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CORE TIP	Induced pluripotent stem (iPS) cells present great promise, both to research and to medicine. However, we know very little regarding the mechanisms that occur throughout the iPS cell reprogramming process and thus the process remains inefficient. In this review, we discuss the 3 stages of reprogramming, initiation, maturation and stabilisation, and clarify the signalling pathways underlying each phase. We draw together the current knowledge to propose a model for the interactions between the key pathways in iPS cell reprogramming with the aim of illuminating this complex yet fascinating process.
KEY WORDS	Pluripotency; Reprogramming; Induced pluripotent stem; Cell signalling; Embryonic stem
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Abstract

Induced pluripotent stem (iPS) cells, somatic cells reprogrammed to the pluripotent state by forced expression of defined factors, represent a uniquely valuable resource for research and regenerative medicine. However, this methodology remains inefficient due to incomplete mechanistic understanding of the reprogramming process. In recent years, various groups have endeavoured to interrogate the cell signalling that governs the reprogramming process, including LIF/STAT3, BMP, PI3K, FGF2, Wnt, TGF β and MAPK

pathways, with the aim of increasing our understanding and identifying new mechanisms of improving safety, reproducibility and efficiency. This has led to a unified model of reprogramming that consists of 3 stages: initiation, maturation and stabilisation. Initiation of reprogramming occurs in almost all cells that receive the reprogramming transgenes; most commonly *Oct4*, *Sox2*, *Klf4* and *cMyc*, and involves a phenotypic mesenchymal-to-epithelial transition. The initiation stage is also characterised by increased proliferation and a metabolic switch from oxidative phosphorylation to glycolysis. The maturation stage is considered the major bottleneck within the process, resulting in very few “stabilisation competent” cells progressing to the final stabilisation phase. To reach this stage in both mouse and human cells, pre-iPS cells must activate endogenous expression of the core circuitry of pluripotency, comprising *Oct4*, *Sox2*, and *Nanog*, and thus reach a state of transgene independence. By the stabilisation stage, iPS cells generally use the same signalling networks that govern pluripotency in embryonic stem cells. These pathways differ between mouse and human cells although recent work has demonstrated that this is context dependent. As iPS cell generation technologies move forward, tools are being developed to interrogate the process in more detail, thus allowing a greater understanding of this intriguing biological phenomenon.

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Core tip: Induced pluripotent stem (iPS) cells present great promise, both to research and to medicine. However, we know very little regarding the mechanisms that occur throughout the iPS cell reprogramming process and thus the process remains inefficient. In this review, we discuss the 3 stages of reprogramming, initiation, maturation and stabilisation, and clarify the signalling pathways underlying each phase. We draw together the current knowledge to propose a model for the interactions between the key pathways in iPS cell reprogramming with the aim of illuminating this complex yet fascinating process.

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INTRODUCTION

Pluripotency, the ability of a single cell to give rise to all cells within an entire living organism, is of great biological interest both in terms of understanding developmental mechanisms as well as the medical potential that pluripotent stem cells possess. However, our understanding of the cell signalling networks underlying this complex process still remains incomplete. The first pluripotent stem cells were isolated from mouse blastocysts simultaneously by 2 groups in 1981^[1,2]. This was replicated 17 years later using human blastocysts^[3]. Embryonic stem (ES) cells have since been isolated

from other species including rhesus monkeys^[4] and rats^[5,6]. Both human and mouse ES cells have provided an invaluable resource to understand the basic biology of the pluripotent state.

A “core circuitry” of homeodomain transcription factors, *Oct4*^[7], *Sox2*^[8] and *Nanog*^[9], governs pluripotency in both mouse and human ES cells^[10]. These transcription factors are expressed both *in vivo* in the inner cell mass (ICM) of the blastocyst and *in vitro*, in pluripotent cells. These 3 factors closely interact within the cell; for example *Oct4* and *Sox2* have been shown to form a heterodimeric transcription complex^[11-13] and all 3 factors share target genes^[14,15]. This interaction facilitates the precise regulation of the core circuitry necessary to maintain the pluripotent state; for instance *Oct4* overexpression leads to endoderm and mesoderm differentiation whereas blockade of *Oct4* induces trophoblast differentiation^[7]. This may be explained by its biphasic role in *Nanog* regulation whereby low levels of *Oct4* result in upregulation of *Nanog* whereas higher levels of *Oct4* result in downregulation of *Nanog*^[15]. Similarly, small increases in *Sox2* expression or ablation of *Sox2* expression both induce multilineage differentiation^[16]. Blockade of *Nanog* does not induce differentiation, thus indicating that *Nanog*'s role in the core circuitry of pluripotency is to stabilise the pluripotent state rather than acting as a housekeeper. However, *Nanog* knockdown does lead to an increased capacity for differentiation into primitive ectoderm^[9]. The core pluripotency circuitry is also autoregulatory since all 3 factors have been shown to regulate the expression of each other as well as themselves^[14,15,17]. Interestingly, SOX2 is dispensable for the activation of *Oct4/Sox2* target genes since forced expression of *Oct4* is able to rescue pluripotency in *Sox2*^{-/-} cells, however, *Sox2* expression is necessary to maintain *Oct4* expression^[8]. Although it is

