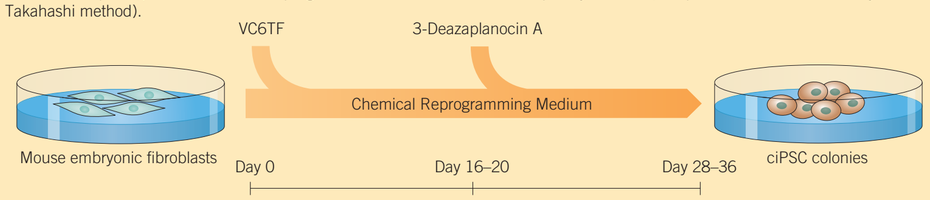
Stem Cell Workflow: Using Small Molecules

Stem cells (SCs) have immense potential as a limitless source of cells and tissues for research and treatment of various diseases, as well as for investigating early embryonic development. Small molecules can be used at all stages of the stem cell work flow, and their use has several advantages over standard techniques: small molecules are chemically-synthesized, versatile, animal-free, and are cell permeable. In addition, their effects are rapid and reversible, so their use can reduce the duration of reprogramming and differentiation protocols.

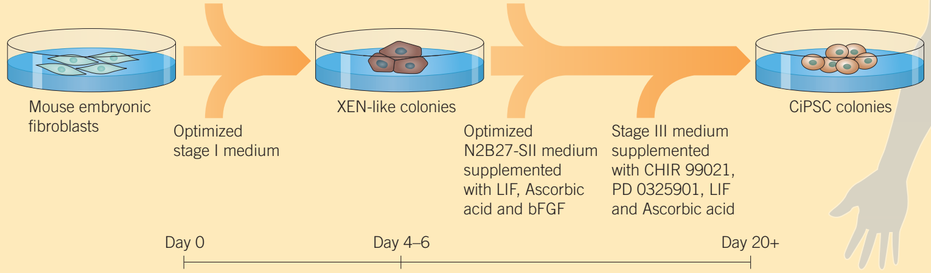
Reprogramming

Reprogramming is the regression of a specialized cell to a simpler state resulting in cells with stem-like properties known as induced pluripotent system cells (iPSCs). Takahashi *et al.* (2007) first reported reprogramming of specialized human adult cells by introducing the transcription factors Oct3/4, Sox2, Klf4 and c-Myc (known as the Yamanaka factors or OSKM) into adult human dermal fibroblasts via retroviral transduction. The resulting iPSCs had similar properties to ESCs and could differentiate into all three embryonic germ layers. Similar techniques are still widely used.

Small molecules can be used to enhance reprogramming efficiency: **Valproic acid** (VPA) can increase reprogramming efficiency by Yamanaka factors by >100 fold. Using a cocktail of small molecules and growth factors to reprogram cells dispenses with the need for retroviral transduction and increases the efficiency of reprogramming. Hou *et al.* (2013) first described the generation of iPSCs using only small molecules. The researchers discovered that a combination of six compounds, **VPA**, **CHIR 99021**, **RepSox**, **Tranylcypromine**, **Forskolin** (these 5 compounds are together known as **VC6TF**) and **3-Deazaneplanocin A**, followed by culture in 2i medium (see Self-Renewal panel), could be used to reprogram mouse somatic cells at a frequency of 0.2% (compared with 0.01 – 0.02% by the Takahashi method).



Modification of this protocol by Zhao *et al.* (2018) led to a highly efficient method for generating ciPSCs from mouse embryonic fibroblasts (MEFs). The three-stage process uses 12 compounds (including VC6TF) plus the growth factors, LIF and bFGF to generate ciPSCs in 16 to 20 days compared with 30 days using Yamanaka factors. This type of chemical reprogramming holds promise for the generation of cells for cell therapy and disease modeling.

