Small molecule-induced cellular conversion†

Debojyoti De, Debasish Halder, ‡ Injae Shin* and Kyeong Kyu Kim*ac

Over the last decade, the development of methods to promote conversion of one type of cell to a specific type of another cell (or change of cell fate) has received great attention in basic biological research and therapeutic applications. A precise, reproducible and safe protocol for inducing this change is a prerequisite for cellular conversion. Although genetic manipulation, which relies on the introduction of specific genes into cells, is a promising approach, the results of initial investigations have highlighted serious safety concerns associated with forced ectopic gene expression with unpredictable side effects. Alternatively, a chemical approach that relies on the use of small molecules to modulate the cell fate has great potential in terms of precise control and clinical safety. In addition, the ease of application, reproducibility and scalability are features that make a small molecule-based approach an extraordinary resource for this purpose. In this review we summarize methods which have been devised to identify small molecules that induce cellular conversion and highlight recent advances made using small molecule modulators to induce changes in the fate of cells.

1. Introduction

Terminal differentiation of cells is critical for the successful regeneration of damaged tissues. Progenitor cells in some organs and tissues can differentiate into adult cells to replace damaged cells. However, certain types of cells, such as neurons, cardiomyocytes, pancreatic cells and nephrons, in vital organs and tissues are difficult to be regenerated under diseased conditions. In this case, cell therapy (or cell transplantation) is required to regain function. In this regard, regenerative medicine which deals with ‘the process of replacing or regenerating human cells, tissues or organs to restore or establish normal functions’ has led to renewed hope.1 Over the last decade, advances in this field have provided fundamental insight into the regulatory mechanisms for cell differentiation and strengthened the promise of therapeutic applications of this cell regeneration strategy.

Initial efforts to produce various cell lineages have been accomplished through inducing differentiation of embryonic

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stem cells (ESCs). However, owing to ethical and immunogenicity issues (i.e., rejection of allogeneic transplants by the host’s immune system) associated with the use of ESCs, induced pluripotent stem cells (iPSCs), generated from adult somatic cells such as fibroblasts, are used for differentiation into specific types of cells. These efforts have not only created the possibility of an effective approach to cell therapy but they have also generated excellent platforms for disease modelling, drug discovery and understanding the intricacy of embryonic development. However, owing to safety concerns such as the onset of teratoma formation, the use of ESCs and iPSCs is restricted to clinical settings. To avoid these issues, multipotent adult stem cells, such as mesenchymal stem cells, obtained from adipose tissue, bone marrow, tonsil and umbilical cord blood, have been studied extensively with respect to their potential to differentiate into various cell types. Although they have been applied for regenerative purpose, the use of multipotent adult stem cells for promoting cell fate changes is also narrowed by the presence of certain limitations such as insufficient amounts of source cells and their limited ability of differentiation. Alternatively, trans-differentiation, a conversion of somatic cells to different lineages without passing through a pluripotent state, is a promising approach for production of patient-specific cells for numerous applications.

Genetic approaches, which rely on introduction of exogenous genetic materials through viral-mediated transduction, have been used to promote cell fate changes. However, they have a potential risk of integrating a foreign DNA fragment into the genome, which leads to unpredictable side effects such as tumor formation (Table 1). In addition, the transient introduction of genes of interest into cells, which is relatively safer compared to viral vector-mediated transduction, has also been employed to induce changes in cell fate, but it has disadvantages such as a low efficiency, a complicated experimental setup and high cost (Table 1). These concerns about the genetic approach restrict its use in clinical applications. Alternatively, utilization of small molecules which modulate changes in cell fate (termed ‘small molecule-based cellular alchemy’) gained impetus more recently. Practical advantages being the precise spatio-temporal control of cell fate changes, rapid response times, reversibility, tunability, reproducibility and scalability (Table 1). Moreover, they can be structurally and functionally optimized to enhance their desired effect through synthetic chemistry. Despite such advantages over the genetic approaches, chemical approaches may suffer from unintended activity against off-targets or multiple targets, which in turn causes side-effects. The clinically feasible strategy for cell fate change could be the small molecule-based approach for converting somatic cells to target cells. One of the early examples of this protocol is differentiation of skeletal muscle cells into neurogenic cells using the small molecule, neurodazine.

The promise held using small molecules to bring about cellular conversion is reflected in a number of recent reports. Continuously expanding efforts in this area suggest that application of the chemical approach to cell therapy has enormous potential. In addition, small molecules that modulate the cell fate can be used as chemical tools to understand molecular networks and signaling responsible for the identities of a particular cell type. In this review, we briefly summarize screening methods that have been developed to identify chemical inducers and highlight recent advances made in promoting chemical-based cellular conversions in three germ layers: ectoderm, mesoderm and endoderm. The reader might refer to several excellent review articles more focused on the mechanism of cellular conversion and its applications to therapeutics and regenerative medicine.

2. Methods to identify chemical inducers to bring about cellular conversion

Small molecules that modulate the fate of cells are identified mainly by employing two broad types of approaches involving

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Inducers. Small molecule libraries, generated from an array of specific marker proteins in order to uncover possible chemical beating or twitching, accumulation of lipid vesicles) or by observing cell-specific phenotypes (e.g.

