Chapter 4

Roles of Tumor Suppressor Signaling on Reprogramming and Stemness Transition in Somatic Cells

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1. Introduction

The pioneering landmark, established by Takahashi and Yamanaka (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) in reprogramming somatic cells into induced pluripotent stem (iPS) cells using the four transcriptional factors of Oct4, Sox2, Klf4, and c-Myc, represents one of the most important paradigm shifts in current stem cell biology. This unprecedented discovery could potentially revolutionize regenerative medicine, cell-based therapy and personalized medicine. Despite recent great advancement in cell reprogramming, there are still considerable technical challenges to circumvent restrictions of applications of reprogramming technology (Kawamura et al., 2009; Saha and Jaenisch, 2009). The utilization of over-expressed transcriptional factors, which of many play oncogenic roles, during somatic reprogramming posts the risk of malignant transformation, thus, limiting its clinical applications. Moreover, the reprogramming process using these factors is still inefficient in some of cell types, and is not always successful in other kinds of cells (Kawamura et al., 2009; Marion et al., 2009; Menendez et al., 2012). Therefore, the underlying mechanisms for signaling control of these factors still need to be further explored.

Somatic cell reprogramming is a complicated cellular process that is controlled by many signaling networks. Accumulated evidence indicated that stemness transition can be detected in some tumor cells following the introduction of relevant signal stimulation, and cancer cells or differentiated cells can be changed into stem cell-like cells that go through less-differentiated stages (Chen et al., 2008; Fodde and Brabletz, 2007; Huang et al., 2009; Liu et al., 2009a).

However, stemness transition may not lead to a full reprogramming of treated cells, which is determined by the delicate controls of signaling network activities in living cells. Interestingly, stemness transition may accompany epithelial-mesenchymal transition (EMT) events in cancer cells, and both programs are closely linked to the core stem cell gene network activities. Not surprisingly, multiple signaling pathways have been reported to be involved in EMT events and generation of stem cell-like cells. Wnt/β-catenin and TGF-β signaling are two potent inducers of EMT during embryonic development and cancer progression (Li et al., 2010; Mani et al., 2008; Morel et al., 2008; Scheel et al., 2011). Other involved pathways in these cellular activities may include BMP/Activin/Nodal, Notch, Hedgehog, Fibroblast growth factor signaling, and others (Chen et al., 2008; Huang et al., 2009; Kang and Massague, 2004; Natalwala et al., 2008; Thiery, 2002, 2003; Wu and Zhou, 2008).

The Wnt/β-catenin signaling pathway, highly conserved among various species and composed of a large family of proteins that control many biological properties (Fodde and Brabletz, 2007; Kikuchi et al., 2009; ten Berge et al., 2008b), may play a central role in the control of reprogramming and stemness process. This pathway includes more than two hundred genes and plays a critical role in modulating the delicate balance among stemness, proliferation, and differentiation in certain stem cell niches and tumor cells (Gu et al., 2010; Katoh, 2007; Lowry et al., 2005; Reya and Clevers, 2005). The established evidence reveals that various levels of Wnt/β-catenin signaling are likely to contribute to distinct cellular activities such as stemness transition, differentiation, carcinogenesis, and the EMT program. Therefore, the cellular activities and fate decisions are determined by this signaling activity in both dosage-dependent and tissue-dependent fashions (Anton et al., 2007; Kikuchi et al., 2009; Lluis et al., 2008; Reya and Clevers, 2005; Slack et al., 1995; Tapia and Scholer, 2010a; ten Berge et al., 2008a; Vermeulen et al., 2010). However, whether and how this signaling pathway has its direct influence on pluripotency gene networks and EMT events is largely unexplored.

