

# Chemicals Regulating Cardiomyocyte Differentiation

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## 1. Introduction

Differentiation toward cardiomyocytes from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) has been studied widely. Many of ESC/iPSC differentiation methods have been developed using embryoid body (EB) methods, in which ESCs/iPSCs spontaneously differentiate in floating aggregates. Various marker genes (early mesoderm or mesoendoderm cells: *Brachyury*, mesoderm cells: *Mesp1*, *Flk1*, and *Pdgfra*, cardiac progenitors: *Nkx2.5*, *Islet1* and cardiomyocytes: myosin chains) have been identified as stage specific markers to recapitulate developmental process in embryo. The clues from developmental biology provided ideas to use growth factors (*Activin A/Nodal*, *Bmp4*, *Cerberus*, *Wnt3a*, and *Wnt11*) to guide cardiac differentiation. Recently these stem cell technologies have been combined with chemical biology, in which small molecules are identified and used to regulate cell fate or modulate cell reprogramming. Several chemicals were reported that they could enhance cardiomyocyte differentiation from embryonic stem cells. However, it is hard to determine how these chemicals work, which cell types they affect, and what are their molecular targets because of heterogeneity in EBs. We have established a novel systemic cardiovascular differentiation method that is based on two dimensional culture and sequential purification using cell surface markers. A mesoderm lineage population, Flk1 expressing cells derived from mouse ESC/iPSC can give rise to vascular endothelial cells, pericytes, hematopoietic cells, and cardiomyocytes. Cardiomyocytes appear after 4 days co-culture of Flk1<sup>+</sup> cells on OP9 feeder cells. A cardiac progenitor population, FCV (Flk1<sup>+</sup>/CXCR4<sup>+</sup>/Vascular endothelial cardherin<sup>-</sup>) cells among the progeny of Flk1<sup>+</sup> mesoderm cells are observed 2 days after Flk1 purification. In our system, Flk1<sup>+</sup> and FCV cells are able to give rise to all cardiovascular cells - cardiomyocytes, endothelial cells, and pericyte even from single cell. We have identified a novel cardiogenic effect of cyclosporin-A (CsA) well-known immunosuppressant. CsA affects on mesoderm cells to drastically increase the differentiation of cardiac progenitors and cardiomyocytes up to 10 fold. In this chapter, we review recent advances of combination of stem cell technologies and chemical biology, especially chemical biology on cardiomyocyte differentiation, and our recent findings. We also describe some information to compare high throughput screening and high content screening in cell based assay.

## 2. Heart development *in vivo* and cardiomyocyte differentiation *in vitro*

To understand how cardiomyocytes differentiate from ESCs/iPSCs, comparison between embryo and ESC/iPSC differentiation would be helpful. In this section, we describe how a heart develops in an embryo, and how cardiomyocytes differentiate from ESCs/iPSCs. (Figure 1 and 2)

### 2.1 Heart development *in vivo*

The heart is the first functional organ in mammals. After fertilization, a Zygote cleaves and forms blastocyst before implantation (-E3.5), which contains inner cell mass (ICM) and trophoblast. ICM cells develop toward whole body, and ESCs are established from ICM cells. ICM cells segregate to epiblast and primitive endoderm lineages in late blastocyst. In epiblast stage, Part of epiblast cells migrate to midline and form primitive streak under induction of *Nodal* secreted from node. In primitive streak, *Brachyury* is expressed. *Wnt3a* and *Wnt5* are secreted in primitive streak and regulate expansion of primitive streak (Takada et al. 1994). Cells migrating through primitive streak differentiate to mesoderm and endoderm. The outer layer cells are called endoderm that is primordia of intestines, liver, pancreas and so on. The rest cells in epiblast, which do not migrate, are called ectoderm that is primordia of neuronal system and skin. Between the layers of endoderm and ectoderm, mesoderm cells differentiate from primitive streak and loose epithelial markers such as E-cadherin. Part of mesoderm cells migrate to lateral side and are called lateral plate mesoderm (LPM). LPM expresses *Flk1* and *Pdgfra* on their surface and *Mesp1* that is a transcriptional factor regulating cardiac mesoderm (Kataoka et al., 1997; Saga et al., 2000). Several secreting factors are known as regulators of LPM and cardiac mesoderm formation. Spatial-temporal expression of *Bmp4* regulates mesoderm fate. Anterior visceral endoderm cells secrete *Noggin*, *Cerberus*, and *Dkk1*, which regulate cardiac mesoderm formation thorough inhibition of BMP and Wnt signaling (Foley and Mercola, 2005). Non canonical Wnt family, *Wnt11* is also known to be involved in cardiac mesoderm to heart development (Pandur et al., 2002). LPM cells migrate anteriorly and forms cardiac crescent, which expresses *Nkx2.5* and/or *Islet1*. *Nkx2.5*<sup>+</sup> population called first heart field and *Islet1*<sup>+</sup> population called second heart field. Cardiac crescent cells migrate and fuse in midline and form heart tube. Then heart tube loops rightward to form four-chambered heart. First heart field mainly contributes to left ventricle and second heart field contributes to atrium and right ventricle.

### 2.2 Cardiomyocyte differentiation *in vitro* – Embryoid body method

In ESC/iPSC differentiation, EB method is widely used (Figure 1). This method is rather easy to differentiate ESC/iPSC toward various cell types. However, it is hard to evaluate exact molecular mechanisms and monitor at single cell level. Using EB method in cardiac differentiation, beating EB in total EB rate is widely used for quantitative evaluation. During differentiation process, some mesoderm (*Brachyury*, *Mesp1*, *Flk1*, and *Pdgfra*) or cardiac markers (*Nkx2.5*, *Islet1*, *actinin*,  $\alpha$ MHC and etc.) are used. These markers were evaluated using RT-PCR or transgenic line in some cases. Other than transgenic line or cell surface antigen, it is hard to evaluate in single cellular level for differentiation step. Some growth factors mentioned above increase cardiac differentiation. *Wnt3a* have been shown to have stage specific and bi-phasic effect (Naito et al., 2006; Ueno et al., 2007). *Wnt3a* treatment increased cardiac differentiation in early stage of differentiation and decreased in late stage.