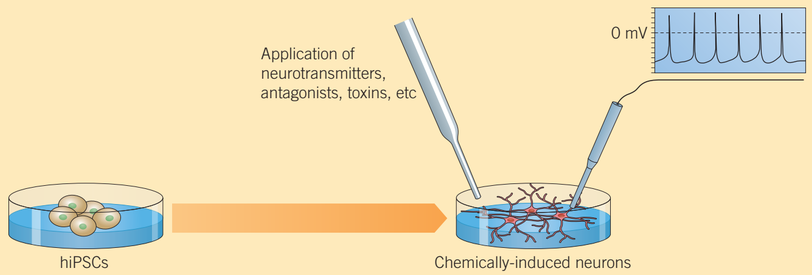


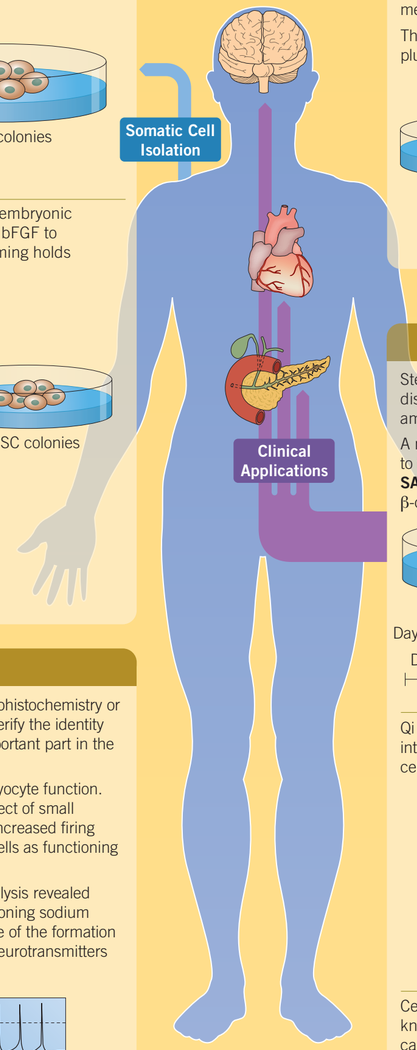
Verification

The characterization of differentiated cells is a necessary step before the cells can be used in assays or therapy. Immunohistochemistry or Western blotting, using antibodies against specific markers expressed by the target cell, are techniques widely used to verify the identity of differentiated cells, as is quantitative RT-PCR, which is used to analyze gene expression. Small molecules play an important part in the verification of cell function, by pharmacological or electrophysiological techniques.

Following conversion of fibroblasts into cardiomyocytes, Cao *et al.* established the presence of genes involved in cardiomyocyte function. Electrophysiological analysis of the cells revealed ventricular-like action potentials. The researchers then looked at the effect of small molecules on action potential firing and found that **Caffeine** and the non-selective β-adrenoceptor agonist **Isoprenaline** increased firing rate, while the muscarinic agonist **Carbachol** slowed the firing rate, helping to establish the identity of the differentiated cells as functioning cardiomyocytes.

Telezhkin *et al.* (2016) describe the verification of functional neurons differentiated from iPSCs. Electrophysiological analysis revealed spontaneous action potential firing, which was eliminated by application of **Tetrodoxin**, indicating the presence of functioning sodium channels. Application of **GABA** induced inhibitory postsynaptic currents that could be blocked by **Bicuculline**, indicative of the formation of a functioning neural network. Calcium imaging using **FURA-2AM**, revealed Ca^{2+}-influx in response to application of neurotransmitters GABA, **Glutamate** or **Glycine**. Techniques such as these provide evidence for the presence of mature functioning cells.



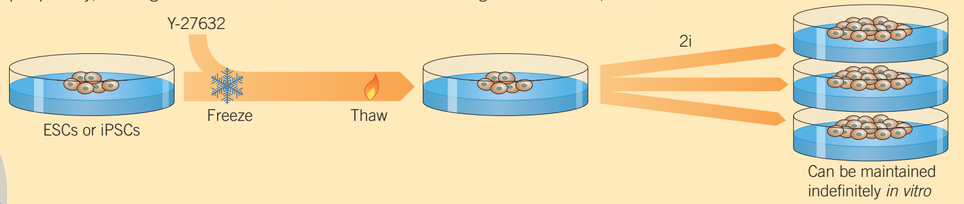


Self-Renewal

Stem cells self-renew and proliferate by the division of a parent cell into two identical daughter cells. Conventional culture methods for PSCs require 'feeder' cells, serum products and growth factors, such as LIF and bFGF. More recently, chemically-defined serum-free media have been developed to replace the requirement for feeder cells and serum products, and small molecules can be used to maintain self-renewal of stem cells.