As mentioned previously, cell fate decisions are controlled by both positive and negative forces in human cells. It has been well-established that tumor suppressor genes (TSGs) are important regulators to control cell proliferation, differentiation and cell death. Not surprisingly, these genes also play important roles in programming, reprogramming, and stemness transition in human cells. The well-studied TSGs, such as *p53*, *p16,* and *RB1*, serve as key regulators for the cell programming (Bonizzi et al., 2012; Hong et al., 2009; Liu et al., 2009b; Marion et al., 2009; Molchadsky et al., 2010; Wenzel et al., 2007). There are a number of reports on *p53* / p21 pathway that are involved in the reprogramming process and stemness transition in somatic cells. It should be noted that Wnt signaling was linked to the *p53* pathway a long time ago, suggesting that both signaling pathways may play interactive and critical roles in cell fate determination (Damalas et al., 1999; Kinzler and Vogelstein, 1996; Lee et al., 2010). Recent findings demonstrated that several mechanisms play a limiting role in somatic reprogramming and cell stemness transition (Figure 1) (Kawamura et al., 2009; Menendez et al., 2012; Menendez et al., 2010; Takahashi, 2010; Tapia and Scholer, 2010b). In most situations, these genes serve as active players or barriers for cell reprogramming. However, many essential questions on the roles of TSGs in cell fate decision remain unclear. For example, whether *p53*-induced inhibition in reprogramming is transient or just in the early stage is still in question (Cox and Rizzino,

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2010; Krizhanovsky and Lowe, 2009; Wahl, 2011). Also, it was reported that the loss of *RB1* is critical for the expansion of the stem cell populations (Liu et al., 2009a; Wenzel et al., 2007). Undoubtedly, there is an urgent need to further elucidate the molecular mechanism and signaling pathways in regulating and controlling the process of somatic reprogramming and stemness transition.

Epigenetic regulation is one of the important mechanisms in the regulation of TSG activities. Recently, epigenetic modification has been shown to influence the reprogramming process, suggesting that many known TSGs may be involved in these cellular activities. Some reports illustrated that a dedifferentiation process of somatic cells to iPS cells involves dynamic epigenetic remodeling. In addition, there seem to be interactions between reprogramming transcription factors and epigenetic modifiers during these cellular activities (Takahashi, 2010).

In this chapter, the role of TSGs in cell reprogramming and stemness process, and regulation of these genes during stem cell renewal will be discussed, as described in Figure 1. We will review the role of TSG-mediated pathways and epigenetics as a barrier in cell fate determinations.

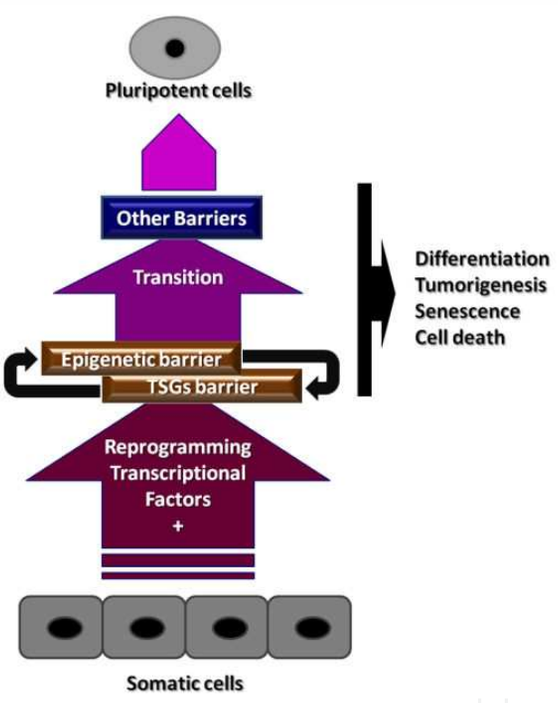

Figure 1. Schematic representative of somatic reprogramming. The reprogramming efficiency is markedly influence by TSG-mediate pathways and epigenetic modifications.

1.1. *CDKN2A (p16^{INK4A} and p14^{ARF})* gene

The *CDKN2A* (*INK4/ARF)* locus encodes two important TSGs, the *p16*^{INK4A} (or *p16*) and *p14*^{ARF}. They are important regulators for two other critical tumor suppressive signaling pathways for controlling cell proliferation, namely *RB1* and *p53*. Utikal et al. reported that secondary murine embryonic fibroblasts (MEFs) were capable of generating iPS cells at early passage, but the

efficiency decreased after serial cell culture passaging and the concomitant onset of cellular senescence (Utikal et al., 2009). This phenomenon was mainly correlated with accumulation of molecular changes in the late passage senescent MEFs (Utikal et al., 2009). Indeed, up-regulation of $p16^{INK4A}$ (*INK4A*), $p14^{ARF}$ (*ARF*), and $p21^{CIP}$ was concurrently observed in the late passage of the MEFs (Utikal et al., 2009). Deficiency and knockdown of *INK*, *ARF*, and *p53* expression resulted in higher efficiency of iPS cell formation. Interestingly, when MEFs were cultured in low oxygen condition (4%), both the expression of *INK4A* and *p53* were reduced. Most importantly, the efficiency of the iPS reprogramming was increased in the low oxygen condition. This further supports the role of *CDKN2A* and *p53* in inhibiting the reprogramming process (Utikal et al., 2009).