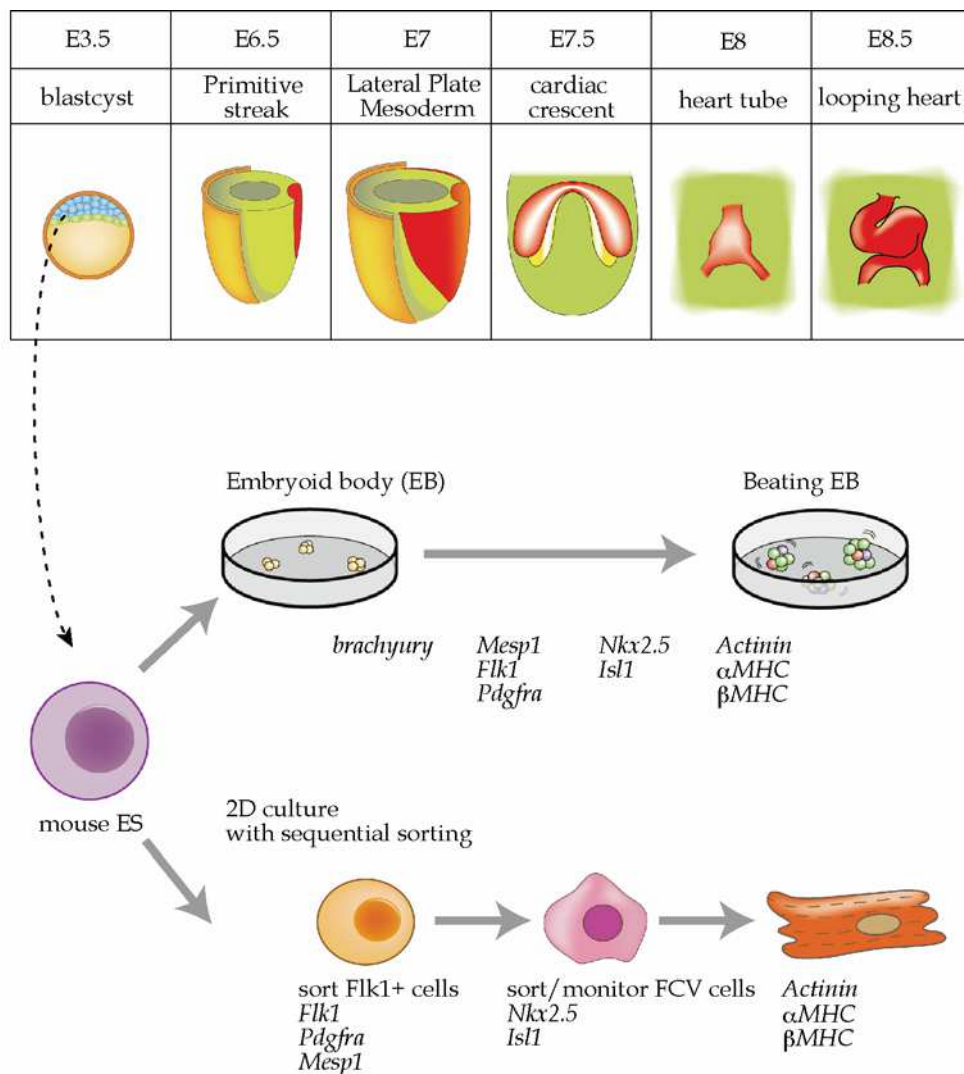


Fig. 1. Heart development in embryo and cardiac differentiation from ESCs in vitro. Cardiac lineage is mainly differentiate through primitive streak, lateral plate mesoderm, and cardiac crescent. Cardiac crescent cells migrate and fuse at midline of embryo and form heart primordial called heart tube. ESCs are established from inner cell mass of blastocyst. When ESCs are cultured in suspension, they form embryoid body, spontaneously differentiate and start beating when cardiomyocyte appear. Another differentiation method using 2D culture with sequential sorting provide us to understand stage specific process of differentiation.

Administration of Wnt11 in late stage of EB increase cardiomyocyte differentiation (Terami et al., 2004). Short term exposure of Noggin also directed ESCs to cardiac differentiation (Yuasa et al., 2005). Recently, directed differentiation methods using Activin A and BMP4 in EBs or high density culture of human ESCs were reported (Buehr et al., 2008; Laflamme et al., 2007). As shown here, some cardiogenic growth factors have been reported similar to embryo development. However these studies are still not clear to show the molecular mechanisms or target cells because of heterogeneity of cells in EBs or high density culture.

### **2.3 Cardiomyocyte differentiation *in vitro* – Two-dimensional culture and sequential differentiation**

To overcome the weak points of EB method, we have developed a novel differentiation method using two-dimensional culture and FACS. In this method, we can systemically induce cardiovascular cells – cardiomyocyte, endothelial cells, pericyte, and hematopoietic cells from single Flk1 positive mesoderm cells (Figure 2). Withdrawal of LIF induces ESC/iPSC differentiation. Flk1<sup>+</sup> mesoderm cells appear during 4-4.5 days after LIF withdrawal. Flk1<sup>+</sup> cells are negative for E-cadherin, an ESC marker, and positive for Pdgfra. As previously reported (Kataoka et al., 1997), Flk1<sup>+</sup>/Pdgfra<sup>+</sup> cells are observed wedge-shaped areas on proximal lateral mesoderm of mid-late streak stage embryo (E7.0) and are also observed in the anterior head fold region of head fold stage embryos (E7.5-7.75), which might consistent with cardiac crescent. Purified Flk1<sup>+</sup> cells give rise to cardiomyocyte after 3-4 days coculture on OP9 feeder cells. Endothelial cells, hematopoietic cells, and pericytes are also observed in Flk1 culture on OP9. At Flkd2, small subset of Flk1 progeny, Flk1<sup>+</sup>/CXCR4<sup>+</sup>/VE-cadherin<sup>-</sup> (FCV) cells appear and almost all cardiomyocytes are from this FCV population (Yamashita et al., 2005). Even single Flk1<sup>+</sup> and FCV cells can give rise to all these cardiovascular cells on OP9 feeder cells. Thus, it let us to know how much Flk1<sup>+</sup> and FCV cells have the differentiation potentials and to observe differentiation process under time lapse imaging (Eilken et al., 2009). Using this system, we can determine differentiation stage specific event and target cells.

