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Transdifferentiation: do transition states lie on the path of development?

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Abstract

The direct conversion of one differentiated cell fate into another identity is a process known as Transdifferentiation. During Transdifferentiation, cells pass through intermediate states that are not well understood. Given the potential application of transdifferentiation in regenerative medicine and disease modeling, a better understanding of intermediate states is crucial to avoid uncontrolled conversion or proliferation, which pose a risk for patients.

Researchers have begun to analyze the transcriptomes of donor, converting and target cells of Transdifferentiation with single cell resolution to compare transitional states to those found along the path of development. Here, we review examples of Transdifferentiation in a range of model systems and organisms. We propose that cells pass either through a mixed, unspecific intermediate or progenitor-like state during Transdifferentiation, which, to varying degrees, resemble states seen during development.

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Transdifferentiation, Reprogramming, Cell conversion, Development, Cell states.

Introduction

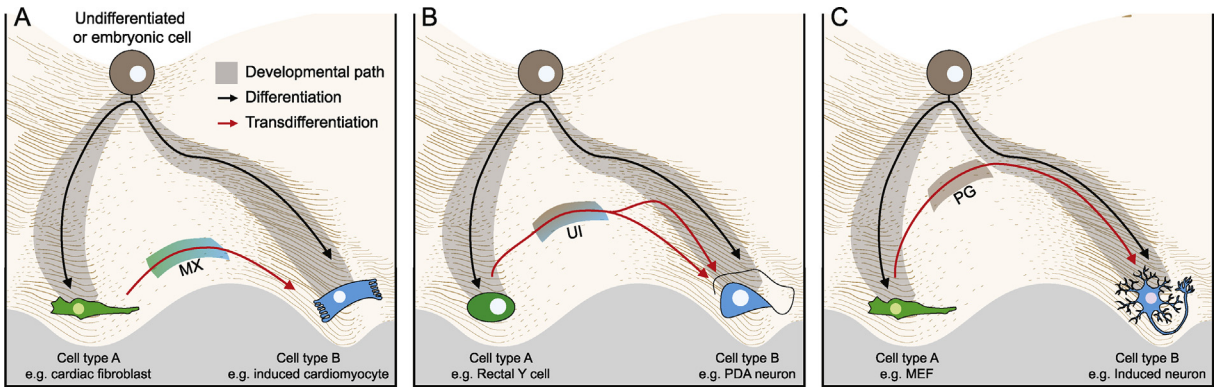
The direct conversion of one differentiated cell fate into another identity is a process known as transdifferentiation (Td). It has been hypothesized, however, that most, if not all, converting cells go through an intermediate state and that the 'direct' aspect of Td is not as clear-cut as previously thought [27]. During Td, cells were thought to pass through transitional states resembling those seen during development. For many examples of Td this holds true as, for instance, fibroblasts undergoing Td to neurons pass a progenitor-like state which, at the level of their gene expression profile, partly resembles a state seen during normal development [41]. Transitional states that are different to those in development also occur. For example, during pancreatic alpha- to beta-cell conversion, mixed-state cells express markers for both alpha- (glucagon) and beta-cells (insulin) following beta-cell ablation *in vivo* [40].

Understanding the states that cells pass through during Td is crucial given its potential application in regenerative medicine and disease modeling. However, one important question pertaining is whether cells pass through an intermediate state and, whether such cells could undergo uncontrolled conversion or proliferation and pose a risk for patients. To answer this, researchers have begun to analyse the transcriptomes of donor, converting and target cells to explore the mechanisms of Td. By doing so, one can also identify the barriers that prevent Td and, if conversion is occurring, the degree to which the original fate is lost. These efforts have been greatly assisted by the development of single cell RNA-sequencing (RNA-seq) technologies. Studies can now compare transitional states occurring during Td to those found along the path of development at a single cell level.

Here, we review several key examples of Td that were studied in a range of model systems and organisms. We propose that cells pass either through a mixed, unspecific intermediate or progenitor-like state during the course of Td, which, to varying degrees, resemble states seen during development (Figure 1).