Table 1 Comparison of genetic with chemical approaches for cell fate changes

<table>
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<tr>
<th>Methods of cell fate change</th>
<th>Mechanism</th>
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<td>Genetic approach</td>
<td>Viral mediated gene delivery</td>
<td>Highly efficient</td>
<td>Genomic integration</td>
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<td>Transient gene delivery</td>
<td>Genes of master transcription factors that regulate expression of genes responsible for cell fates are delivered to target cells by transducing virus particles. Cell fate changes take place by expressing key genes responsible for the cell fate.</td>
<td>Non-integrative</td>
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<td>Small molecule-based approach</td>
<td>Small molecules enter target cells through diffusion, and regulate activity of proteins which are involved in the cell fate change. Small molecules can also bind to plasma membrane receptors. Chemicals induce cell fate changes by activating or inhibiting proteins responsible for cell fates.</td>
<td>Highly efficient</td>
<td>Clinically not feasible</td>
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Fig. 1 Screening of chemicals that induce the cellular conversion. Lineage-committed, multipotent or pluripotent stem cells, either untransfected or transfected with a reporter, are incubated with members of small molecule libraries (phenotypic screening) or known chemical regulators of signaling pathways (target directed screening). Alternatively, chemically treated cells are incubated with a fluorescent probe that responds to specific types of cells (fluorescent probe based screening). Once chemical inducers to change the cell fate are identified, the chemical induced cells are further characterized using immunostaining, RT-PCR and/or DNA chip analyses.

Phenotype- and target-based screenings (Fig. 1). The phenotype-based screening approach does not require a priori knowledge of regulatory pathways and it employs unbiased screening for chemicals capable of inducing cellular conversion by visibly detecting phenotypes of target cells. In this approach, lineage-committed cells, multipotent adult stem cells, ESCs or iPSCs are incubated with members of small molecule libraries followed by observing cell-specific phenotypes (e.g. cell morphology, cell beating or twitching, accumulation of lipid vesicles) or by immunostaining with antibodies that can detect the cell-type specific marker proteins in order to uncover possible chemical inducers. Small molecule libraries, generated from an array of reactive building blocks, are intensively utilized to develop biologically active molecules or drug-like small molecules. For high-throughput phenotypic screening, stably transfected cells with a reporter gene (e.g. green fluorescence protein (GFP)), which is placed under the control of the promoter of the target cell marker and thus can be activated during cellular conversion, are treated with members of small molecule libraries. Small molecules which promote the activation of a reporter gene are selected as tentative chemical inducers using a high-content imaging instrument. Once selected through initial screening, the chemical inducers are confirmed as being active using other techniques such as immunostaining and RT-PCR analyses. If necessary, lead optimization is then carried out using structure–activity relationship (SAR) studies. Furthermore, a target protein of the chemical inducer can be explored using affinity chromatography, drug affinity responsive target stability (DARTS) or other techniques in order to elucidate its mode of action of cellular conversion. Because target protein identification of a selected chemical is not always possible, the effect of a chemical inducer on signaling pathways can also be investigated using DNA chips or/and regulators of signalling pathways which are involved in cellular conversion.

A target-based approach, which relies on a priori knowledge of signalling pathways, involves incubation of cells with small molecules whose mechanisms of action are known. In this method, lineage-committed cells, multipotent or pluripotent stem cells, either untransfected or transfected with a reporter, are incubated with a single or a cocktail of small molecule modulators of the signaling pathways. Bioactive components that have capability of changing the cell fate are identified by examining the marker expression using immunostaining with lineage-specific antibodies or by monitoring the expression of a reporter in the transfected cells. This approach has proven to be very useful for uncovering chemical inducers that change the fate of cells. However, it has a major limitation as a consequence of the fact that it requires a priori knowledge of the molecular pathway involved in the cellular conversion.

In addition to these approaches, another attractive method for high-throughput screening to identify chemical inducers is
to use a fluorescent probe that responds to a specific type of cells. For example, the nonfluorescent probe FM1-43 exhibits fluorescence when it enters neurons which possess a high concentration of K^+. This probe has been employed to identify small molecules that induce neurogenesis of skeletal muscle cells and mouse P19 embryonic carcinoma cells. However, cell type-selective fluorescent probes are rare, and thus more probes are needed to be developed for use in the high-throughput screening method. Finally, the functional properties of target cells induced by chemicals are examined. In particular, functional activities of induced neurons and cardiomyocytes can be examined by measuring their specific electrophysiological properties.

3. Small molecules that promote cellular conversion

During the development of vertebrates, a gastrula with three germ layers (ectoderm, mesoderm and endoderm), formed from a blastula, is differentiated into various types of cells (Fig. S1, ESI†). Following gastrulation, the endoderm, which corresponds to the inner layer of the embryo, gives rise to endodermal cells such as pancreatic cells, hepatocytes and intestinal epithelial cells. The mesoderm, which corresponds to the middle layer of the embryo, forms cardiac, skeletal muscle, smooth muscle, bone, fat and blood cells as well as chondrocytes. The ectoderm, which originates from the outer layer of the embryo, differentiates into neurons, astrocytes, oligodendrocytes, microglial cells and Schwann cells. In this review, we highlight recent findings arising from studies of small molecule-induced cellular conversion corresponding to three germ layers (Table S1, ESI†).

3.1 Ectoderm lineages

The central and peripheral nervous systems are primarily composed of neurons and glial cells. Neural stem cells, which is derived from the ectoderm, differentiate into neuronal restricted and glial restricted progenitors. The former cells are further differentiated into neurons, and the latter into glial cells such as astrocytes, oligodendrocytes and microglial cells. Neurons have a function to generate electrical signals, called action potentials, which allow quick transmission of information to target cells. Glial cells play a role in supporting and protecting neurons and also supply nutrients and oxygen to neurons in the nervous system. In this section, we highlight investigations focussing on chemical-induced differentiation of pluripotent cells into ectoderm lineages, such as neurons, astrocytes, oligodendrocytes and Schwann cells, and chemical-promoted reprogramming of lineage-committed cells to multipotent neural progenitors or neural stem cells.