In the heart, there are some subsets of cardiomyocytes, ventricular and atrial cardiomyocytes, conduction system cells, and pacemaker cells. Some molecular markers are known for these cardiomyocytes, such as myosin light chain (*Mlc2v* and *Mlc2a*), gap junction protein, and ion channels (Hyperpolarization-Activated Cyclic Nucleotide-Gated Channels, L and T type Ca channels, Na channels and K channels). Electrophysiological study clearly distinguishes them in single cellular level. In early differentiation stage (such as Flkd9.5), ESC derived cardiomyocytes show almost homogeneous phenotype, like heart tube stage. Cardiomyocytes have automaticity, higher maximum diastolic potential, and slower maximum stroke velocity (dV/dt). On the other hand, in late differentiation stage (such as Flkd23.5), cardiomyocytes show diverse character – (1) automaticity+, higher maximum diastolic potential and slower dV/dt (2) automaticity-, deep resting membrane potential, and faster dV/dt (Yanagi et al., 2007).

### **2.4 2D culture for EC differentiation**

Flk1 positive cells are also able to differentiate to endothelial cells and pericyte on collagen IV dish with VEGF or in Collagen gel as well as on OP9 cells (Yamashita et al., 2000). Administration of cAMP with VEGF enhanced arterial endothelial cell differentiation (Yurugi-Kobayashi et al., 2006). Furthermore, this system clearly reconstituted downstream signals of cAMP. PKA enhance endothelial differentiation through enhancing VEGF signaling by

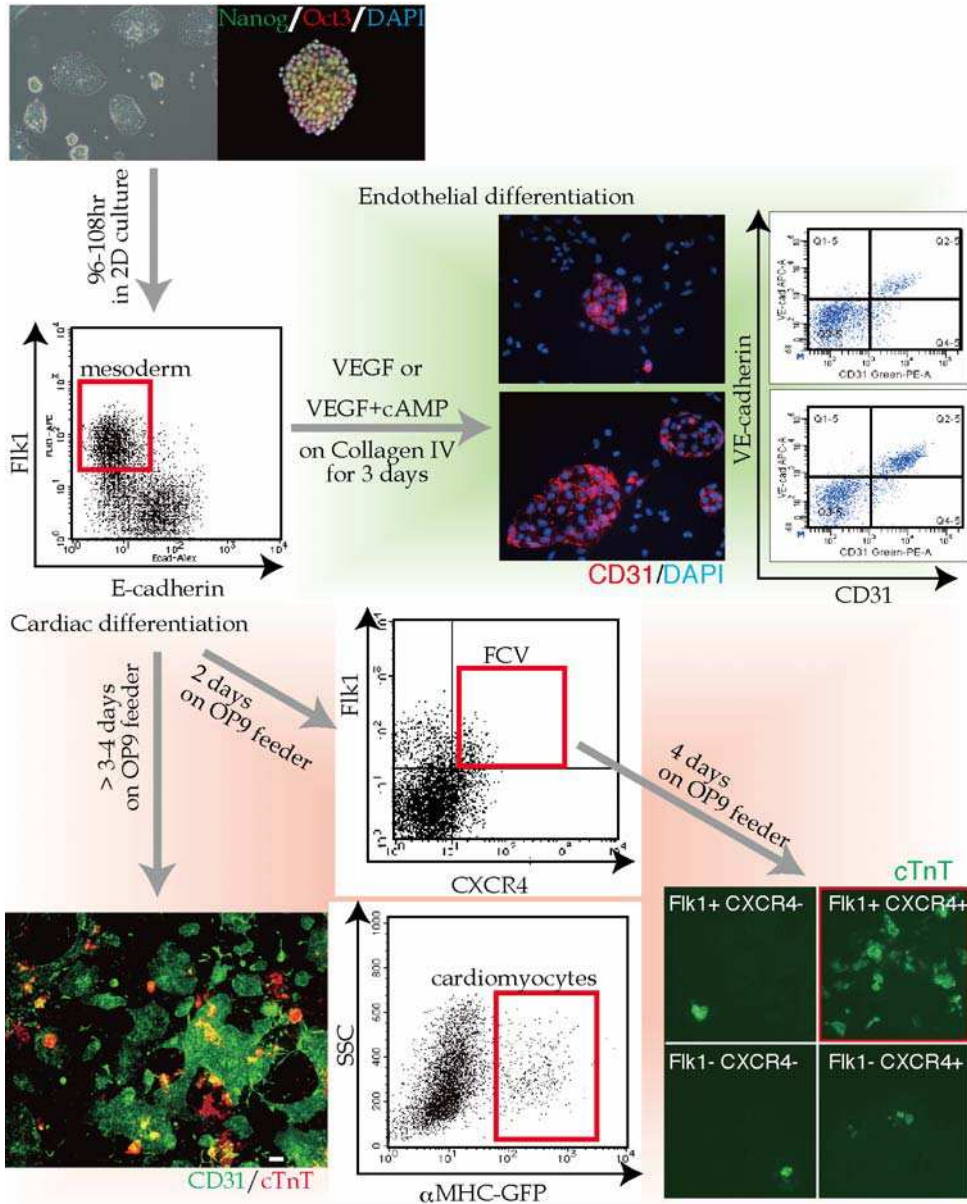


Fig. 2. Cardiac lineage differentiation from Flk1+ mesoderm cells. Cardiomyocytes are differentiated from Flk1+ cells on OP9 feeder cells. Self-beating cardiomyocyte appears 3-4 days after Flk1+ cells are cultured on OP9 cells (Flkd3-4). At Flkd2, cardiogenic potential is restricted in Flk1+ /CXCR4+ /VE-cadherin (FCV) population. Endothelial cells are differentiated from same Flk1+ cells on OP9 cells and on Collagen IV coated dish with VEGF. (modified from Yamashita et al., 2005)