Concurrently, Li et al. also worked on the role of *INK4*/*ARF* locus which encodes three TSGs, $p16^{IN4A}$, $p14^{ARF}$, and $p15^{INK4B}$ on the reprogramming of differentiated cell into iPS cells. They showed that the locus is completely silenced in iPS and embryonic stem cells. The three transcription factors, Oct4, Klf4, and Sox2 repressed the gene expression of $p16^{INK4A}$, $p14^{ARF}$, and $p15^{INK4B}$ with concomitant appearance of iPS cells. In addition, genetic knockdown of the *INK4*/*ARF* locus improved the efficiency of iPS cell generation. In mouse cells, *ARF* played more significant role as compared to *INK4A*. In contrast, the *INK4A* function was more prominent than the *ARF* in human cells (Li et al., 2009). Interestingly, ageing up-regulated the gene expression of the three genes at the *INK4*/*ARF* locus and, in turn, led to less efficient reprogramming in cells from old organisms; this defect can be rescued by genetically inhibiting the *INK4*/*ARF* locus. Taken together, these findings provide strong evidence that supports the role of *CDKN2A* in regulating cell reprogramming in iPS cells.

The epidermis is a tissue that undergoes continual and rapid self-renewal, and which is dependent on the presence of stem cells and transient amplifying keratinocytes. In primary human keratinocytes, *INK4A* also plays an important role in regulation of their stemness properties (Maurelli et al., 2006). The *INK4A* inactivation enabled the primary human keratinocytes to escape replicative senescence and blocked clonal evolution and maintained keratinocytes having the stemness phenotypes. A persistent *INK4a* inactivation is necessary for maintenance of immortalization of the keratinocytes, which was accompanied by reactivation of B cell-specific Moloney murine leukemia virus site 1 *(Bmi-1)* expression and telomerase activity. *Bmi-1* expression is necessary to maintain the immortalization induced by *INK4a* inactivation. In contrast, the *INK4a* inactivation in the transient amplifying keratinocytes did not undergo immortalization but senescence. Thus, *INK4a* inactivation appears to selectively inhibit clonal conversion in highly proliferative somatic cells. Interestingly, inactivation of *INK4a* up-regulated the *ARF*/*p53*/$p21^{Waf1}$ pathway but this up-regulation of the *p53* pathway was unlikely to suppress the cell proliferation. The *p53* pathway was necessarily inactivated during immortalization of human keratinocytes. This study clearly indicates the regulation of keratinocyte clonal evolution by *INK4a* regulation and its inactivation in epidermal stem cells is necessary for maintaining the stemness phenotypes (Maurelli et al., 2006).

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1.2. *RB1* gene

RB1 (*pRB1* family members: *RB1, RBL1*, and *RBL2*) was identified as a TSG in patients with inherited retinoblastoma. It is one of the well-studied TSGs. It involves in cell cycle G_1/S transition regulation and binds to an important transcription factor family, E2F. Based on the Knudson two-hit hypothesis, loss of single copy of *pRB1* gene is not sufficient to induce tumor formation, loss of another copy is necessary for inducing tumor formation (Knudson, 1971). Mouse *pRB1*was found to be crucial during embryonic development; loss of two copies of *RB1* gene in mouse embryo is lethal (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Wu et al., 2003). Trophoblasts are cells forming the outer layer of a blastocyst, which provide nutrients to the embryo and develop into a large part of the placenta. Specific loss of mouse *pRB1* gene in trophoblast stem cells resulted in an overexpansion of trophoblasts, profound placental abnormalities, and eventually fetal death (Wenzel et al., 2007). Loss of *pRB1* resulted in an increase of *E2F3* expression and the combined depletion of *pRB1* and *E2F3* in trophoblast stem cells rescued the *pRB1* mutant phenotypes by restoration of placental development and by extending the lifespan of embryos. As can be seen, the *pRB1* pathway plays a critical role in the maintenance of a mammalian stem cell population for proper development of both extra-embryonic and fetal tissues.