3.1.1. Neuron cell generation. Over the last decade, a number of chemical strategies to induce neuronal differentiation have been developed. In an early effort using a phenotype-based screening method, Schultz and his co-workers identified TWS119 as a chemical inducer of neuronal differentiation of mouse P19 embryonic carcinoma cells and mouse ESCs (Fig. 2). TWS119 acts as a neurogenic inducer by inhibiting glycogen synthase kinase 3β (GSK-3β) which plays a suppressive role in neuronal differentiation by blocking the Wnt signaling pathway. In addition, the same group uncovered neuropathiazol that has an activity of inducing differentiation of primary hippocampal neural progenitor cells (HCNs) into neurons (Fig. 2). The results of the SAR study showed that KHS101 displays a higher induction activity and improved pharmacokinetic properties compared to those of neuropathiazol (Fig. 3). The results of mechanistic studies showed that KHS101 directly interacts with the transforming acidic coiled-coil containing protein 3 (TACC3) that controls the nuclear localization of the aryl hydrocarbon receptor nuclear translocator 2 (ARNT2), a nervous system-specific transcription factor which plays a role in promoting neuronal differentiation. Consequently, KHS101 promotes neurogenesis of HCNs by enhancing the nuclear localization of ARNT2 through blocking interaction between ARNT2 and TACC3. Interestingly, upon administration into rats, KHS101 distributes to the brain where it promotes a significant increase in neurogenesis in vivo.

In parallel to the effort described above, Hsieh’s group discovered, by employing a phenotype-based screening method, the isoxazole derivative, ISX9, that promotes neurogenesis of HCNs (Fig. 4). This substance not only induces neurogenesis of HCNs but also it enhances neuronal differentiation in P19 cells, subventricular zone progenitors, and the whole brain of an adult mouse. However, ISX9 blocks astrogenesis promoted by the treatment of the leukemia inhibitory factor (LIF) and bone morphogenetic protein-2 (BMP-2) in HCNs, indicating that it is a selective neurogenic inducer. The observations made in the mechanistic study suggest that ISX9 induces neuronal differentiation via Ca^{2+} influx into the nucleus. Upon an increase
in the concentration of Ca\(^{2+}\) in the nucleus, Ca\(^{2+}\)-activated CaM kinase phosphorylates and mediates nuclear export of histone deacetylase-5 (HDAC-5), a corepressor of myocyte enhancer factor 2 (MEF2). As a consequence, MEF2 which is an important regulator of cell differentiation retrieves activity and in turn activates the expression of neuronal genes in order to promote neuronal differentiation of adult neural progenitor cells.

Small molecules, which have the ability to convert readily available and simply manageable somatic cells into neurons without the use of heterologous genetic materials or proteins, have also been explored. In a first attempt to uncover compounds with neurogenesis inducing activity, mouse C2C12 myoblasts were incubated with members of an imidazole library for 5 days and then treated with FM1-43.\(^7,19\) From this effort the synthetic small molecule, neurodazine (Nz), was identified as a chemical inducer causing conversion of myoblasts to neurogenic cells (Fig. 5). Nz-treated C2C12 cells neither progress along their myogenic lineage nor are they converted to glial cells. In an attempt to convert them into neurogenic cells, myotubes differentiated from C2C12 cells were initially treated with myoseverin in order to generate mononucleates.\(^20\) Isolated mononucleates were then incubated with Nz. The Nz-treated mononucleates were found to be converted into neurogenic cells. Furthermore, Nz treatment leads to conversion of the mononucleates and satellite cells, obtained from human single muscle fibers, into neurons. These results showed that a small molecule like Nz promotes neuronal differentiation of non-pluripotent myoblasts and the cells derived from mature human skeletal muscles into neurons. Subsequent studies showed that Nz and its analog, neurodazole (Nzl), selectively promote differentiation of P19 cells into neurophysiologically active neurons by activating Wnt and Shh signaling pathways (Fig. 6).\(^13\) It was also shown that Nz and Nzl enhance neurogenesis of NIH3T3 fibroblasts and neuroblastoma cells (SHSY5Y and Neuro2A).\(^21\)

A target-based approach for determining the effects of small molecules on cell fate changes concentrates on compounds whose biological activities and mechanisms of action are characterized. For example, human fibroblasts were directly converted into neuronal cells using a cocktail of small molecule modulators of multiple signaling pathways (Fig. 7).\(^22\) In this study, human fibroblasts were incubated with a mixture of valproic acid (VPA), CHIR99021, repsox, forskolin, SP600125, GO6983 and Y-27632 to induce neurogenesis. The treated cells were further incubated for more than 2 weeks in a maturation medium containing CHIR99021, forskolin and dorsomorphin. The chemical-induced neurons generate action potentials, indicating that they are electrophysiologically active. Importantly, this chemical approach produced neuronal cells from familial Alzheimer’s disease patients, showing the success of this protocol in generating patient-specific neuronal cells.

In a similar manner, mouse fibroblasts are also directly converted into neuronal cells using a cocktail of small molecules, forskolin, ISX9, CHIR99021 and I-BET151 (a bromodomain and extra-terminal motif protein (BET) family inhibitor).\(^23\) This mixture induces cell reprogramming via disruption of the fibroblast program using I-BET151 and induction of neuronal cell fate mostly by ISX9. Importantly, the chemical-induced neurons have the ability to generate action potentials and fast, inactivating
inward and outward currents, showing that they exhibit electrophysiological properties. In addition, when they were co-cultured with primary astrocytes or primary neurons in maturation media, the chemical-induced neurons form functional synaptic connections with each other or with the pre-existing primary neurons.

