

# Stem Cell Workflow: Using Small Molecules

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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 workflow, 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.

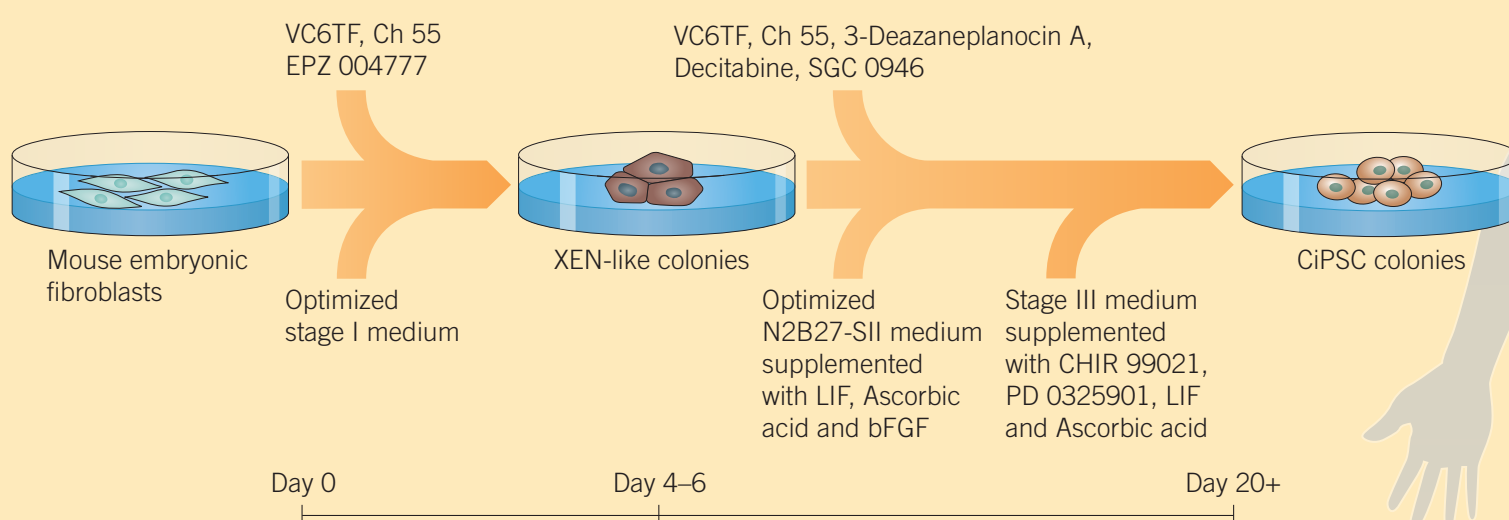
## Reprogramming

Reprogramming is the regression of a specialized cell to a simpler state resulting in cells with stem-like properties known as induced pluripotent stem 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 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-Deazaplanocin 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).

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-Deazaplanocin 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.

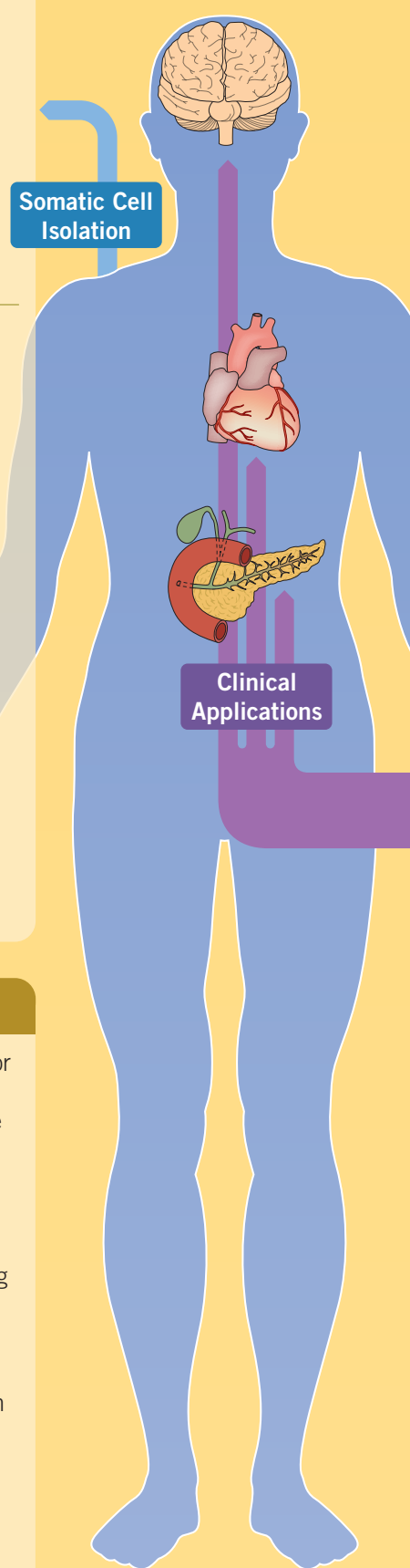
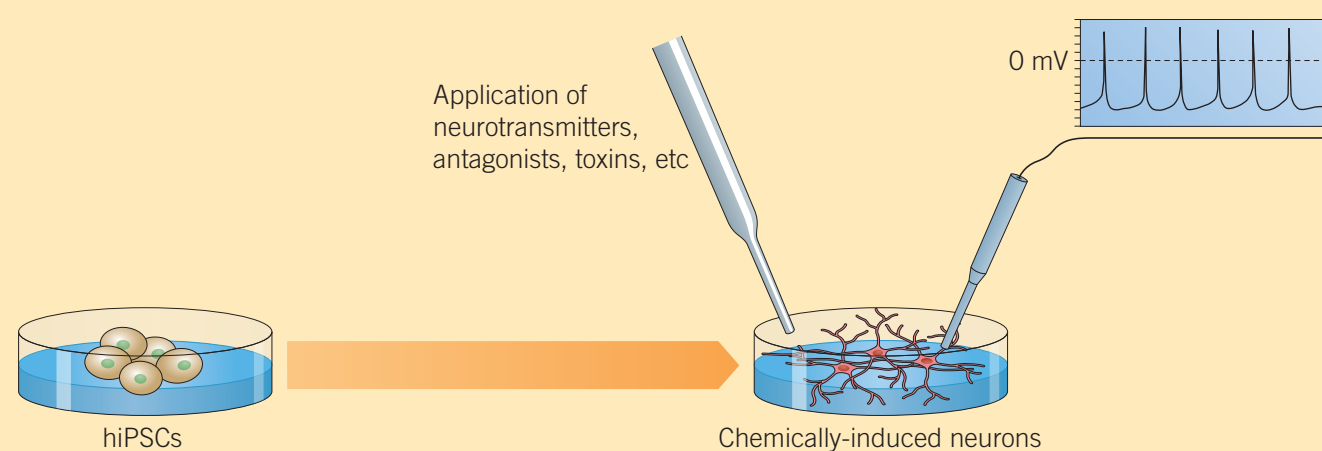