up-regulation of *Flk1* and *Nrp1*, which forms VEGF receptor complex (Yan et al., 2009). Administration of cAMP also activate Notch and  $\beta$ -catenin, which form transcriptional complex to increase arterial endothelial cell differentiation (Yamamizu et al., 2010).

### 3. Chemical biology in stem cell biology

ESC Differentiation methods have been developed based on embryology as described above. Recently, combined with chemical biology, it has come to next stage. In this section, we review chemical biology and show some examples of chemical biology combined with stem cell biology

#### 3.1 What is chemical biology?

Chemical biology or chemical genetics is the study to elucidate biological systems with small molecules that bind directly to proteins to alter protein function. There are two ways of chemical biology: forward chemical genetics and reverse chemical genetics similar to conventional genetics. "Forward" means phenotype based, so random mutation is used in conventional genetics and screenings of small molecules, which induce phenotype of interest, are used in chemical genetics. "Reverse" means target based, so that gene-targeting and transgenic animals, and RNA interference technologies are used in conventional genetics, and target-specific small molecules, such as kinase inhibitors, are used in chemical genetics. The concept itself is not so much different; however, chemical genetics have great advantages and some disadvantages compared to conventional method. Small molecules have great advantages in temporal control, rapid inhibition or activation, and regulation of functionally overlapping targets, compared to conventional method. However, they also have some disadvantages in target specificity and off-target effect. To verify or confirm the targets of small molecules are more difficult.

When chemical biology is combined with stem cell biology, some screening system is required to screen chemical libraries. High throughput screening (HTS) is one of conventional and highly efficient method to screen chemicals, in which such as plate reader is used to measure biological output (luciferase activity, etc.). Recent advances of imaging system provide a novel screening method, named high content screening (HCS), which is based on microscopes. In HCS, immunostained wells are photographed and scored using computer automatically to measure immunoreactive area or to count number of cells that show appropriate phenotypes.

#### 3.2 Chemical biology in cardiomyocyte differentiation

In this section, we describe some example of cardiogenic small molecules.

Ascorbic acid is a firstly identified chemical that increased cardiomyocyte differentiation from ESC (Takahashi et al., 2003). Ascorbic acid was identified from "The FDA2000 Drug Library", which contains 880 of bioactive compounds approved for human use. In this screen, the authors used an ESC line carrying  $\alpha$ MHC-EGFP. The only compound found to reproducibly induce differentiation in monolayer culture was ascorbic acid. Interestingly, ascorbic acid showed no significant effect in cardiac differentiation using EB formation.

Cardiogenol was identified from 100,000 compound library using P19, embryonic carcinoma cell line, stably transfected rat atrial natriuretic factor promoter-luciferase (ANF-luc) vector (Wu et al., 2004). In this ANF-luc reporter assay, Approximately 80 compounds were

identified and 35 of 80 compounds induced MHC expression in P19CL6. 4 diaminopyrimidines, cardiogenol A-D were the most potent in inducing MHC expression. Wu et al. also tested R1, a mouse ESC line, to confirm the effects of cardiogenol. R1 cells were cultured in extremely high density (10,000cells/well in gelatin coated 384-well plate) with cardiogenol treatment in first 3 days of 7 days culture period. Although the authors did not mention about the molecular machineries or differentiation stage specificity, R1 cells were treated in early differentiation stage indicating cardiogenol may induce cardiogenic mesoderm.

Sulfonylhydrazone (Shz-1) was identified from 147,000 compound library using P19CL6, stably transfected bacterial artificial chromosome (BAC) of Nkx2.5 locus, which luciferase is knocked-in (Nkx2.5-luc) (Sadek et al., 2008). 3,000 strong positive hits from the primary screen were selected and 1,600 compounds were still positive for the secondary screen using dose response strategy. The authors clustered these hits into 10 lists of families with chemically distinct, synthetically tractable core structural motifs. Shz was one of the strongest lead chemicals. 48 hr treatment of Shz-1 at 5 $\mu$ M increased Nkx2.5-luc activity and 72hr Shz1 treatment increased sarcomeric  $\alpha$ -tropomyosin expression in P19CL6. They also tested SM1 ES cells. Because 48hr after treatment of Shz-1 endogenous myocardin and Brachyury expression level was increased, Shz-1 might also increase cardiac mesoderm differentiation. They tested various signal cascades to identify Shz-1 target molecules but failed. They also demonstrated Shz1 increased cardiac differentiation from human M-PBMCs *in vitro*. Shz1 treated M-PBMCs rescued Cryoinjured rat heart.

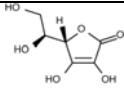
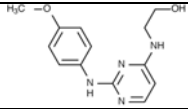
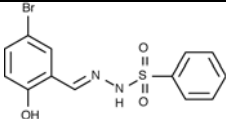
Compound	structure	Assay	Screened compound number	Molecular mechanisms
Ascorbic acid		$\alpha$ MHC-EGFP ESC	880	Unknown
Cardiogenol C		ANF-Luc P19	100,000	Unknown
Shz-1		Nkx2.5-Luc P19CL6	147,000	Unknown

Table 1. Cardiogenic chemicals

### 3.3 Chemical biology in maintenance and generation of pluripotent stem cells

Efficient maintenance and generation method of ESCs and iPSCs are also important for cardiac differentiation and for stem cell biology. Here we describe recent advances of chemical biology in maintenance and generation of ESC/iPSC.