Sox2 is a key regulator in the maintenance of pluripotency and stemness. It was shown that suppression of its function abrogates stemness of pluripotent cells and leads to induction of differentiation into several types of cells.24 To selectively and efficiently promote neurogenesis of pluripotent cells, a protocol was developed which relies on forced suppression of Sox2 temporarily in the initial phase followed by lineage-specific induction of neurogenesis using small molecules (Fig. 8A).25 Specifically, P19 cells were transduced with TAT peptide-conjugated Skp protein (an inhibitor of Sox2) during embryoid body (EB) formation and then treated with a neurogenic inducer, Nz or Nz1, in the course of differentiation. The P19 cells are selectively converted into electrophysiologically active neurons without generation of astrocytes and cardiac cells. This study demonstrates that a combination of stemness suppression and exclusive lineage-specific activation can be utilized to obtain lineages with high efficiencies of commitment.

Sirtuin 1 (SIRT1), a mammalian homologue of the yeast HDAC Sir2, is known to restrict differentiation of stem cells and neural stem cells into neurons. The selective SIRT1 inhibitor, EX-527, was found to convert P19 cells into functional neurons without generation of cardiac cells and astrocytes (Fig. 8B and C).26 Inhibition of SIRT1 by EX-527 enhances the expression of neuronal markers, while decreasing the expression of Hes1, a crucial transcriptional co-repressor of SIRT1, which is responsible for maintaining pluripotency and preventing neurogenesis.

More recently, it was shown that a cell permeable synthetic peptide, which inhibits interactions between a repressor element-1 silencing transcription factor (REST) and Sin3A, promotes differentiation of P19 cells into electrophysiologically active neurons by impeding REST activity that represses multiple neuronal genes in non-neuronal cells.27

Astrocytes have been utilized to generate neuronal cells. In one effort, human astrocytes were sequentially exposed to a cocktail of small molecule modulators (LDN193189, SB431542, TTNPB, thiazovivin, CHIR99021, VPA, DAPT, SAG, and purmorphamine) of signaling pathways to induce neuronal differentiation (Fig. 9).28 This chemical reprogramming protocol epigenetically silences glial genes and concomitantly activates neuronal genes. When neurons, derived from astrocytes by utilizing this protocol,
were further incubated in a maturation medium containing several neurotropic factors such as the brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and insulin-like growth factor 1 (IGF-1), they created functional synaptic networks with synchronous burst activities. Furthermore, when the astrocyte-derived neurons were transplanted into mouse brain, the grafted neurons established synaptic connections with host neurons.

3.1.2. Glial cell generation. Glial cells, the non-excitable supporting cells of the nervous system, primarily function to regulate the internal environment of the brain, especially the fluid surrounding neurons and their synapses, and to supply nutrients to neurons. Glial cells, such as astrocytes, oligodendrocytes and microglial cells, are generated from neural stem cells in the central nervous system. In addition, Schwann cells, another type of glial cells that are derived from neural crest cells, play roles in the maintenance and regeneration of axons of the neurons in the peripheral nervous system. The genetic manipulation approach has normally been used to convert other cells into glial cells, but a few cases exist where the small molecule approach has been explored.

For example, a neural stem cell line and primary neural stem cells have been differentiated into astroglial cells using the AMP-activated protein kinase (AMPK) activator, AICAR (Fig. 10).29 However, the MAPK activator, metformin, does not act as an astrocyte inducer of neural stem cells although both the AICAR and metformin activate the AMPK signaling pathway in neural stem cells. The results of further mechanistic studies showed that the AICAR induces astroglial differentiation of neural stem cells by activating the Janus kinase (JAK)/Signal transducer and the transcription 3 (STAT3) pathway. In contrast, metformin does not activate this pathway. This finding suggests that astrogenesis of neural stem cells induced by the AICAR takes place via activation of the JAK/STAT3 pathway.

Schwann cells were generated from human epidermal neural crest cells by manipulating signaling pathways as well as by treatment with growth factors.30 This study showed that incubation with sonic hedgehog, CHIR99021, β-mercaptoethanol, all-trans retinoic acid, SB431542, fibroblast growth factor 2 (FGF2), platelet-derived growth factor-BB (PDGF-BB), forskolin and neuregulin 1 leads to conversion of human epidermal neural crest cells into Schwann cells, which express markers of myelinating Schwann cells that are conducive to nerve repair. The induced Schwann cells are functionally active, as reflected by the fact that they form a myelin sheath around the axon of the ganglion neuron co-cultured with rodent dorsal root ganglion neurons.

3.1.3. Reprogramming of lineage-committed cells to multipotent neural progenitors or neural stem cells. To date, several chemical protocols have been developed to obtain multipotent neural progenitors and neural stem cells from lineage-committed cells. An early example of a small molecule approach is the conversion of oligodendrocyte precursor cells (OPCs) to neural stem-like cells (Fig. 11).31 In this study, primary rat OPCs were transfected with a reporter construct that uses the Sox2 promoter to drive EGFP expression. Because transcription factor Sox2 is highly expressed in neural stem cells but not in OPCs, a small molecule-mediated activation of Sox2 reprograms lineage-restricted OPCs into neural stem-like cells that have multipotent differential potential. In order to identify small molecules that activate Sox2 expression in OPCs, the reporter-transfected OPCs

![Fig. 10](image-url)
were incubated with members of a chemical library. An assay using Sox2-driven GFP expression enabled identification of four compounds that inhibit HDAC activity, such as sodium butyrate, trichostatin A (TSA), MS-275 and apicidin. These substances were found to convert lineage-committed OPCs into multipotent neural stem cells, as judged by observing that the chemical-induced cells are capable of differentiating into neurons, astrocytes and oligodendrocytes.