Chen *et al.* were the first to show that a small molecule, SC 1 (also known as Pluripotin), which inhibits differentiation, can be used to maintain pluripotency in the absence of growth factors. Ying *et al.* (2008) subsequently identified a combination of two small molecules (known as **2i**), the MEK inhibitor **PD 0325901** and GSK-3 inhibitor **CHIR 99021**, which can sustain ESC self-renewal. ESCs grown in 2i medium show lower levels of spontaneous differentiation compared with standard SC culture methods. 2i can also be used to rescue cultures that have started to deteriorate.

The ROCK inhibitor **Y-27632** also has an important role in stem cell maintenance as it increases cloning efficiency of ESCs without affecting pluripotency, enabling the survival of stem cells in culture over the long-term (Watanabe, 2007).



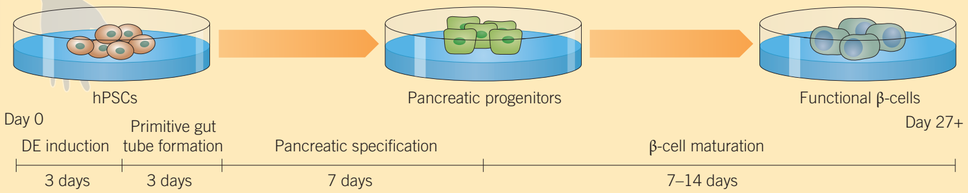
Storage

Cryopreservation is used to store cells, including stem cells, for *in vitro* culture, however after freeze-thawing the survival rate of cells can be poor. Li *et al.* (2008) found that treatment of hESCs with the ROCK inhibitor Y-27632 prior to freezing, significantly increases the viability of cells after thawing. Y-27632 also improves the survival of cells that have been differentiated from PSCs, facilitating their use in disease modeling and therapy.

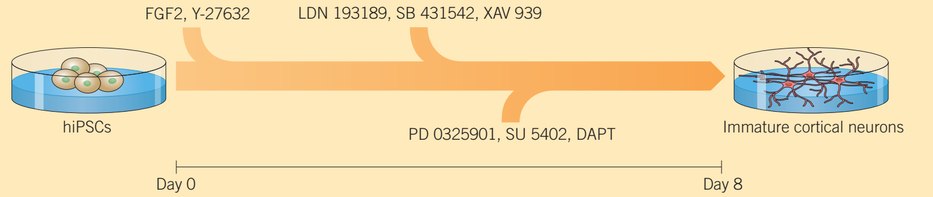
Differentiation

Stem cells can be differentiated into numerous cell types with a variety of potential uses, including drug screening, toxicity testing and disease modeling. They also hold promise for treating conditions such as neurodegenerative diseases, diabetes, and traumatic injury, among others. Small molecules are versatile tools to control stem cell fate and direct differentiation toward specific cell types.

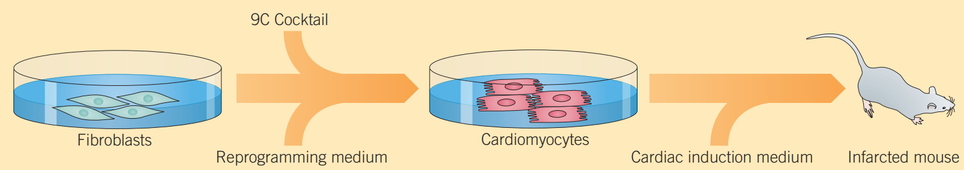
A method to derive functional human pancreatic β-cells from hPSCs designed by Pagliuca *et al.* (2014) presents the possibility of a new way to treat diabetes. Their six-stage protocol uses a combination of 11 small molecules and proteins (**Activin A**, **CHIR 99021**, **Retinoic Acid**, **SANT-1**, **LDN 193189**, **Phorbol 12,13-dibutyrate**, **T3**, **Compound E**, **RepSox**, **Heparin**, **Betacellulin** and **KGF**) and produces functional β-cells in 28 days, which when transplanted into diabetic mice are found to ameliorate hyperglycemia.