It is important to note that the Td transition states defined here broadly reflect a complex process and cells undergoing Td may lie at the intersection of these definitions. The nature and complexity of transitional states observed during Td are likely to be influenced by donor- and target-cell types, as well as the environmental context. We also briefly discuss the role of terminal selector transcription factor(s) (TFs) and highlight the need to carefully assess the degree to which the original fate is turned off during Td. Overall, our understanding of Td at the single-cell level will be crucial for characterising transitional states observed during Td.

Figure 1



Converting cells can deviate from the path of development. Cells undergoing Td can pass through (A) mixed 'MX' (B) unspecific intermediate 'UI' or (C) progenitor-like 'PG' transition states. Figures are modified versions of the Waddington landscape [4].

Mixed states during Td

With the concomitant loss of one fate and the gain of another, a converting cell will pass through a mixed state where both fates are present. How a cell navigates this change in identity, and emerges as a differentiated cell with a new fate, is quite remarkable given the fact that this state would be likely absent during development. Moreover, many Td events are initiated by only a single or few TFs which initiate a program leading to target fate acquisition. For example, expression of Mef2c, Gata4 and Tbx5 (MGT) leads to Td of mouse cardiac fibroblasts to induced cardiomyocytes (iCMs) via several states defined by Liu et al., as intermediate fibroblast, pre-iCM and iCM [19]. Single-cell transcriptome analysis suggests that the pre-iCM state is unstable and represents a 'mixed' state where both cardiomyocyte and fibroblast-specific markers are expressed [19]. This observation was mirrored at the protein level. After induction of mouse fibroblast to iCM conversion upon MGT expression [36], a mild decrease, of fibroblast-specific genes Col1a1 and Col1a2 was observed at the 48–72 h time point [36]. If these cells access developmental programs to achieve conversion, one would expect to detect markers for a progenitor-like state. During fibroblast to iCM conversion, the early cardiac progenitor marker Isl1 and the pan-cardiovascular progenitor marker Mesp1 were not activated, suggesting that Td did not pass through a progenitor-like state [11]. However, in other cases of Td, a mixed state with precursor-like properties exists. For instance, endogenous 'hybrid' $CD4^+$ T cells produce cytokines characteristic of different lineages and, from these cells, multiple cell fates arise as reviewed elsewhere [20].

Interestingly, a mixed state during Td might be the result of a mechanism where the original fate is switched off passively. That is, as the new fate is established, cells fail to maintain the original fate and it 'fades' away over time. This likely occurs because the gene expression program of the target fate dominantly recruits the transcriptional machinery to genes specific to its own fate. To address this hypothesis and tease apart mechanisms of Td, mixed states must be characterized in more detail with single-cell resolution.

Unspecific intermediate states during Td

During Td, cells may lose their original identity prior to acquiring a new fate and this can be interceded by an intermediate state that does not resemble donor nor target fates. Unspecific intermediate states may display aspects of stemness, but do not revert completely to a stem cell-like state. In the nematode *Caenorhabditis elegans*, cells can be traced easily due to its transparent body and highly invariant lineage. In-depth characterization of an endogenous Td event has begun in the Jarriault laboratory where a post-mitotic and functionally differentiated epithelial Y cell of the rectum disengages from the rectal tube, migrates and finally converts to a motor neuron termed PDA [13,48]. During Td, the Y cell de-differentiates, passes through an intermediate state and then redifferentiates into a motor neuron termed PDA. The observed intermediate state does not show marker expression for either the rectal Y (LIN-26) nor the neuronal PDA fate (*cog-1*) [34]. To test whether these cells reverted to a bona fide pluri or multipotent state, they were challenged with transient expression of the cell fate-inducers *hlh-1* (muscle), *end-1* (endoderm), *lin-26* (epithelial) and *unc-30* (GABAergic neurons), but no detour to a new identity was observed [34]. Interestingly, it was later shown that NODE (Nanog and Oct4-associated deacetylase) activity was required for Y cell-to-PDA Td in *C. elegans* [15]. The homologs of NODE complex members, including CEH-6/Oct4 and SOX-2/Sox, are known pluripotency factors in mammals [39]. It is therefore possible, that intermediate cells observed during Y cell-to-PDA Td have reverted to a progenitor-like state with restricted potential, but are distinct from bona fide progenitor-like intermediates.