Neural progenitor cells were also induced from somatic cells using a chemical approach. For example, neural progenitor cells were produced from mouse embryonic fibroblasts (MEFs) by treatment with a chemical cocktail containing VPA, CHIR99021 and repsox, under physiological hypoxic conditions.32 Neural progenitor cells generated by utilizing this protocol possess the self-renewal property. Importantly, the chemical-induced neural progenitor cells (ciNPCs) exhibit multipotency because they become astrocytes when placed in astrocyte inducing media as well as neurons when placed in neural media. In addition, the neurons derived from ciNPCs generate repetitive action potentials and exhibit postsynaptic current. ciNPCs were also generated from different somatic cells (mouse tail-tip fibroblasts) and cells from different species (e.g. human urinary cells) using the same chemical cocktail.

More recently, MEFs were also converted to neural stem-like cells using a nine component cocktail including LDN193189, A83-01, CHIR99021, basic fibroblast growth factor (bFGF), Hh-Ag 1.5, retinoic acid, RG108, parnate and SMER28 (Fig. 12).33 The chemical-induced neural stem-like cells (ciNSLCs) were differentiated into neurons, astrocytes and oligodendrocytes. In particular, differentiated neurons were observed to generate repetitive trains of action potentials, and inactivating inward and outward currents. In addition, they exhibit strong spontaneous synaptic network activities, which suggest the formation of functional synapses. When injected into mouse pups, the ciNSLCs efficiently differentiated into neurons, oligodendrocytes and astrocytes. As a consequence, ciNSLCs closely resemble primary neural stem cells. The results of a mechanistic analysis of the chemical-derived NSLC formation showed that reprogramming specific transcription factors, such as Elk1 and Gli2, leads to activating endogenous master neural genes to specify neural identity.

3.2 Mesodermal lineages

Among mesodermal lineages, skeletal and cardiac muscles are of special interest because of their potential in cell therapies for the treatment of degenerative and genetic muscle diseases. In addition, renewed interest in other types of mesodermal lineages, including adipocytes, osteocytes and chondrocytes, has also grown in recent years owing to their therapeutic potential. Because BMP4, Wnt, Notch and Nodal pathways are known to play major roles in the induction of mesodermal lineages, these
pathways have been targeted to modulate the promotion of cell fate changes to mesodermal lineages using small molecules. In this section, several key molecules used for inducing the cell fate changes of pluripotent stem cells and lineage-committed cells into mesodermal lineages are discussed.

3.2.1. Cardiac cell generation. Owing to the limited ability to regenerate cardiac cells in the heart, implantation of differentiated cardiac cells is the only feasible therapeutic option for the treatment of several heart diseases. Precise temporal control of the Wnt signaling pathway, activation in the early differentiation stage and suppression at a later stage, is known to be required for the efficient cardiogenesis. As a result, in the small molecule based-protocol, human iPSCs are initially treated with a GSK-3β inhibitor (CHIR99021 or BIO(6-bromoindirubin-3-oxine)) to activate the Wnt signaling pathway, which consequently initiates cardiomyogenesis. Then, the treated cells are exposed to a small molecule inhibitor (IWP2 or IWP4) of the Wnt signaling pathway in the later stage of differentiation.

Using a phenotype-based high content screening protocol, small molecules that promote cardiac differentiation of human iPSCs under cytokine and serum free conditions were identified. For screening of cardiogenesis inducers, monkey ESCs, stably transfected with a GFP reporter driven by the cardiac specific α-MHC promoter, were treated with members of a chemical library for 8 days from day 6 to day 14. The initial screening of 9600 chemicals led to identification of N11474 as an active cardiogenesis inducer and a following SAR study provided its optimized compound, KY02111, with enhanced activity (Fig. 13). By employing KY02111, human iPSCs were differentiated into electrophysiologically active cardiomyocytes including ventricular and pacemaker cells. The results of a microarray analysis revealed that KY02111 is a suppressor of Wnt signaling. Accordingly, an optimized cardiac differentiation protocol of iPSCs involves the treatment of cells with CHIR99021 and BIO (Wnt signaling activators) in the initial phase and with KY02111 and XAV939 (Wnt signaling suppressors) in the later phase under cytokine and serum free conditions. This treatment procedure leads to generation of 98% cardiac cells.

Ding and coworkers developed a semi-chemical method, using small molecules combined with genetic factors, to reprogram MEFs into cardiomyocytes. A small molecule pool comprised of pluripotency enhancers, signaling modulators and cardiogenesis inducers has been utilized for the initial screening of chemical inducers. Small molecules that enhance cardiogenesis induced by the expression of pluripotency transcription factors Oct4, Sox2 and Klf4 were chosen by counting the number of spontaneously beating colonies produced upon chemical treatment as a measure of differentiation efficiency. It was found that treatment with CHIR99021, SB431542, pate and forskolin, together with genetic factors (Oct4, Sox2 and Klf4), gives rise to the highest cell conversion efficiency. Because these four chemicals were found to bring about the same effect as do Sox2 and Klf4 using further studies, Oct4 is used as the sole genetic factor combined with four chemicals in the optimized protocol for promoting cardiogenesis. However, the precise role of each small molecule in inducing cardiac cell fate is unknown.