Qi *et al.* (2017) have developed a protocol using a cocktail of six small molecules to derive cortical neurons from hiPSCs. When transplanted into postnatal mouse cortex at day 8 of differentiation the resulting neurons are functional and establish long term connections. In addition, cells remaining in culture exhibit functional electrophysiological properties by day 16.



Cells can also be reprogrammed directly from one specialized cell type to another, without first being converted to ciPSCs, a process known as transdifferentiation or direct-lineage reprogramming. Cao *et al.* (2016) described a method to convert human fibroblasts into cardiomyocytes using a cocktail of nine small molecules: **CHIR 99021**, **A83-01**, **SC1**, **OAC-2**, **Y-27632**, **BIX 01294**, **A 8351**, **SU 16f** and **JNJ 10198409 (9C)**. The 9C-treated cells were subsequently cultured in cardiac induction medium and transplanted into mice where they converted into cardiomyocyte-like cells.



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Reprogramming
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3-Deazaneplanocin A
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DBZ
Thiazovivin
(±)-Bay K 8644
Trichostatin A
RepSox
Valproic acid, sodium salt
Kenpaullone
Alsterpaullone
SMER 28
L-Ascorbic acid
BIX 01294
Crotonic Acid
Tranylcypromine

Differentiation
LDN 193189
XAV 939
DAPT
SAG dihydrochloride
Dibutyryl-cAMP, sodium salt
Forskolin
SU 5402
IWP 2
IWP 4
IDE 1
IBMX
Fluoxetine
Metformin
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ISX 9
Dexamethasone
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Proliferation and Cell Viability
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Prostaglandin E2
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MB 05032
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Go 6983
SB 203580
A 769662
LY 294002
CH 223191
Pluripotin
SB 216763
BIO
PD 98059
PD 173074
Troglitazone
Cyclopamine
Mitomycin C

GMP Small Molecules
Y-27632
CHIR 99021
SB 431542
DAPT

Abbreviations

bFGF	Basic fibroblast growth factor
ciPSC	Chemically-induced pluripotent stem cell
ESC	Embryonic stem cell
GSK	Glycogen synthase kinase 3
KGF	Keratinocyte growth factor
LIF	Leukemia inhibitory factor
MEK	Mitogen-activated protein kinase kinase (MAP2K)
PSC	Pluripotent stem cell
ROCK	Rho-kinase
RT-PCR	Reverse transcription polymerase chain reaction
T3	Triiodothyronine
XEN	Extraembryonic endoderm

References
Cao, N *et al.* (2016) *Science* **352** 6290
Chen, S *et al.* (2006) *Proc.Natl.Acad.Sci.USA* **103** 17266
Hou, P *et al.* (2013) *Science* **341** 651
Ichikawa, H *et al.* (2011) *Cryo Letters* **32** 516
Li, X *et al.* (2008) *Stem Cells Dev.* **17** 1079
Pagliuca, FW *et al.* (2014) *Cell* **159** 428
Takahashi, K *et al.* (2007) *Cell* **131** 861
Tamm, C *et al.* (2013) *PLoS One* **8** e81156
Telezhkin, V *et al.* (2016) *Am.J.Physiol.Cell Physiol.* **310** C520
Watanabe, K *et al.* (2007) *Nat.Biotechnol.* **25** 681
Wilson, HK *et al.* (2016) *Tissue Eng. Part C, Methods* **22** 1085
Ying, QL *et al.* (2008) *Nature* **453** 519
Zhao, T *et al.* (2018) *Cell Stem Cell* **23** 31

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