## Verification

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  $\beta$ -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.

Telezkin *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 **Tetrodotoxin**, 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.

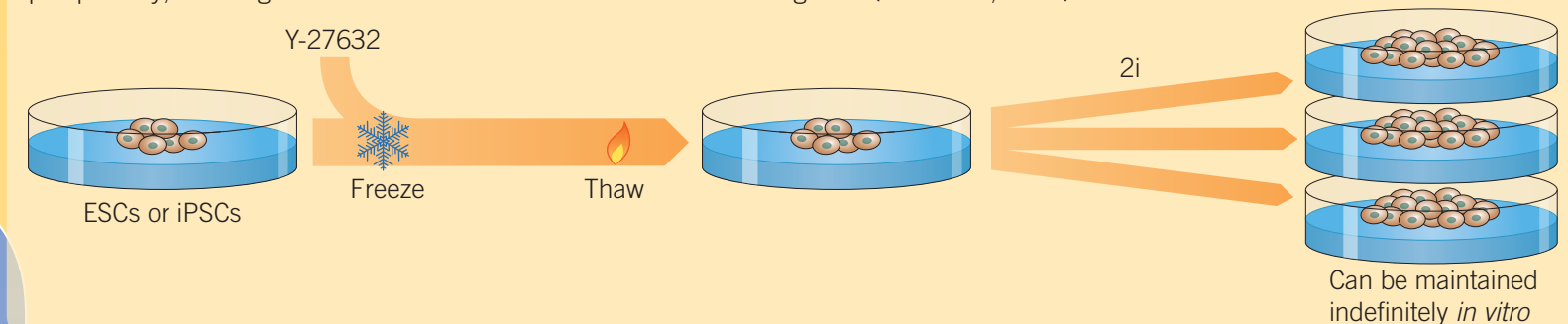


## Self-Renewal

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).



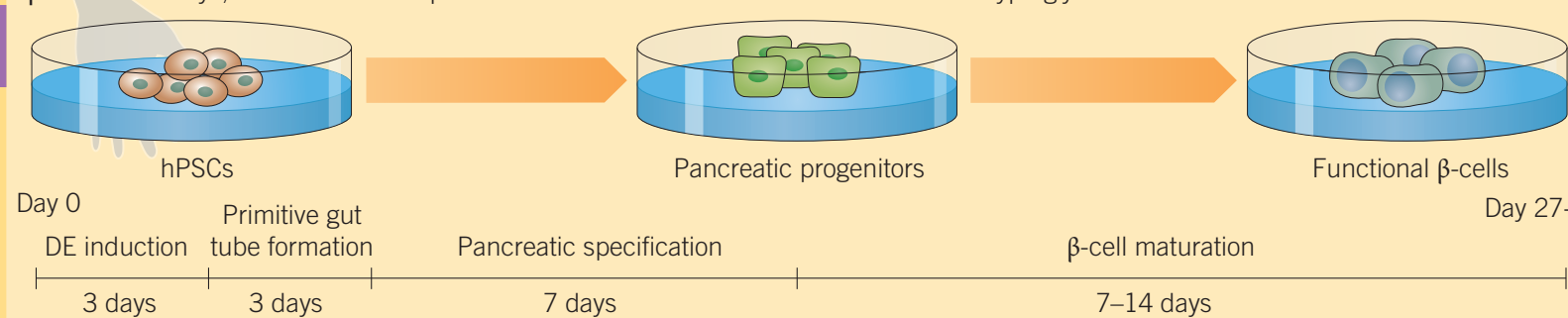
## Storage

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.