clear that OCT4, SOX2 and NANOG occupy the top level of the pluripotency hierarchy, these core factors also regulate a wide range of genes associated with pluripotency signalling networks including *Stat3*, *Zic3*, *TdGF1*, *Lefty/Ebaf*, *Dkk1* and *Frat2*^[14].

With the emergence of this complex molecular inter-play of dosage dependency between hierarchical transcription factors in the maintenance of the somewhat unstable pluripotent ground state, it seems surprising that simply over-expressing these factors in somatic cells can induce the pluripotent state. However, the collective seminal studies of Yamanaka and Thomson show this to be feasible in their descriptions of reprogramming somatic cells to induced Pluripotent Stem (iPS) cells^[18-20].

The original iPS cell reprogramming strategy published by Takahashi *et al*^[19] 7 years ago remains robust and largely unaltered to the present day. The “Yamanaka factors”, *Oct4*, *Sox2*, *Klf4* and *cMyc* were constitutively expressed using genome integrating retroviruses in both mouse^[18] and subsequently human^[19] fibroblasts, and under ES cell culture conditions were able to induce pluripotency. To date, this methodology is still widely used, however, various adaptations to the method of vector delivery and reprogramming factors (Table 1) have been made. Advances in vector delivery have generally been made to either improve efficiency or safety, by preventing integration of the transgenes into the genome. For example, iPS cells have now been successfully generated using episomal plasmids^[21], Sendai viruses^[22] and piggyBac transposons^[23] to deliver the reprogramming factors and even proteins^[24] or small molecules^[25] alone. Many divergent cell-types have been successfully reprogrammed to pluripotency including neural stem cells^[26], neural progenitor cells^[27], keratinocytes^[28], B lymphocytes^[29], meningeal

membrane cells^[30], peripheral blood mononuclear cells^[31] and pancreatic β cells^[32]. Often the minimal factors necessary to reprogram a cell depend on the endogenous “stemness” of the starting cell, for example, neural stem cells can be reprogrammed using *Oct4* alone since they express high levels of the other Yamanaka factors^[26].

The common aspiration is that iPS cells will provide an autologous source of cells for a multitude of regenerative medicine therapies in the future and clinical trials using iPS cells have begun^[33]. However, the most immediate utility of iPS cell technologies is the ability to study patient-derived cells in the lab. iPS cells present the opportunity to study a range of diseases in novel ways by isolating and reprogramming patient-specific cells and then differentiating them into the cell type of interest. For example, iPS cells have been generated from patients suffering from a wide range of disorders including Duchenne muscular dystrophy, Parkinson’s disease, Huntington’s disease, type I diabetes and Down’s syndrome (reviewed in^[34]). In addition, cells such as disease-specific cardiomyocytes, which would be difficult to obtain from patients, can also be generated and used to test specific drugs^[35]. In summary, the generation of iPS cells has stimulated the growth of a hugely active new area of research with promise to revolutionise medicine. However, the reprogramming process remains extremely inefficient and the basic molecular understanding of a process that does not appear to readily occur in nature is only just being unravelled. A greater understanding of the basic biology will lead to more efficient methodologies for iPS cell reprogramming *in vitro* and also potentially lead to strategies to therapeutically manipulate differentiated cells *in vivo* to become stem cells and repair or

regenerate diseased tissues.

