This is associated with a high residual fibroblast GRN status of the resultant cells and unwanted GRNs established during the direct conversion process. Although cells directly converted might mimic gene expression patterns of their in vivo counterparts, numerous genes are differentially expressed (Caiazzo et al., 2011; Sekiya and Suzuki, 2011; Yu et al., 2013). Two aspects of their heat map analysis reflect the cell-type-specific memory of the starting cells and an exogenous factor-mediated off-target effect (Caiazzo et al., 2011; Sekiya and Suzuki, 2011; Yu et al., 2013). The first aspect is that (1) genes are inactive in both the starting and resultant cells but highly expressed in the in vivo counterparts, and (2) genes are highly expressed in both the starting and resultant cells but inactive in the in vivo counterparts. Consistent with these findings, CellNet convincingly detected considerably higher donor cell GRNs in the resultant cells. The second aspect is that genes are expressed in the resultant cells, which had not been active in the in vivo counterparts and starting cells. Such a scenario is possible if the factors used in the conversion can actually turn on different cell-type-specific GRNs. As CellNet accurately detects unexpected GRNs from converted cells, one could go back and address such problems. An important lesson here is that a minimal set of transcription factors employed in the direct conversion process might be insufficient for faithfully recapitulating the properties of the in vivo counterparts in the resultant cells.

So far, direct conversion of fibroblasts into cells faithfully recapitulating the properties of in vivo counterparts has proven difficult. Morris et al. (2014) attempt to alleviate this difficulty using CellNet. They first identify that macrophages directly converted exhibit a high B cell GRN, with only a partial macrophage identity (Figure 1D). Knockdown of Pou2af1 and Ebf1 in macrophages, as suggested by CellNet, significantly improves the cells' similarity and functionality. However, B-cell-specific GRNs still remain intact after knockdown. The authors also used the so-called induced hepatocytes (iHeps) as another model (Figure 1E). CellNet analysis of iHeps identifies both liver and intestine GRNs. The coexistence of the two cell fates in one cell type suggests that the functional