Another interesting example is the CCAAT/enhancer binding protein alpha (CeBPα) induced conversion of pre-B cells into macrophages. During this Td, cell-surface marker combinations that are characteristic for hematopoietic stem and progenitor cells, such as c-Kit and FMS-like tyrosine kinase 3, were essentially not observed and expression of pluripotency factors Oct4, Nanog and Sox2 was not detected [5]. Cells undergoing pre-B cell-to-macrophage Td show expression of genes specific for the B cell (*Cd19*) and macrophage (*Mac1*) fates being present and, therefore, also display aspects of a mixed transitional state [5,46].

The question of whether, and to what degree, the original fate must first be erased prior to acquisition of the target cell fate may depend on how similar the donor and target cell fates are. Regardless of how alike donor and target cells are, transdifferentiating cells may have to travel along strict development-like paths to achieve conversion. Intermediate states, however, may be distinct from those found during development and unique to Td.

Progenitor-like states during Td

To achieve Td, converting cells may revert to a progenitor state with partial or complete pluripotency before following developmental paths for redifferentiation. Pluripotent-like states can be induced by exogenous expression of the Yamanaka factors OCT4, SOX2, KLF4 and MYC (OSKM) [39]. Interestingly, use of OCT4 was shown to increase the efficiency of directly reprogramming fibroblasts to blood cells through enhancing the induction of haematopoietic progenitor states [38]. In some instances, partial reversion to a progenitor-like state may be sufficient for re-differentiation. For example, Ascl1-induced conversion of mouse embryonic fibroblasts (MEFs) into induced neuronal (iN) cells shows a continuum of intermediate states [41]. Using single cell RNA-seq, Treutlein and colleagues compared cells at intermediate positions along the MEF-to-iN trajectory with neural precursor cell (NPC) bulk transcriptomic data. They found that several NPC markers including *Gli3*, *Sox9*, *Nestin*, *Fabp7*, *Hes1* but not canonical NPC marker genes such as *Sox2* and *Pax6*, were expressed in converting cells [41]. Their data suggests a state which is distinct from donor and target cell fates that has similarities to a progenitor-like state, in this case, NPCs. However, the observed state also deviates from the NPC state seen during development as canonical NPC marker genes are missing [41]. In further support of a progenitor-like state resembling the natural one seen during development, Ascl1 was found to bind the same sites as in neural progenitor cells during fibroblast to induced neuronal (iN) conversion [45].

A progenitor-like state resembling developing neurons was also observed *in vivo* during the TF-induced conversion of germ cells into neuron-like cells in *C. elegans*. Following knockdown of the histone chaperone LIN-53 (homolog of CAF-1p48/RBBP7 in mammals) and overexpression of the Zn-finger TF CHE-1, germ cells undergoing conversion to neurons express the bHLH gene *hlh-2*/Daughterless thereby indicating a state of immature neurons [18,30]. This *in vivo* Td phenomenon further suggests that cells can follow a development-like path to achieve identity conversion.

During regeneration, cells might transdifferentiate to replace lost cells and tissues and, like in the examples below, undergo dedifferentiation, proliferation and redifferentiation to do so. Limb regeneration is possible following amputation in the salamander *Ambystoma mexicanum* (axolotl) [17]. The blastema, a mass of undifferentiated cells from which a new limb is formed, was shown to contain cells in a progenitor-like state with restricted potential [17]. Using CRISPR-derived lineage tracing, it was recently suggested that axolotl limb regeneration recapitulates development of the original limb [8]. However, an important factor for limb regeneration, *kazald1*, is not expressed in the progenitor cells of the developing limb bud suggesting a Td-specific path [1].