IPS REPROGRAMMING IS A STEPWISE PROCESS

Much progress has been made in recent years to define the molecular mechanisms involved in iPS cell reprogramming. This has led to the general acceptance of the model proposed by Samavarchi-Tehrani *et al*^[36] that reprogramming consists of 3 phases: initiation, maturation and stabilisation (Summarised in Figure 1). Throughout reprogramming various changes occur not only to the cell phenotype but also to gene and non-coding RNA expression, epigenetic status and metabolism. In this review we will focus on cell signalling during the 3 stages of iPS cell reprogramming whilst other aspects are reviewed elsewhere by Papp *et al*^[37] and Jia *et al*^[38].

INITIATION

The initiation phase of reprogramming occurs in virtually all successfully transfected cells^[39] and is characterised by somatic genes being switched off by methylation, an increase in cell proliferation, a metabolic switch from oxidative phosphorylation to glycolysis, reactivation of telomerase activity and a mesenchymal-to-epithelial transition (MET)^[40]. MET is a feature of both mouse^[41] and human^[42] somatic cell reprogramming and involves the loss of mesenchymal characteristics such as motility and the acquisition of epithelial characteristics such as cell polarity and expression of the cell adhesion molecule E-CADHERIN, perhaps explaining why *E-cadherin* can replace *Oct4* in the reprogramming process^[43]. MET and the opposite transition, epithelial-to-mesenchymal transition (EMT), are key features of embryogenesis^[44], tumour metastasis^[45] and both mouse^[46] and human^[47] ES cell differentiation. Interestingly, the MET

that marks the initiation of cellular reprogramming is reversible since removal of the reprogramming factors from mouse “pre-iPS” cells after induction of reprogramming has been shown to lead to reversion of the cells to a mesenchymal phenotype^[36], thus demonstrating that continued transgene expression is necessary to allow cells to progress to the maturation stage.

Mechanistically, *Sox2* suppresses expression of *Snail*, an EMT inducer^[48], and *Klf4* induces *E-cadherin* expression, thus promoting MET^[41]. In addition, Maekawa *et al*^[49] have shown that the Glis family zinc finger 1 protein *Glis1* can substitute *cMyc* in the reprogramming cocktail by inducing MET, thus initiating iPS cell reprogramming. MET can also be induced by chemicals, for example, various groups have demonstrated the ability of transforming growth factor (TGF) β inhibition to enhance the initiation stage of both mouse^[50,51] and human^[42] somatic cell reprogramming. This observation is supported by the finding that addition of recombinant TGF β abrogates iPS cell formation^[42] and is likely due to the EMT-inducing action of TGF β signalling, which then prevents the MET that is critical to successful iPS cell reprogramming. TGF β signalling promotes EMT *via* a wide variety of mechanisms, including mediating the disassembly of junctional complexes, reorganising the cell cytoskeleton, and EMT gene activation^[52]. Various TGF β inhibitors have been used to promote reprogramming, including A-83-01^[41,53], E616452^[25,50] (also known as RepSox) and SB431542^[42] (Table 2). In addition to promoting MET, TGF β inhibitors promote *Nanog* expression^[50], thus providing 2 potential mechanisms for their ability to enhance reprogramming. Mitogen-activated protein kinase (MAPK) signalling,

activated by TGF β , further induces the expression of mesodermal genes^[52]. Inhibitors of MAPK signalling such as PD0325901 have therefore been used in combination with TGF α inhibitors to promote MET^[42].

Bone morphogenetic protein (BMP) signalling also plays an important role in the initiation stage of mouse iPS cell reprogramming by promoting MET *via* upregulation of epithelial genes such as *E-cadherin*, *Occludin* and *Epithelial cell adhesion molecule*^[36]. Chen *et al*^[54] have shown that BMPs can replace *Klf4* in the reprogramming cocktail, allowing mouse embryonic fibroblasts (MEFs) to be reprogrammed using *Oct4* alone. However, constitutive BMP activation prevents human somatic cell reprogramming. This was discovered through the observation that a naturally occurring *Alk2* mutation, which causes fibrodysplasia ossificans progressiva in humans, prevents iPS cell reprogramming and that this blockade can be rescued by inhibition of the ALK2 receptor^[55].

Increased proliferation has been observed in cells undergoing reprogramming as early as 3 d after induction of reprogramming^[56] and is likely to be initiated by *cMyc* transgene expression^[57]. *Lin28* expression and *p53* knockdown also increase the efficiency of iPS cell reprogramming by stimulating cell proliferation^[39]. Specifically, LIN28 has been shown to regulate cell cycle genes such as *Cyclin A*, *Cyclin B* and *Cdk4*^[58] whilst *p53* induces cell cycle arrest *via* *p21* and thus *p53* knockdown promotes proliferation^[59].