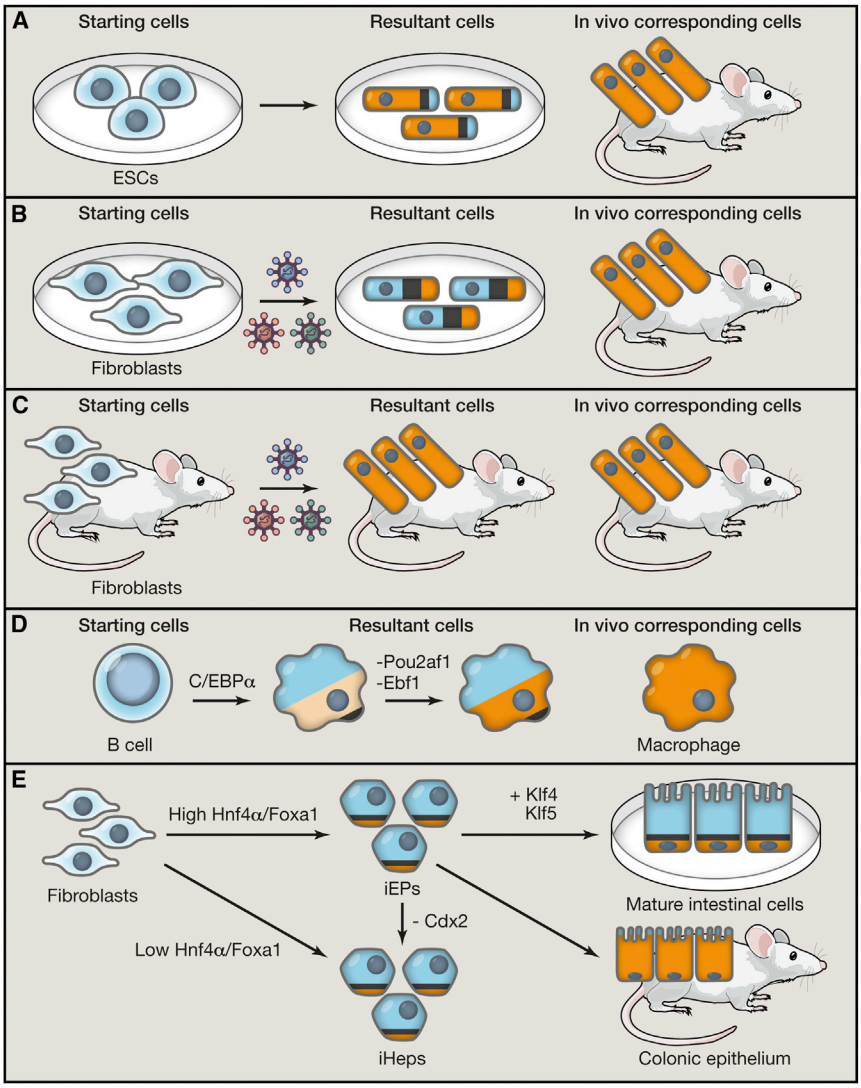

Figure 1. The CellNet Platform Assesses the Fidelity of the Resultant Cells from Direct Differentiation and Direct Conversion

(A) In vitro direct differentiation of embryonic stem cells (ESCs). The resultant cells have incompletely established their in vivo counterpart GRNs (orange). Residual ESC GRNs are incompletely silenced (blue). Unexpected GRNs are partially or transiently established (black).

(B) In vitro direct conversion by ectopic expression of lineage-specific transcription factors. The resultant cells exhibit persistently high donor-cell-specific GRN status (blue). Their in vivo counterparts GRNs are partially established (orange). Unintended GRNs unexpectedly emerge during direct conversion (black).

(C) In vivo direct conversion by ectopic expression of lineage-specific transcription factors. The resultant cells exhibit complete establishment of in vivo counterparts GRNs (orange). The donor-cell-specific GRNs are completely silenced.

(D) Direct conversion of B cells into macrophages by ectopic expression of C/EBPα. The resultant cells exhibit incomplete establishment of macrophage-specific GRNs (light orange). Residual B-cell-specific GRNs remained active (blue). Hematopoietic stem cell (HSC)-specific GRNs are partially established (black). Knockdown of Ebf1 and Pou2af1 in macrophages improves macrophage identity and functionality (orange). However, donor-cell-specific GRNs remain intact (blue). The intensity of the orange color reflects the fidelity of the resultant cell identity.

(E) Direct conversion of fibroblasts into endoderm-progenitor-like cells (iEPs) and hepatocyte-like cells (iHeps). Higher levels of Hnf4α and Foxa1 drive the cells toward iEPs, which have both intestinal (orange) and hepatic (black) fates. Lower levels of Hnf4α and Foxa1 promote conversion into cells of the hepatic fate. Knockdown of Cdx2 in iEPs allows for transition of the cells toward iHeps. Klf4 and Klf5 need to be overexpressed in iEPs for achieving mature intestinal cells in vitro. Donor-cell-specific GRNs remain intact (blue). iEPs can differentiate into cells resembling colonic epithelium in vivo after they are transplanted into the colon. Donor-cell-specific GRNs are almost extinguished in engrafted cells.

maturation of one fate can be suppressed by another. Indeed, knockdown of CellNet-prioritized Cdx2, a regulator of the intestinal fate in iHeps, fortifies the cells' liver similarity and functionality. However, neither liver classification nor liver GRN status is improved following knockdown.

CellNet efficiently selects candidate factors, and their manipulation in the cells somewhat enhances their similarity and functionality. However, donor-cell-specific GRNs appear to be tightly locked and can hardly be extinguished (Figure 1). Furthermore, partial classification scores and unsatisfactory GRN status are almost never improved. These phenomena do not occur in two instances—in the direct conversion in vivo of cardiac fibroblasts into cardiomyocytes (Cahan et al., 2014), and in the differentiation in vivo of iHeps/iEPs into cells resembling colonic epithelium after transplantation into the colon (Morris et al., 2014). Conversion could, therefore, be improved by using the instructive signals of the endogenous cell niche. An important task is to define in vitro conditions that closely resemble such instructive signals, so cells can be derived that faithfully recapitulate the properties of in vivo counterparts.