Mouse ESCs require LIF for their maintenance. Pluripotin was identified for the replacement of LIF from 50,000 compound library as activating Oct3/4-GFP transgene in ESC (Chen et al., 2006). Using affinity chromatography, ERK1 and RasGAP were identified as the molecular targets of pluripotin. Using similar Oct3/4-GFP transgenic mice, E-616452 a

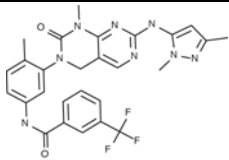
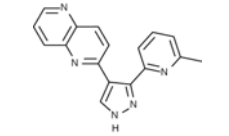
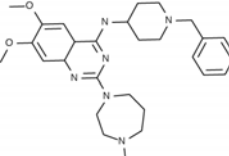
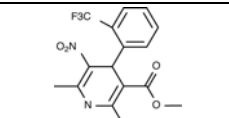
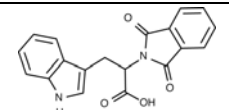
Compound	structure	Primary Assay	Screened compound number	Molecular mechanisms
Pluripotin		Oct3-GFP	50,000	ERK/Ras-GAP inhibition
E-616452		Oct3-GFP	800	Tgfr1 inhibition
BIX01294		ALP positive colony number	2,000	G9a/GLP inhibition
BayK8644				L type Ca channel agonist
RG108				Dnmt inhibition

Table 2. Small molecules identified for maintenance of ESC/iPSC and generation of iPSC using forward chemical genetics.

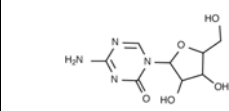
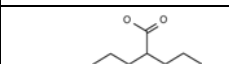
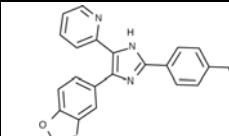
Compound	structure	Primary Assay	Molecular mechanisms
5-azacytidine		Oct3-GFP (%) from partially reprogrammed cells.	DNA methylation inhibition
Valproic acid		Oct3-GFP colony number	HDAC inhibition
SB431542		ALP positive colony number	Promoting Mesenchymal-epithelial transition through TGFβ signal inhibition

Table 3. Small molecules identified for maintenance of ESC/iPSC and generation of iPSC using reverse chemical genetics



Tgfb $\beta$ 1 inhibitor was identified as an alternative of Sox2 transgenes in generation of iPSCs from 800 compounds (Ichida et al., 2009). BIX01294 (G9a/GLP inhibitor), BayK8644 (L type Ca channel agonist) and RG108 (Dnmt inhibitor) were also identified that they could enhance reprogramming through screening of 2,000 bioactive molecules. These chemicals are identified through phenotype-based screening, so that forward chemical genetics was used for identification of them.

Using reverse chemical genetics, some important findings for maintenance and generation of ESCs/iPSCs were reported. As FGF4/ERK signaling is a promoter signal to induce differentiation of mouse ESCs, inhibition of FGF4/ERK using SU5402 and PD184352 suppressed ESC differentiation without LIF. In this condition, apoptosis was relatively high. To reduce apoptosis and increase ESC growth, CHIR99021 a specific GSK3 inhibitor was combined with SU5402 and PD184352. This three-inhibitor combination SU5402+PD184352+CHIR99021 (3i) support the maintenance of ESCs without Stat3 that is indispensable downstream target of LIF (Ying et al., 2008). Furthermore, 3i supported to generate rat ESCs (Buehr et al., 2008).

Generating iPSCs is robustly studied using small molecules such as 5-azacitidine (Mikkelsen et al., 2008), valproic acid (Huangfu et al., 2008) and TGF $\beta$  inhibitor (Lin et al., 2009) with reverse genetics to elucidate the machineries of reprogramming.

### 3.4 Chemical biology in other lineage differentiation

As like cardiogenic chemicals and small molecules for maintenance and generation of ESC/iPSC, Douglas A. Melton and his colleagues reported some small molecules induce definitive endoderm and pancreatic lineage differentiation using HCS (Borowiak et al., 2009; Chen et al., 2009). Inducers of definitive endoderm (IDE) 1 and 2 were identified from 4,000

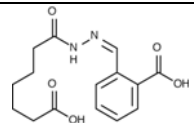
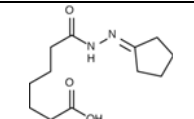
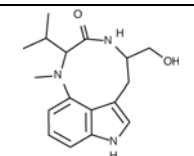
Compound	structure	Assay	Screened compound number	Molecular targets
IDE1		Sox17-DsRed	4,000	Trans-activate TGF $\beta$ /nodal signaling
IDE2				
Indolactam V		Pdx1 staining	5,000	Activate PKC signaling

Table 4. Small molecules using endodermal lineage induction from ESC/iPSC

compounds using Sox17-dTomato mESC line to replace Activin A for the initial induction of endoderm. IDE-1 and 2 transactivate TGF $\beta$ /Activin A/nodal signaling through unknown machinery. (-)-Indolactam V was identified from 5,000 compounds as inducing differentiation of Pdx1<sup>+</sup> pancreatic progenitor from definitive endoderm derived from human ESCs using high content screening. Activation of PKC signaling is the major pathway of the induction of Pdx1<sup>+</sup> pancreatic progenitor using Indolactam V.

#### **4. An immunosuppressant cyclosporin-A induced cardiac progenitor and cardiomyocyte differentiation**

We reported cyclosporin-A, an immunosuppressant specifically and efficiently induce cardiac progenitors and cardiomyocytes from mesoderm cells (Yan et al., 2009). In this section, we summarize our results (Figure 3 and 4).