Fibroblast growth factor (FGF) signalling has also been implicated at the initiation stage^[60]. Araki *et al*^[61] show that *Fgf4* is upregulated on day 3 after induction of reprogramming in MEFs and Jiao *et al*^[60] show that FGF2 can improve the reprogramming efficiency in the early phases of mouse somatic cell reprogramming, whereas it has

adverse effects in the later stages. Mechanistically, this group have shown that FGF2 promotes the early stages of reprogramming through accelerating cell proliferation, facilitating MET and eliminating extracellular collagens. In addition to an increased proliferation rate, the minority of cells that undergo successful reprogramming also exhibit resistance to apoptosis and senescence, by transgene expression^[56]. Recent studies have shown that miR-302 expression allows cells to overcome reprogramming-induced senescence^[62] and that silencing of the INK4/ARF locus is also likely to be involved, since INK4/ARF blockade improves reprogramming efficiency^[63,64]. The INK4/ARF locus encodes tumour suppressor genes that activate the retinoblastoma and p53 pathways. Its inactivation therefore blocks apoptosis and senescence and facilitates reprogramming.

The initiation phase is also characterised by a metabolic switch from oxidative phosphorylation to glycolysis^[65] that occurs around 7 d after induction of reprogramming^[66] and involves phosphatidylinositol-3-kinase (PI3K)/AKT signalling^[53,67]. For example, Chen *et al*^[67] have demonstrated that the PI3K/AKT pathway was activated during reprogramming in parallel with the upregulation of glycolytic gene expression, showing specifically that AKT activated 2 key glycolytic regulators, AS1060 and PFKFB2. Zhu *et al*^[53] have also shown that PS48, an activator of the PI3K/AKT pathway, is able to enhance reprogramming by upregulating glycolytic genes. By switching their metabolism from oxidative phosphorylation to anaerobic glycolysis, pre-iPS cells assume an ES cell-like phenotype^[68]. ES cells are likely to have developed this form of metabolism as an adaptation to the hypoxic *in vivo* environment of the early embryo^[69]. Interestingly, various groups have shown that iPS cell reprogramming is enhanced

by hypoxia^[70,71], likely due to the acceleration of this metabolic shift.

MATURATION

Tanabe *et al*^[72] have recently identified the maturation stage of iPS cell reprogramming as being a major bottleneck in the process, which is likely to account for the low efficiency of the process generally. They demonstrate that LIN28, but not NANOG, shp53 or CYCLIN D1, promotes maturation of iPS cells. During maturation, epigenetic changes occur allowing expression of the first pluripotency-associated genes^[40]. These genes include *Fbxo15*, *Sall4*, *Oct4*, *Nanog* and *Esrrb*. Interestingly, *Esrrb* has been shown to be sufficient to reprogram MEFs in collaboration with *Sox2* and *Oct4*^[73].

LIF/STAT3 signalling is required for the maturation phase of mouse iPS cell reprogramming^[74]. Interestingly, pre-iPS cell colony formation has been observed in the absence of LIF, however, beyond day 6 of reprogramming these colonies detach. This is likely due to the requirement that cells undergoing the reprogramming process have for LIF signalling to maintain *cMyc* expression^[75]. In addition, Tang *et al*^[74] demonstrate that LIF/STAT3 activation induces earlier formation of an increased number of pre-iPS cell colonies. Mechanistically, this group demonstrate that LIF/STAT3 signalling is required for demethylation of pluripotency-associated gene promoters. Specifically, STAT3 signalling was shown to directly block the action of the DNA methyltransferase DNMT1 and Histone deacetylases 2, 3 and 8.

Wnt signalling also enhances the maturation phase of mouse somatic cell reprogramming whereby exogenous stimulation of the pathway using Wnt3a between days 6 and 9 after induction of

reprogramming enhances the formation of *Nanog* positive colonies^[76]. Various groups have suggested that expression of *Nanog* is necessary for cells to advance from the maturation phase to the stabilisation stage^[39,77] and thus, Samavarchi *et al*^[36] suggest that *Nanog* expression alone is responsible for mediating the transition from pre-iPS cells to stably reprogrammed cells. This group demonstrate that removal of the reprogramming factors from mouse iPS cells at day 9 after induction of reprogramming did not induce phenotypic reversion. Other groups, however, have reported different time points for the stabilisation stage, including day 11^[78,79] and day 16^[80], suggesting that this can vary depending on discrete protocols and culture variations. It is clear that there remains substantial information to be learned regarding this critical intermediary step but NANOG appears to play a pivotal role in iPS cell maturation.