In addition, a chemical cocktail for converting human fibroblasts to cardiomyocytes was uncovered using a phenotype-based screening method (Fig. 14). In this screening, human fibroblasts virally transfected with a cardiac specific αMHC-GFP reporter gene were incubated for 6 days with individual members of 89 compounds, consisting of signaling modulators, epigenetic modifiers, metabolic modulators, cell death inhibitors and stress relievers, in combination with a chemical cocktail (SB431542, CHIR99021, pate and forskolin) that was identified using a semi-chemical method to bring about cardiac reprogramming. The cells were incubated for additional 5 days in the presence of cardiogenic molecules (activin A, BMP4, VEGF and CHIR99021), and their differentiation into cardiomyocytes was then monitored by determining the level of the expression of GFP. This screening procedure showed that a mixture of CHIR99021, A83-01, BIX01294, AS8351, SCI1, Y27632 and OAC2 serves as an effective chemical cocktail for generating cardiac cells from fibroblasts. The results of an additional screening of 300 chemicals composed of signaling modulators (inhibitors of kinases, phosphatases and receptors) showed that SU166F and JNJ10198409 (inhibitors of the platelet-derived growth factor pathway) are active substances and that their inclusion into the verified cocktail greatly enhances cardiac differentiation of human fibroblasts. Importantly, the cardiomyocytes generated using this protocol exhibit electrophysiological properties as well as a genetic signature that matches adult cardiomyocytes.
Another successful protocol for converting MEFs to cardiomyocytes includes the use of a chemical cocktail consisting of valproic acid, repsox, CHIR99021, parnate, forskolin and TTNPB. Although these six chemicals were the part of chemical cocktails that were previously employed for conversion of MEFs to iPSCs, treatment of MEFs with these chemicals led to generation of spontaneously beating cardiac cells. The functional properties of the chemical-induced cardiomyocytes were confirmed by measuring the action potential signature similar to that of atrial and ventricular-type cells as well as their genetic signature similar to that of mouse adult cardiomyocytes.

3.2.2. Skeletal muscle cell generation. Skeletal muscle is another important mesodermal lineage target for cell fate change because it has therapeutic potential in the treatment of many degenerative muscle diseases accompanied by the muscle damage and loss. Similar to conditions employed to induce cardiac cells, activation of Wnt signaling is necessary for muscle generation. Accordingly, human iPSCs are differentiated into myogenic precursor cells by activating the Wnt signaling pathway using CHIR99021. Subsequently, the precursor cells are expanded by incubating with fibroblast growth factor 2 (FGF-2) and are differentiated into mature skeletal muscles by culturing in a medium containing the N2 supplement. This supplement was originally formulated for neural stem cell expansion but it was also known to enhance terminal differentiation of myoblasts and myocytes.

3.2.3. Adipocyte (fat cell) generation. Adipogenesis is the process of generation of fat cells which are important for energy homeostasis. White adipocytes act as energy storage depots whereas brown adipocytes play a role in generating body heat in a process called thermogenesis. Therefore, brown adipocytes have a beneficial health effect in controlling obesity and reducing body weight. Dysfunction of adipocytes leads to many metabolic disorders, such as cardiovascular diseases, type 2 diabetes and obesity. Thus, an understanding of the mechanisms involved in adipogenesis and methods for generation of adipocytes will aid in the development of therapeutic approaches to several metabolic diseases.

A phenotype-based screening protocol was used to identify chemicals that convert myoblasts to brown adipocytes. In this investigation, myoblasts were exposed to members of a 20,000 small molecule library. The formation of lipid droplets, observed by staining cells with oil red O that detects triglycerides and lipids, was employed as a readout of the conversion efficiency. The results of this screening demonstrated that bexarotene, an agonist of the retinoic acid X receptor, stimulates adipogenesis of myoblasts (Fig. 15). This finding suggests that retinoid X receptors are key regulators of brown adipocyte differentiation. Genes associated with brown fat synthesis were observed to be highly expressed in brown adipocytes induced by bexarotene from myoblasts. Furthermore, mice, orally administered with bexarotene, displayed a lower gain of body weight despite having the same level of food intake as do vehicle-treated mice.

3.2.4. Osteocyte (bone cell) generation. Although bone is regenerated minimally after slight fractures or damage, its self-healing is insufficient to remedy most serious bone related diseases such as fractures, osteoporosis, osteogenesis imperfecta and osteoarthritis. Thus, transplantation of bone cells with autologous origin is necessary to treat serious bone related diseases. However, implanting bone grafts using the same origin is generally not feasible because of the limited amount of tissue sources. As a result, regenerative approaches serve as excellent treatment alternatives.

Small molecules that convert mouse ESCs, mouse iPSCs and human iPSCs into osteoblasts (bone forming cells) were uncovered by employing a target-based approach. For this purpose, pluripotent cells were converted to osteoblasts via a stepwise differentiation promoted by four chemicals including CHIR99021, cyclopamine, SAG and TH (Helioxanthin derivative) without hormones and growth factors (Fig. 16). Because it is known to be an inhibitor of hedgehog signaling that is relevant to the ectodermal commitment, cyclopamine was expected to play a
role in suppressing ectodermal differentiation. Therefore, treatment with CHIR99021 and cyclopamine in the initial phase of differentiation of pluripotent cells led to preferential mesodermal commitment that is required for conversion to osteoblasts. SAG and TH contribute to committing the mesoderm to osteoblasts in the induction phase. Further maturation of osteoblasts was conducted by growing cells in a medium containing phospho-ascorbic acid, $\beta$-glycerophosphate and dexamethasone, and differentiation of osteoblasts was confirmed by assessing the extent of calcium accumulation in cells by utilizing alizarin red staining.