Humans and other mammalians are unable to regenerate large portions of lost limbs or other internal organs after traumatic injury or surgical excisions. In contrast, lower vertebrates are able to regenerate entire limbs, the lens of the eye, and portions of the heart (Brockes and Kumar, 2008; Poss et al., 2002; Tanaka and Weidinger, 2008). The difference can be explained in part by the observation that inactivation of *pRB1* alone in lower vertebrates was sufficient to induce skeletal muscle regeneration by reversing differentiation and post-mitotic arrest in the muscle cells (Tanaka et al., 1997). In mammalian muscle cells, suppression of *pRB1* alone was not sufficient to reverse the post-mitotic arrest and terminal differentiation (Camarda et al., 2004; Huh et al., 2004; Pajcini et al., 2010). The tumor suppressor *ARF* which is present in mammals, but absent in regenerative vertebrates, is a regeneration suppressor in addition to *pRB1* (Pajcini et al., 2010). Concurrent inactivation of both *ARF* and *pRB1* resulted in mammalian muscle cell cycle re-entry cell proliferation and dedifferentiation (Pajcini et al., 2010). These results indicate that suppression of both *pRB1* and *ARF* will result in the ability of skeletal muscle cells to lose their differentiated characters, and the skeletal muscle cells will then proliferate and dedifferentiate in a manner that mimics the regenerative lower vertebrate cells. Furthermore, *pRB1* is not only restricted to serve as a cell cycle regulator, but also to impact differentiation and tissue-specific gene expression directly by binding histone deacetylase 1 (HDAC1) and promoting activation of muscle genes such as the myogenic activator MyoD (Puri et al., 2001).

The *pRB1* gene family plays an important regulatory role in neuronal differentiation (Slack et al., 1995). When treated with retinoic acid, the embryonal carcinoma p19 cells were induced to differentiate into cultures primarily consisting of neurons and astrocytes. During this neuroectodermal differentiation, a dramatically increase of pRB1 protein levels was observed. When the pRB1 family proteins in the p19 cells were inactivated by the E1A mutant, the differentiating p19 cells underwent apoptosis. The dying cells were those committed to the

neural lineages because neurons and astrocytes were lost from the differentiating cell culture. The results suggest that the pRB1 family proteins are essential for the neural lineage development and the absence of functional pRB1 activities will trigger cell death of the differentiating neuroectodermal cells.

The *pRB1* pathway is also critical for inducing the cellcycle arrest that mediates cell-cell contact inhibition in fibroblasts; when all three *pRB1* family members, *RB1, RBL1*, and *RBL2*, were inactivated by triple knockouts (TKOs), the fibroblasts escaped from contact inhibition and grew into 3D colonies or stacks in cell culture (Dannenberg et al., 2000; Sage et al., 2000). The outgrowth of TKO MEFs into spheres triggered reprogramming to produce cells with cancer stem cell properties. Whereas the fibroblasts with a single *pRB1* mutation retained contact inhibition, when this inhibition was bypassed by forcing the cells to form outgrowth spheres, the fibroblasts were reprogrammed to generate cells with a cancer stem cell phenotype (Liu et al., 2009a). These findings suggest a potential mechanism for generation of cancer stem cells from differentiated somatic cells as a result of tumor outgrowth.

1.3. *p53* gene

p53, as the "guardian of the genome" (Lane, 1992), plays a pivotal role in regulating the delicate balance of cell proliferation and cell death (Molchadsky et al., 2010). Since its discovery more than three decades ago, the role of *p53* in suppressing tumor initiation and progression is well established. It is, therefore, not surprising that *p53* is lost, inactivated, or mutated in the majority of cancers. In respond to external stress stimuli, *p53* prevents cancer development by inducing cellcycle arrest, DNA repair, senescence, and apoptosis.

Researchers have newly identified roles played by *p53* including regulation of pluripotency and dedifferentiation, as a potent barrier in reprogramming. (Hong et al., 2009). Undoubtedly, the function of *p53* is now far more complex than just simply playing the role as the classical tumor suppressor (Bonizzi et al., 2012; Kawamura et al., 2009; Marion et al., 2009; Menendez et al., 2010; Molchadsky et al., 2010; Tapia and Scholer, 2010a; Wahl, 2011; Zhao and Xu, 2010). This provides us with a new insight on the complexity of *p53* signaling in controlling cell fate decisions. Despite accumulating effort in deciphering the diversified roles played by *p53*, the cellular and molecular mechanism underlying the acquisition of "stemness" involved in the *p53* signaling is still largely unexplored.