STABILISATION

Only around 1% of cells that initiate reprogramming make it to the stabilisation stage^[72]. This can be explained by the observation made by Golipour *et al*^[81] that not all cells are “stabilisation competent”. This group identify a gene expression signature that distinguishes stabilisation competent and stabilisation incompetent cells and show that stabilisation competent cells require transgene repression to enter this stage. Since the stabilisation stage is characterised by transgene independence, only cells that have activated endogenous pluripotency gene expression are able to maintain pluripotency at this late stage. Endogenous pluripotency gene expression is facilitated by demethylation of pluripotency gene promoters, thus explaining why various DNA and histone methyltransferase inhibitors

have been shown to accelerate iPS cell reprogramming, amongst other small molecules (Table 2). This may also explain the ability of the H3K27 demethylase UTX to substitute for some of the original reprogramming factors^[82].

The end-point of iPS cell reprogramming is a matter of some controversy. For example, the stabilisation stage of mouse iPS cell reprogramming involves X chromosome reactivation whereas human iPS cell reprogramming does not^[83]. X chromosome inactivation is a process that occurs as female embryonic cells, which have 2 active X chromosomes, commit to differentiation. This feature of human ES and human iPS cells, amongst others (reviewed in^[84]), means that they represent the primed pluripotent state. Human iPS cells generated in the presence of ACTIVIN/NODAL and FGF2 ligands are stabilised in this primed state whereas mouse iPS cells reprogrammed in the presence of LIF and BMP4 can be fully reprogrammed to the uncommitted naïve ground state (Figure 2). Interestingly, human dermal fibroblasts (HDFs) have been shown to give rise to naïve human iPS cells when reprogrammed in the presence of LIF, FGF2 and TGF β 1 plus inhibitors of c-Jun NH2-terminal kinase, p38, MAPK and glycogen synthase kinase 3 (3i)^[85], thus demonstrating that the cell signalling context is critical to the determination of naïve and primed pluripotency rather than the two states representing a species difference. The derivation of various novel stem cell lines, including intermediate epiblast stem cells which exhibit dual responsiveness to LIF and ACTIVIN/NODAL signalling^[86], has challenged the concept of 2 distinct pluripotent states, instead suggesting that a spectrum of pluripotency exists, an idea we develop in Hawkins *et al*^[87]. Thorough investigation into this spectrum of pluripotency, and therefore the transition from

pluripotent cells to differentiated cells, should accelerate the delineation of mechanisms occurring throughout the reverse process, from a somatic cell to an iPS cell.

CONCLUSION

A proposed model for the signalling networks required for the various stages of mouse and human iPS cell reprogramming can be found in Figure 1. However, this knowledge is still vastly incomplete. New technological advances are required to thoroughly interrogate the contribution of a wide range of signalling pathways to somatic cell reprogramming. One of the limitations of many current approaches is the inability to track reprogramming cell signalling in real-time since cells must be sacrificed to obtain data, for example for microarray analysis^[36], fluorescence-activated cell sorting or protein extracts^[78] at various time points. Some advances have been made to track reprogramming cells in real-time, for example, Smith *et al*^[88] carried out time-lapse imaging with the aim of tracking single cells undergoing the reprogramming process. However, they concluded that this was virtually impossible. We are currently interrogating the role of cell signalling networks in iPS cell reprogramming using a range of GFP reporter HDF lines activated by transcription factors involved in relevant cell signalling pathways. This allows us to monitor signalling pathway activity throughout an entire iPS cell reprogramming experiment in real-time. We anticipate this will enable us to temporally map the contribution of a wide range of signalling pathways to iPS cell reprogramming, thus illuminating this enigmatic biological phenomenon.