##### **4.1 CsA induced cardiac progenitors and cardiomyocyte differentiation from mesoderm cells.**

Addition of CsA (1-3 $\mu$ g/mL) to Flk1<sup>+</sup> cells on OP9 showed a striking effect to increase cardiomyocyte differentiation at Flkd6. Cardiac troponin T positive area was increased more than 10 times and 60% of Flk1<sup>+</sup> cell-derived cells became  $\alpha$ MHC-GFP<sup>+</sup> cardiomyocytes in maximum. To clarify stage specificity, we added CsA during the differentiation stage of undifferentiated ESC-Flkd0, Flkd0-d2, and Flkd2-d6. CsA most potently showed the cardiogenic effect during Flkd0-2. Thus, we evaluated CsA effect on FCV cardiac progenitor differentiation and demonstrated that CsA treatment potently increased cardiac progenitor differentiation more than 20 times. Interestingly, CsA treatment strongly suppressed endothelial or hematopoietic differentiation suggesting that CsA may shift the cell fate from hemangiogenic to cardiogenic.

##### **4.2 Character, expanded cardiac progenitor**

Expanded FCV progenitor cells express *Flk1*, *Cxcr4*, *Gata4*, *Nkx2.5*, and *Islet1* but not myosin chains nor ion channels. *Nkx2.5* and *Islet1* are the markers for the first and second heart field respectively, which were reported as cardiac progenitor markers in ESC differentiation (Moretti et al., 2006; Wu et al., 2006). In FCV cells, approximately 24% of FCV cells were positive for *Nkx2.5*, 42% were positive *Islet1*, and 14% were double positive for *Nkx2.5* and *Islet1*. This efficient expansion of the rare FCV progenitor cells allowed us to confirm their cardiogenic potential *in vivo*. We performed transplantation of CsA-induced FCV cells to rat chronic myocardial infarction model. At 2 weeks after the injection, transplanted FCV cells were successfully differentiated into cardiomyocytes and integrated in the infarct heart. This result indicates that CsA-expanded FCV cells can show highly cardiogenic potentials also *in vivo* after cell transplantation.

Cardiomyocytes induced using CsA express various cardiac markers such as *Gata4*, *Tbx3*, *Nkx2.5*, *Islet1* and multiple myosin chains. Cardiomyocytes also have apparent sarcomere structure, and pacemaker like potential with spontaneous beating or quiescent ventricular type potential.

##### **4.3 Molecular mechanism of cyclosporin A**

Cyclosporin A is known as inhibitor of Calcineurin-NFAT signaling. To elucidate whether NFAT cascade is important to inducing cardiomyocyte differentiation, we tested various