### 3.3 Endodermal lineages

Endodermal lineages, such as pancreatic cells, hepatocytes, lung progenitors and thymus, are also important in cell therapy. The Activin/Nodal pathway is known to play a key role in endoderm commitment. As a result, the cytokine member of the TGF-$\beta$ superfamily, Activin A, was added to differentiation media in order to convert pluripotent cells (human and mouse ESCs and iPSCs) into definitive endoderm. Moreover, activation of the Wnt signaling pathway using CHIR99021 and BIO has been found to be effective for endoderm formation.

The first effort to obtain the definitive endoderm from ESCs without using growth factors or hormones utilized a screening of 4000 small molecules using mouse ESCs transfected with a tdTomato-fluorescent gene driven by an endoderm specific Sox17 promoter. The results showed that two TGF-$\beta$ signaling activators, IDE1 and IDE2, act as chemical inducers of definitive endoderm (Fig. 17). The resulting definitive endodermal cells were further differentiated into pancreatic progenitor cells by treatment with $(-)$-indolactam V, a pancreatic progenitor inducer, whose discovery is described in Section 3.3.1.

Adult somatic cells were also converted to endodermal progenitors using small molecules. Observations made in

![Fig. 15](https://example.com/f15.png)

**Fig. 15** Conversion of myoblasts to brown adipocytes by a small molecule. (A) Scheme of stepwise conversion of myoblasts to adipocytes. (B) Chemical structure of bexarotene (Bex). (C) Cells treated with Bex exhibit oil red O staining but untreated cells do not. Reproduced with modification from ref. 41, with permission from Cell Press, copyright 2017.

![Fig. 16](https://example.com/f16.png)

**Fig. 16** Differentiation of human iPSCs to osteoblasts. (A) Scheme of stepwise conversion of human iPSCs to osteoblasts. (B) Function and (C) chemical structures of small molecules used in this study. Structures not shown here are shown in previous figures and Table S1 (ESI†). (D) Expression of osteoblast specific markers Runt-related transcription factor 2 (Runx2, green) and SP7 (red) confirmed by immunostaining (scale bar = 100 $\mu$m). (E) Alizarin red staining demonstrated the accumulation of calcium salts of the differentiated osteocyte cells on days 5, 19, and 23 during the course of human iPSC differentiation into osteoblasts. Reproduced with modification from ref. 42, with permission from Cell Press, copyright 2017.

![Fig. 17](https://example.com/f17.png)

**Fig. 17** Conversion of ESCs into the definitive endoderm and into pancreatic progenitors by small molecules. (A) Scheme of stepwise conversion of ESCs to pancreatic progenitors. (B) Function and (C) chemical structures of small molecules used in this study. Structures not shown here are shown in previous figures and Table S1 (ESI†). (D) Definitive endodermal cells obtained from mouse and human ESCs using IDE1 and IDE2 were immunostained with antibodies against endoderm specific markers, sex determining region Y-box 17 (Sox17, green) and forkhead box A2 (FoxA2, red). (E) The chemical-induced definitive endodermal cells from ESCs were differentiated into pancreatic progenitors by indolactam V, which was confirmed by positive immunostaining with an antibody against pancreatic progenitor specific marker, pancreas/duodenum homeobox protein 1 (Pdx1, red). Reproduced with modification from ref. 45, with permission from Cell Press, copyright 2009.
screening small molecules that were previously known to promote the cellular fate change showed that a cocktail containing BIX01294, RG108, SB431542 and (−/−)-Bay K8644 induces conversion of human gastric epithelial cells to multipotent endodermal progenitors in the presence of a gastric subepithelial myofibroblast feeder layer (mesenchymal feeder cells) (Fig. 18).

The functionality and multipotent nature of the chemically derived endodermal progenitors were demonstrated by differentiating into hepatocytes, pancreatic cells and intestinal epithelial cells.

3.3.1. Pancreatic cell generation. Pancreatic progenitor cells have a multipotent capability of differentiation into exocrine (acinar and ductal cells) and endocrine (α, β, δ, ε-cells and pancreatic polypeptide cells) lineages. Because of the potential of developing a cell therapy for diabetes mellitus, differentiation of pancreatic cells into β-cells has received considerable attention. The first small molecule to induce differentiation of definitive endodermal progenitors in the presence of a gastric subepithelial myofibroblast feeder layer (mesenchymal feeder cells) (Fig. 18). The functionality and multipotent nature of the chemically derived endodermal progenitors were demonstrated by differentiating into hepatocytes, pancreatic cells and intestinal epithelial cells.

In this study, CHIR99021 and Activin A induced the differentiation of human iPSCs into definitive endoderm, which were further converted into pancreatic progenitors in the presence of the keratinocyte growth factor, retinoic acid, SANT1, LDN193189 and phorbol 12,13-dibutyrate (PDBu). To uncover substances that promote the differentiation of pancreatic progenitors into β-cells, the effect of over 150 different combinations of 70 small molecules, which were previously reported to play a role in pancreatic biology, was explored. A cocktail comprised of retinoic acid, Alk5i II, SANT1, XXI, heparin, triiodothyronine, and betacellulin (an EGF family member protein) was found to be optimal for the high-content screening system to identify those that display enhanced expression of Pdx1 with immunostaining. This screening showed that (−)-indolactam V, a PKC activator, not only enhances the conversion of human ESCs to pancreatic progenitors but also promotes expansion of pancreatic progenitors. In addition, (−)-indolactam V displays the synergistic effect of expanding the pancreatic progenitors with FGF-2.