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FIGURE LEGENDS

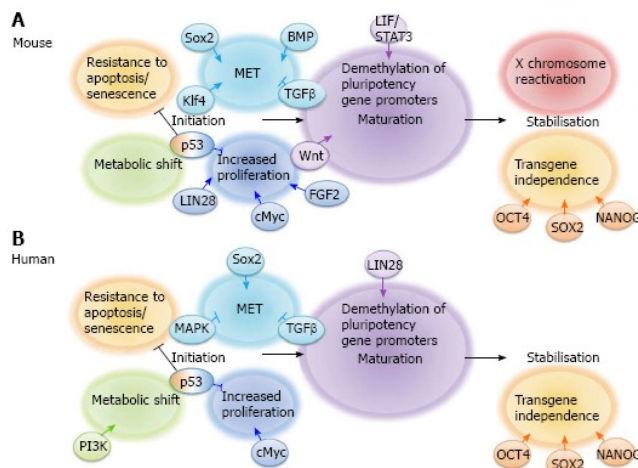


Figure 1 The key stages in (A) mouse and (B) human induced pluripotent stem cell reprogramming and the signalling pathways that regulate them.

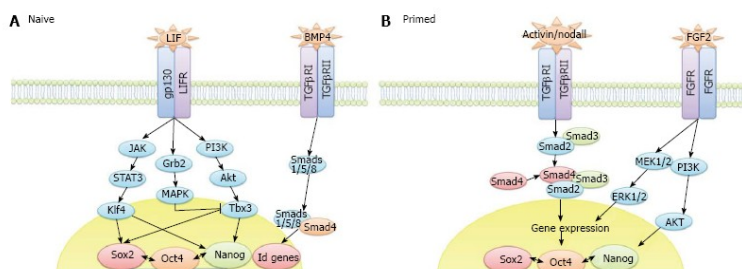


Figure 2 The core signalling networks that maintain pluripotency in (A) naive and (B) primed pluripotent cells.

Table 1 Factors that have been shown to achieve induced pluripotent stem cell reprogramming

Reprogramming factor	Human/mouse	Ref.
Oct4	Both	Takahashi <i>et al</i> ^[18,19]
Sox2	Both	Takahashi <i>et al</i> ^[18,19]
cMyc	Both	Takahashi <i>et al</i> ^[18,19]
Klf4	Both	Takahashi <i>et al</i> ^[18,19]
Nanog	Human	Yu <i>et al</i> ^[20]
Esrrb	Mouse	Feng <i>et al</i> ^[73]
Glis1	Both	Maekawa <i>et al</i> ^[49]
E-cadherin	Mouse	Redmer <i>et al</i> ^[43]
shp53	Both	Hanna <i>et al</i> ^[39]
Lin28	Both	Hanna <i>et al</i> ^[39]
UTX	Both	Mansour <i>et al</i> ^[82]

Table 2 Small molecules that enhance induced pluripotent stem cell reprogramming

Small molecule	Function	Ref.
BIX-01294	Histone methyltransferase inhibitor	Shi <i>et al</i> ^[51]
Bayk8644	Calcium channel agonist	Shi <i>et al</i> ^[51]
RG108	DNA methyltransferase inhibitor	Shi <i>et al</i> ^[51]
5-Aza-2'-Deoxycytidine	DNA methyltransferase inhibitor	Huangfu <i>et al</i> ^[89]
Dexamethasone	Steroid glucocorticoid	Huangfu <i>et al</i> ^[89]
Valproic acid	HDAC inhibitor	Huangfu <i>et al</i> ^[89]
Trichostatin A	HDAC inhibitor	Huangfu <i>et al</i> ^[89]
SAHA	HDAC inhibitor	Huangfu <i>et al</i> ^[89]
PD0325901 + CHIR99021	MAPK inhibition and GSK3 inhibition	Shi <i>et al</i> ^[51] , Silva <i>et al</i> ^[77]
SB 431542+ PD032590 1	TGFβ inhibitor And MAPK inhibitor	Lin <i>et al</i> ^[42]
A-83-01	TGFβ inhibitor	Li <i>et al</i> ^[41] , Zhu <i>et al</i> ^[53]
E616452	TGFβ inhibitor	Ichida <i>et al</i> ^[90]
AMI-5	Protein arginine methyltransferase inhibitor	Yuan <i>et al</i> ^[13]
Kenpaullone	Unknown "novel function"	Lyssiotis <i>et al</i> ^[91]

Adapted from Feng *et al*^[73]. SAHA: Suberoylanilide hydroxamic acid; AMI: Arginine N-Methyltransferase Inhibitor.