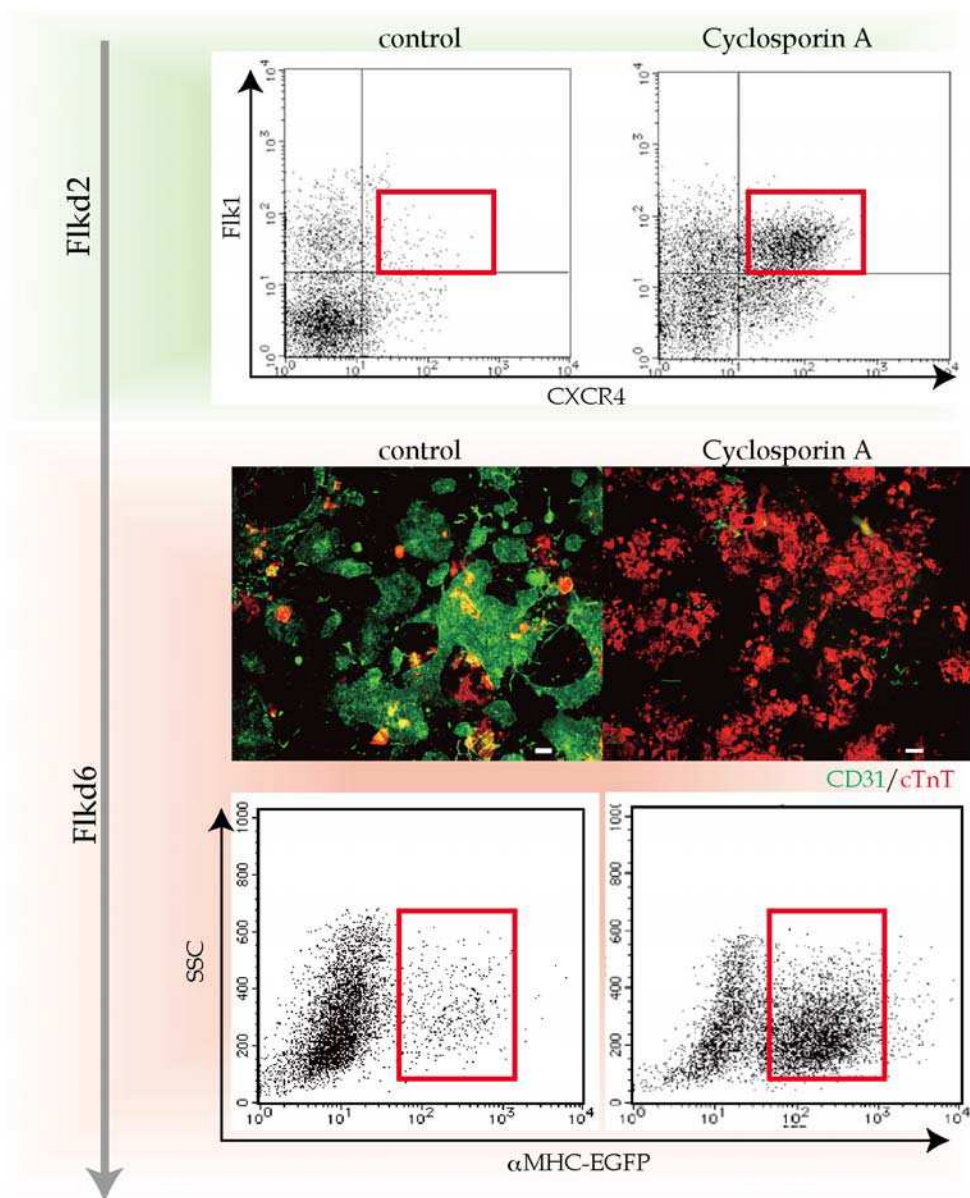


Fig. 3. An immunosuppressant cyclosporin A strikingly induce differentiation of FCV cardiac progenitor and cardiomyocytes. Administration of cyclosporin A increased FCV cardiac progenitor differentiation about 20 fold and cardiomyocyte differentiation about 10 fold with reciprocal inhibition of endothelial differentiation. (modified from Yan et al., 2009)

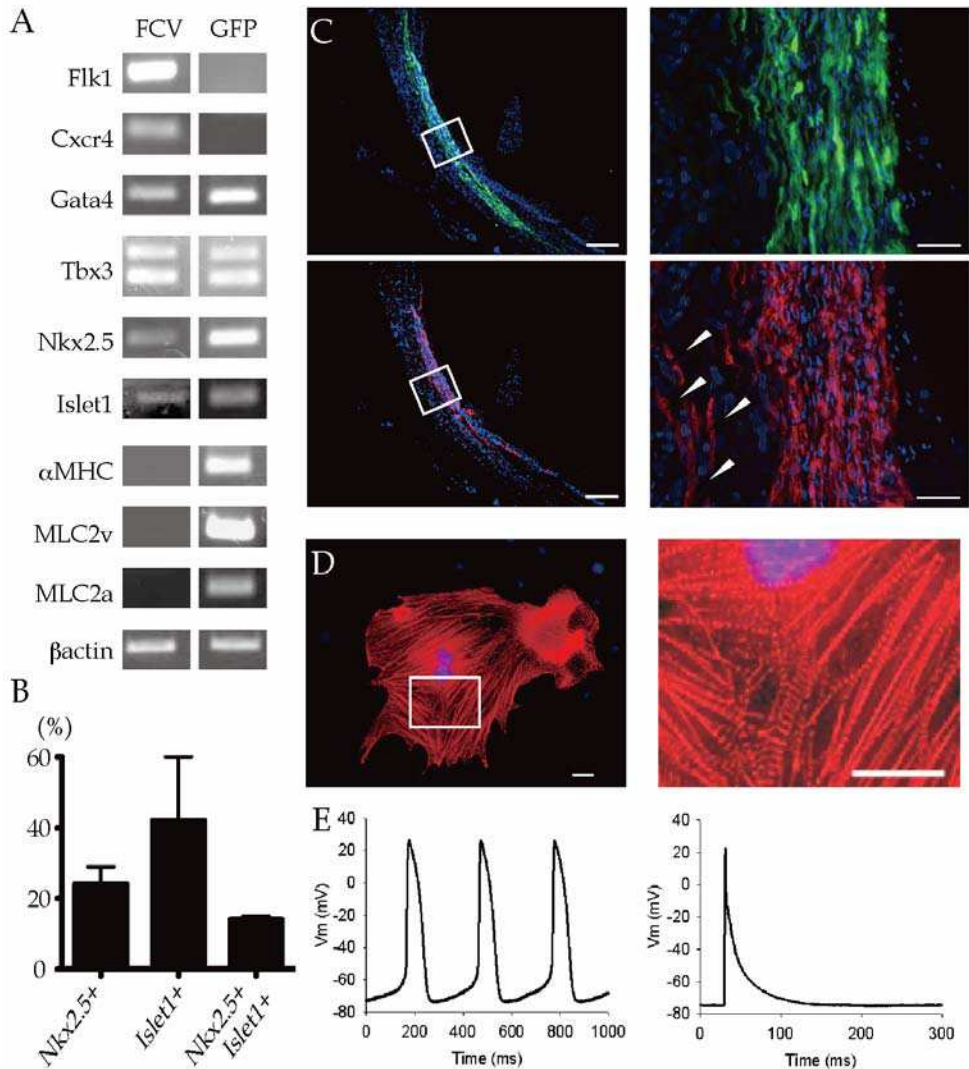


Fig. 4. Characters of expanded FCV cardiac progenitor cells and cardiomyocytes. (A) mRNA expression in purified FCV cardiac progenitor cells and  $\alpha$ MHC-GFP<sup>+</sup> cardiomyocytes. (B) Quantitative analysis of immunostaining for Islet1 and Nkx2.5 in purified FCV cells. (C) Cardiogenic potential of FCV cells in vivo. Purified FCV cells were injected into rat chronic myocardial infarction model. Green:  $\alpha$ MHC-EGFP<sup>+</sup> donor derived cardiomyocytes, Red: cardiac troponin T+ donor and recipient cardiomyocytes, Blue: DAPI (D) Appearance of isolated cardiomyocyte. Apparent sarcomere structure was observed. Red: Actinin, Blue: DAPI. (E) Action potential of isolated cardiomyocyte. Both of pacemaker like potential with spontaneous beating and quiescent ventricular type potential were observed. (modified from Yan et al., 2009)