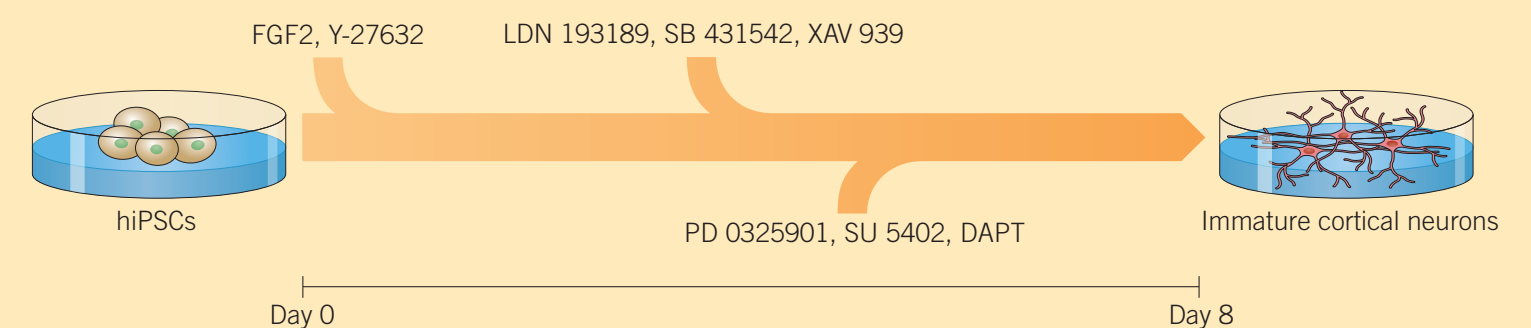
## Differentiation

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.

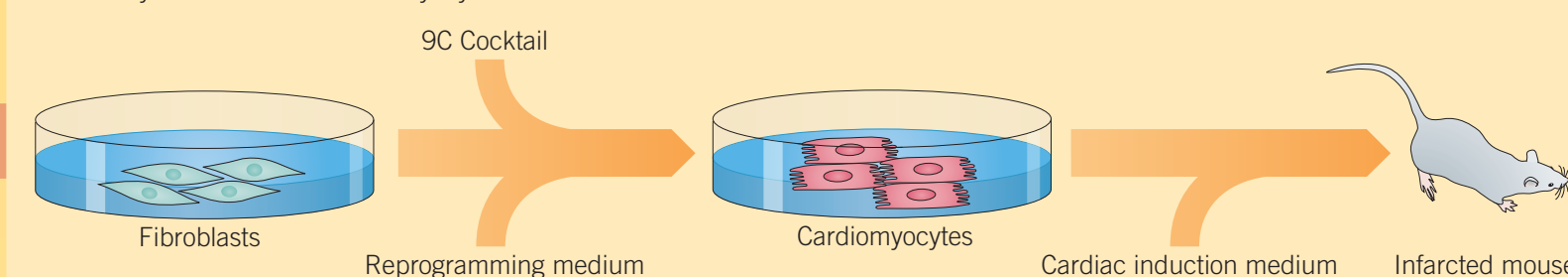
A method to derive functional human pancreatic  $\beta$ -cells from hPSCs designed by Pagliuca *et al.* (2014) presents the possibility of a new way to treat diabetes. Their six-stage protocol uses a combination of 11 small molecules and proteins (**Activin A**, **CHIR 99021**, **Retinoic Acid**, **SANT-1**, **LDN 193189**, **Phorbol 12,13-dibutyrate**, **T3**, **Compound E**, **RepSox**, **Heparin**, **Betacellulin** and **KGF**) and produces functional  $\beta$ -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 161** 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.



## Products available from Tocris

### Reprogramming

CHIR 99021  
PD 0325901  
SB 431542  
A 77-01  
3-Deazaplanocin A  
(S)-(+)-Dimethindene  
DBZ  
Thiazovivin  
(+)-Bay K 8644  
Trichostatin A  
RepSox  
Valproic acid, sodium salt  
Kempellone  
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  
Dorsomorphin  
Wnt-C59  
1-EBIO  
ISX 9  
Dexamethasone  
Zebularine

### Proliferation and Cell Viability

Y-27632  
A 83-01  
Prostaglandin E2  
SB 202190  
Epiblastin A  
MB 05032  
U0126  
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 (MAP2K)  
PSC Pluripotent stem cell  
ROCK Rho-kinase  
RT-PCR Reverse transcription polymerase chain reaction  
T3 Triiodothyronine  
XEN Extraembryonic endoderm

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