For zebrafish heart regeneration, cardiomyocytes have been suggested to follow a path distinct from cardiac development to convert into heart muscles [14]. Converting cardiomyocytes lose characteristic features such as the sarcomere, and expression of sarcomeric genes including ventricular myosin heavy chain (vmhc), suggesting reversion to a progenitor-like state. However, markers for precardiac mesoderm, RNAs of *nkx2.5* and *hand2*, were not significantly upregulated [14,37].

Transdifferentiating pigment epithelial cells of the dorsal iris pass through a progenitor-like state during regeneration of the lens upon lentectomy in newts [7,32,42,47]. During lens regeneration, pluripotency factors Sox2 and Klf4 [22] and the oocyte-type linker histone B4, which is expressed during oogenesis and early embryogenesis, are detected [21]. However, converting cells demonstrate constrained potential as they still form lens tissue when transplanted in other regions of the newt [12,26,33]. These results suggest that transdifferentiating cells can follow development-like paths to achieve regeneration, as previously mentioned [42].

The regenerative capabilities of plants exemplify another transition through a progenitor-like state during cellular conversion. Following excision of the root meristem from *Arabidopsis*, which contains the stem cell niche and supporting cells (quiescent center (QC)), endodermal cells acquired a progenitor-like state prior to replacing the lost tissue [6]. Single cell transcriptomics of a population of 'stele' cells from uncut

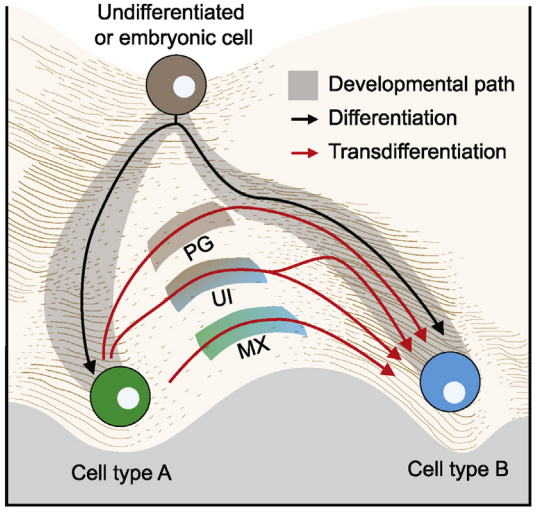
and regenerating roots showed that converting cells lost their stele identity and expressed a mixture of distal identities, including QC, columella and epidermal, before generating progenitors with distinct identities. Additionally, the mixed-identity cells express genes characteristic of the hypophysis, a precursor cell, which gives rise to progenitors for the QC and columella as studied [3,6,29], and previously reviewed [10].

For the *in vivo* examples above, progenitor-like transition states do not appear to fully recapitulate a stem cell-like state and, instead, show restricted potential (Figure 2). Limiting the potential of these intermediate states *in vivo* may ensure that no unexpected cell types are formed, thereby ensuring robust Td. For therapeutic applications of Td, providing an *in vivo*-like context with appropriate intrinsic and extrinsic signals, may constrain deleterious transition states during Td to a point that it would be considered robust and, therefore, safe.

Constraining transitional states during Td

Environmental context has a significant impact on Td. By recreating aspects of the *in vivo* environment during development, transitional states observed during Td may be constrained and Td efficiency improved. As mentioned in previous reviews, some reprogramming strategies include exposing cells to the *in vivo* niche and environment which provide additional inputs to aid maturation [9,27]. For example, mouse myotubes were endowed with the regenerative-like capabilities of the newt after being exposed to an 'extract isolated from regenerating newt limbs'. Following exposure, the myotubes showed reduced expression of muscle-specific genes, myoblast determination (MyoD) and myogenin, and started proliferation [25]. Additionally, the robustness of inducing cardiomyocyte-like cells by GMT overexpression is improved *in vivo* compared with *in vitro* conditions [11,31].

Figure 2



A summary of the different modes of Td. Transdifferentiating cells may pass through mixed 'MX', unspecific intermediate 'UI' or progenitor-like 'PG' transition states. Figure is a modified version of the Waddington landscape [4].