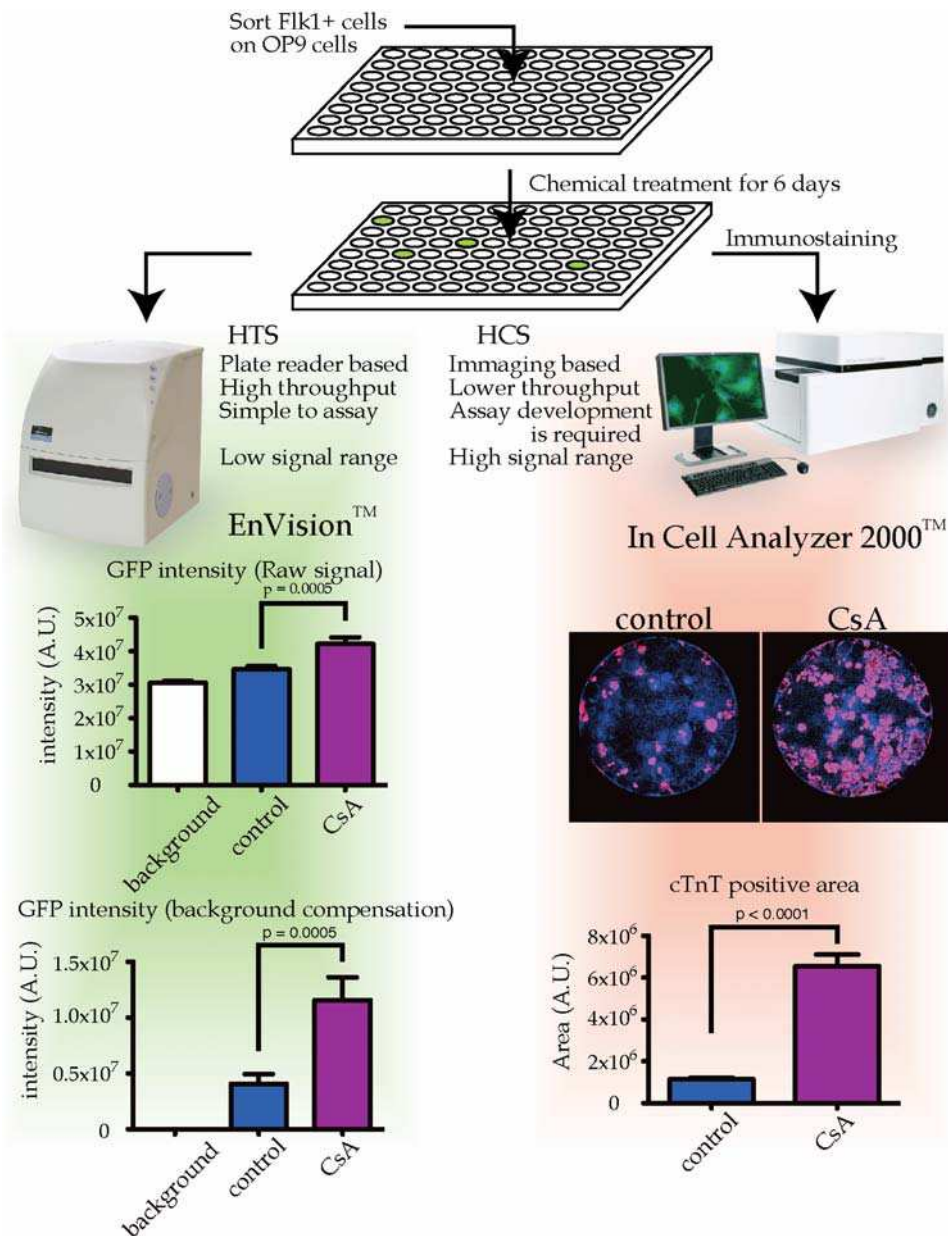


Fig. 5. Application of 2D differentiation system to HTS and HCS. Flk1+ cells are sorted directly on OP9-plated 96 well dish and treated with each chemical from chemical library. After 6 days, high throughput screening (HTS) or high content screening (HCS) is carried out for measuring cardiomyocyte differentiation. HCS provide clear and efficient results than HTS, which show high background level.

NFAT signal inhibitors – FK506, VIVID (NFAT inhibitor) and cypermethrin. All of NFAT inhibitors failed to induce cardiomyocyte differentiation. On the other hand, NFAT inhibitors strongly inhibited endothelial differentiation. These results indicate that CsA effect on cardiomyocyte induction is through unknown pathway, and CsA effect on endothelial induction is through NFAT signaling.

## 5. Extension to HTS and HCS

The finding of CsA prompted us to apply our system to HTS and/or HCS (Figure 5). We first developed HTS using  $\alpha$ MHC-GFP ESCs to determine cardiac differentiation. We found this system could work but it showed high background signals and low signals to noise ratio because of lower dynamic range of GFP (Willems et al., 2009). Thus, to validate the result we needed to reanalyze using immunostaining or flow cytometry. We then developed HCS using  $\alpha$ MHC-GFP and cTnT staining, next. HCS required us some development for imaging and analyzing for the assay and it took much more time for staining and imaging than HTS. As expected, it showed lower background and higher signal to noise ratio than HTS. The balance between the data quality and cost and/or time should be considered to choose HTS or HCS.

Recently we have identified novel cardiogenic effects of natural compounds that strongly promote cardiac differentiation from Flk1<sup>+</sup> mesoderm cells using this HTS combined with HCS for validation of the results (Fukushima et al., unpublished). We also have identified some cardiomyocyte proliferating chemicals using HCS to directly count cardiomyocyte number and analyze cell cycle (Uosaki et al., unpublished).

## 6. Conclusion

Cardiac development *in vivo* and *in vitro*, chemical biology combined with stem cells, and comparison of HTS and HCS are described here. In chemical biology, it is of course important to identify and clarify how chemicals work and what molecular mechanisms are. Indeed it is difficult in some case, chemical biology would greatly improve and accelerate stem cell biology, which has been based on embryonic development.

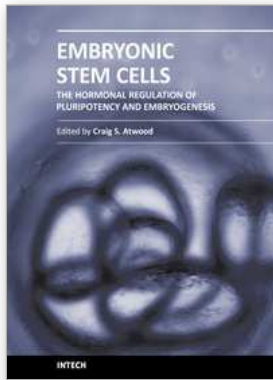
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