Recently, it was shown that a stepwise differentiation process, utilizing several small molecules and growth factors, produces fully functional pancreatic progenitors and β-cells (Fig. 19). In this study, CHIR99021 and Activin A induced the differentiation of human iPSCs into definitive endoderm, which were further expanded into pancreatic progenitors by small molecules. A cocktail comprised of retinoic acid, Alk5i II, SANT1, XXI, heparin, triiodothyronine, and betacellulin (an EGF family member protein) was found to be optimal for the

Fig. 18 Conversion of human gastric epithelial cells into multipotent endodermal progenitors by small molecules. (A) Scheme of stepwise conversion of gastric epithelial cells to cells with endodermal origin. (B) Function and (C) chemical structures of small molecules used in this study. Structures not shown here are shown in previous figures and Table S1 (ESI†). Endodermal progenitors derived from human gastric epithelial cells using four compounds were differentiated into (D) pancreatic cells, (E) intestinal epithelial cells and (F) hepatocytes. Differentiated cells were confirmed by immunostaining with antibodies against lineage specific markers: Pancreatic markers, NK6 homeobox 1 (Nkx6.1) and Pro-insulin, pancreas/duodenum homeobox protein 1 (Pdx1) and glucagon (CCG); intestinal epithelial cell markers, mucin 1 (Muc1), Vilin 1 (Vil1) and caudal type homeobox 2 (CDX2); hepatocyte markers, albumin and α-feto protein (AFP1). Indicyanin green uptake was also examined to confirm hepatocyte generation (scale bar = 50 μm). Reproduced with modification from ref. 46, with permission from Cell Press, copyright 2016.

Fig. 19 Differentiation of human iPSCs into pancreatic progenitors and subsequently to β-cells. (A) Function and (B) chemical structures of small molecules used in this study. Structures not shown here are shown in previous figures and Table S1 (ESI†). (C) iPSCs-derived β-cells (upper panel) and primary β-cells (lower panel) were immunostained with pancreatic specific marker NK6 homeobox 1 (Nkx6.1, red), β-cell specific c-peptide (green) and α-cell specific glucagon (red). Reproduced with modification from ref. 48, with permission from Cell Press, copyright 2014.
conversion of pancreatic progenitors to endocrine cells which were further subsequently differentiated to β-cells in the presence of Alk5i II and triiodothyronine. By monitoring insulin secretion in response to glucose challenge, it was found that the iPSC-derived β-cells display an activity that is comparable with that of physiologically relevant primary β-cells.

### 3.3.2. Hepatocyte (liver cell) generation

Hepatocytes, which constitute a major part of the liver, are involved in a number of vital functions including protein synthesis, detoxification, and metabolism of lipids and carbohydrates. Although regeneration of a damaged liver is possible in most cases, in some situations liver transplantation is the only feasible alternative for the cure of several liver-related congenital diseases and other metabolic disorders. Liver transplantation on a moderate scale is restricted as a result of the shortage of adequate donors and immunogenicity. In this context, generation of functional hepatocytes is required for transplantation and restoration to restore liver functions. FGF and BMP4 signaling pathways are important for committing hepatocytes from the definitive endoderm.

Chemical-based conversion of human iPSCs to hepatocytes was successfully achieved without using any recombinant growth factor. In this study, a three step-conversion of human iPSCs to hepatocytes, involving definitive endoderm formation, hepatic specification or induction, and hepatic maturation, was developed. Definitive endodermal cells were generated from iPSCs by activating the Wnt signaling pathway with CHIR99021 (Fig. 20). The hepatic specification of this definitive endoderm was accomplished by treating the cells with DMSO, which is known to induce hepatic progenitor formation. In the final stage of the protocol, hepatic maturation is performed by treating cells with N-hexanoic-Tyr Ile-(6) aminohexanoic amide (dihexa) combined with dexamethasone (an agonist of glucocorticoid receptor), because glucocorticoid is known to modulate the proliferation and function of hepatocytes. It was also shown that inhibition of a chaperone protein HSP90β using a small molecule CCT-018159 leads to induction of hepatic differentiation by affecting the turnover and stability of HNF4A, a transcription factor which is known to be required for hepatic differentiation from pluripotent stem cells.

### 4. Conclusions

Investigations of the use of small molecules that have the capability of modulating cell fate have yielded exciting methods for induction and differentiation of pluripotent/multipotent stem cells as well as lineage conversion of somatic cells. In particular, small molecule regulators of signaling pathways and epigenetic modification are useful to manipulate the fate, state and function of cells. New and exciting directions are now being followed in the area of small molecule-based cellular conversion. Furthermore, elucidation of the underlying mechanisms for cell fate changes occurring upon chemical treatment will lead to advances in the application of chemical principles and small molecule tools. Therefore, the observations made in this effort will enable researchers to expand the understanding of how small molecules modulate cell fate changes. Ultimately, this effort will pave a way to use the chemical-induced cells in the treatment of many degenerative and incurable diseases such as spinal cord injuries, neurodegenerative disease, cardiac ischemic damage, muscular dystrophy, lung and gastrointestinal diseases. However, to achieve this goal, fine-tuning of the number and concentration of small molecules is required to develop safe regenerative medicine-based treatments for various diseases. Moreover, because chemical inducers exert a range of effects on signaling pathways and gene transcription, more investigations are needed to define precise molecular mechanisms underlying small molecule-induced cellular conversion.

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### Notes and references