To date, the cells directly converted from fibroblasts by ectopic expression of Hnf4α and Foxa1 have been considered to be iHeps (Sekiya and Suzuki, 2011). A surprising finding of the study by Morris et al. (2014) is that iHeps actually appear to be endoderm progenitors (iEPs) (Figure 1E). They have the capacity to differentiate in vitro into either mature hepatocyte-like cells, if Cdx2 is depleted, or intestine-like cells, if the CellNet-prioritized factors Klf4 and Klf5 are overexpressed. Mature cells resembling colonic epithelium are also generated when iEPs are transplanted into the colon—even without Klf4 and Klf5. Furthermore, CellNet, along with other analyses, reveals that cells possessing mature hepatocyte properties can be generated from fibroblasts if low levels of Hnf4β and Foxa1 are supplied. Now, it would be interesting to compare the GRNs of iEPs to those of other iHeps generated by different transcription factors (Vallier, 2014) or to those of the bipotential hepatic stem cells generated from fibroblasts by transduction with Hnf1α and Foxa3 (Yu et al., 2013).

According to Cahan et al. (2014), reprogramming somatic cells into induced pluripotent stem cells (iPSCs) is the most complete and successful pluripotency manufacturing procedure. Indeed, the GRNs of iPSCs and ESCs are essentially indistinguishable. Reprogramming success apparently depends on a well-established protocol with a combination of well-defined transcription factors, culture conditions, and selection makers. The resultant iPSCs comprise a homogeneous population with a high clonality, as a single cell readily expands into an individual clone. Furthermore, current culture conditions, for example 2i, support the growth of only undifferentiated cells. The question remains how well reprogrammed cells correspond to the in vivo counterparts, namely the inner cell mass (ICM) of blastocysts. They might not correspond, because the culture conditions during the reprogramming process determine whether the cells eventually acquire a naive or a primed pluripotent state (Han et al., 2011). Moreover, a recent description of abnormalities in human pluripotent cells generated by reprogramming (Ma et al., 2014) suggests that there is considerable room for improvement in this field as well.

Taken together, the two studies discussed here have established that assessment of the fidelity of in-vitro-manufactured cells and improvement of their quality using CellNet is feasible. This achievement marks a compelling step forward in the production of relevant cells for regenerative medicine. However, several points remain to be addressed. CellNet cannot distinguish between distinct cell subtypes, as it operates largely based on bulk tissues. Cells cultured in vitro often exhibit specific characteristics imposed upon them by their culture environment (Han et al., 2011), whereas the majority of the training data in CellNet is generated from in vivo tissues. Those factors may increase false-positive rates and in turn lead to inaccurate conclusions. Employing single-cell RNA sequencing data from distinct cell types that have been actively cultured in vitro may resolve the issues. Future studies should aim to transfer this technology to cells from patients with genetically inherited disorders for screening aberrant networks and finding ways to correct these networks by means of genetic manipulation.

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Lights, X-Rays, Oxygen!

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Photosystem II uses metal ions to oxidize water to form O_2. Two recent papers employ the new technique of serial femtosecond crystallography utilizing X-ray free-electron lasers and nanocrystals to obtain initial structures of intermediate states of photosystem II catalysis at the site of oxygen production.

Walking through the green of summer should remind us of the unseen molecular machinery that plants, algae, and cyanobacteria use to harness light for the conversion of water and carbon dioxide into sugars and oxygen. Photosynthesis maintains Earth's oxygen levels and provides the basis of our food chain (Blankenship, 2014). Membrane-bound multiprotein complexes, photosystems I and II, catalyze the light-driven reactions at the heart of this process. Two recent articles use serial femtosecond crystallography to capture time-resolved snapshots of changes in the oxygen-evolving center of photosystem II (Kern et al., 2014; Kupitz et al., 2014). These studies not only provide insight into structural events occurring