The environmental context also includes a cell's history. During Y cell-to-PDA neuron conversion in *C. elegans*, the number of neighboring cells did not affect Td, however, Notch signalling (*lin-12*) is required at the time of Y cell specification, but not when Td occurs *per se* [13]. Understanding which aspects of the cellular environment and history regulate Td *in vivo* will be of great importance to overcome the current limitations of Td including low efficiency, fate immaturity and incomplete suppression of the original identity.

Understanding development to improve cellular maturation in Td

In a global sense, specification and differentiation during Td are similar to that observed during development. This is particularly clear during later stages of Td where, similar to development, transcriptional programs initiated during earlier stages are strengthened and morphological changes occur. For instance, a 'maturation stage' was observed during MEF to iN induction where synapse-related genes were switched on [41]. During development, completing cellular differentiation often involves terminal selectors, TFs that activate expression of cell-type-specific genes. It isn't surprising, therefore, that terminal selectors also play a key role in Td. The terminal selector UNC-3 is required for Y cell-to-PDA Td in *C. elegans*, as redifferentiation fails in *unc-3* mutant animals [34]. Ectopic UNC-3 expression also initiates the conversion of germ cells into cholinergic neurons in *C. elegans* [43]. As mentioned before, the terminal selector CHE-1 is sufficient to induce the neuronal fate in germ cells and drives neuronal maturation during both Td and normal development [43,44]. Understanding the role of terminal selectors during development, with particular focus on those involved in both specification and maturation, may improve current or enable novel modes of Td.

Failure to activate the appropriate developmental gene regulatory networks during Td and to deactivate the specific gene expression program of the differentiated cell can result in immature or off-target identities and incomplete suppression of the original fate. In this context, the increased efficiency during MEF to iN conversion by combining the neuron fate-inducing TF Ascl1 with the Myt1-like (Mytl1) TF, is based on Mytl1-mediated suppression of fibroblast and myocyte genes [23,41]. Furthermore, enhanced reprogramming of

human and mouse fibroblast cultures to cardiomyocytes can be achieved when, in combination with GMT plus Mesp1 and Myocd (GMTMM) or GMT, respectively, the micro RNA miR-133 is overexpressed, which represses fibroblast gene expression [28]. Hence, suppressing the original donor cell identity can improve Td, which is an important aspect that needs to be considered when aiming to increase efficiency of successful cell fate conversion.

Concluding remarks

Conversion factors engage developmental gene regulatory networks to induce Td, however, Td can deviate from development and use Td-specific mechanisms. For instance, in *Drosophila*, the conversion of imaginal disc cells to wing cells by *wingless* (leg-to-wing) and eye-to-wing Td by *winged eye*, may utilise a common Td-specific gene set including *dilp8*, *upd* and *Mmp1*, which are not expressed during normal differentiation of the converted region [16,24].

An alternative path to acquire a new fate might be treaded upon by removal of specific factors that act as reprogramming barriers, such as chromatin remodelers. Depleting barrier genes may assist different reprogramming events, without a change in cellular state, by mimicking an artificial but permissive epigenetic landscape. Returning to a 'precursor state of chromatin' organization which resembles that of developing cells may favour reprogramming, as previously hypothesized [9]. Such instances may be the knockdown of *lin-53* (CAF-1p48/RBBP4) in order to reprogram germ cells to neurons in *C. elegans* [43] and depletion of CAF-1 during reprogramming of MEFs to induced pluripotent stem (iPS) cells or iNs [2].

Another recently discovered natural Td event, which depends on the sex of the animal, might represent an additional path of cell fate conversion. Td of a specific glial cell, known as AMso in *C. elegans*, into a neuron, occurs solely in sexually maturing males. Further investigation may reveal through which states and paths the AMso glial cells pass in order to successfully convert to a neuron [35].

Here, we have used Td to describe a range of cellular conversion events whilst acknowledging that the 'direct' aspect, formerly associated with Td, may need redefining as mentioned elsewhere [27]. Overall, Td is a complex process and our understanding of transitional states at the single-cell level is still limited. The environmental context and higher order contexts such as gender, age and other physiological conditions may impact on the process of cellular conversion.

Conflict of interest statement

Nothing declared.

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