During somatic cell reprogramming, the *p53* pathway is activated, thus disrupting iPS reprogramming (Kawamura et al., 2009). *p53* may act as a limiting factor in the iPS reprogramming efficiency. Inhibition of the *p53* pathway either by mutating, deleting or knocking down *p53* or its target gene , *p21*, further enhances the reprogramming efficiency (Kawamura et al., 2009; Liu et al., 2009b; Marion et al., 2009; Tapia and Scholer, 2010b).

The *p53*/*p21* pathway was reported to suppress the iPS cell generation. Suppression of *p53* increased the efficiency of the generation of iPS cells (Hong et al., 2009). A dominant negative *p53* mutant, P275S, was used to study the effect of *p53* on regulating the iPS cell generation. Results suggested that inhibition of *p53* function by introducing the dominant negative *p53* mutant into the MEFs increased GFP-positive colonies in the *p53*-heterozygous MEFs (Hong

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et al., 2009). Similar experiments were also performed in terminally differentiated somatic cells (T-lymphocytes from Nanog-GFP reporter transgenic mice with either *p53* wild-type or null genotype). In this study, the four important stem cell reprogramming factors, *Oct4*, *Sox2*, *Klf4*, and *c-Myc* were introduced into the T-lymphocytes. No GFP-positive colony can be observed in the *p53* wild-type T lymphocytes (Hong et al., 2009). On the other hand, GFP-positive colonies can be observed in *p53*-null lymphocytes and the cells were expandable and have a similar morphology with the mouse ES cells (Hong et al., 2009). The increased GFP-positive cells can also be observed in the adult human dermal fibroblasts (HDFs) by introducing the dominant negative *p53* together with the reprogramming factors into the HDFs (Hong et al., 2009), suggesting the importance of *p53* in regulating the iPS cell reprogramming.

The function of *p53* in regulating stem cell multipotency was confirmed in germ-line stem cells (GSCs). Depletion of *p53* function in the GSCs increased the efficiency of reverting GSC multipotency status (Kanatsu-Shinohara et al., 2004). This finding can also be observed in a *p53* knockout mouse study (Lam and Nadeau, 2003). Hanna et al. suggested that depletion of *p53* function in clonal B cells can only enhance the kinetics of reprogramming somatic cells into iPS cells with a higher cell division rate (Hanna et al., 2009). However, it does not regulate the overall efficiency (Hanna et al., 2009). A *p53* mutant, R172H, which induces conformation change of the *p53* protein, was reported to associate with higher reprogramming efficiency than WT *p53* in the MEFs (Lang et al., 2004). Lang et al. showed that reprogramming efficiency in that particular *p53*-mutated MEFs, which was induced by utilizing a two factor system (Oct4 and Sox2), is higher than the *p53* knockout MEFs that was induced by using the three factors system (Oct4, Sox2, and Klf4) (Lang et al., 2004), suggesting the importance of *p53* in regulating the reprogramming process.

Cicalese et al. suggested that the function of *p53* in stem cells is critical to maintain a constant number of stem cells by imposing an asymmetric mode of self-renewing division. In the $p53^{-/-}$ and *ErbB2* tumor mammospheres, up-regulation of Nanog is observed. These studies also revealed the importance of *p53* in regulating the stem cell polarity, and the loss of *p53* induces increased frequency of symmetric division and tumor initiation and growth (Cicalese et al., 2009).

The suppression of the reprogramming efficiency of iPS cells by *p53* can be associated with the maintenance of genomic integrity of iPS cells. Deficient *p53* resulted in shorter telomeres in the reprogramming MEFs (Marion et al., 2009), suggesting the low efficiency of reprogramming in the WT *p53* cells to prevent the spreading of cells upon DNA damage and to ensure iPS cell genomic integrity (Marion et al., 2009).

Another barrier affecting the reprogramming is the *INK4A*/*ARF* tumor suppressor locus, as described previously. A recent report by Li and colleagues illustrated that the *INK4A*/*ARF* locus was suppressed during the early stage of reprogramming, leading to inactivation of the *p53* and *pRB1* pathways (Li et al., 2009). Interestingly, cells with $p16^{INK4A}$ knockdown alone are sufficient to enhance the reprogramming efficiency (Li et al., 2009). Together, these observations indicate that both *p53* and *pRB1* may work synergistically as barriers in somatic cell reprogramming (Li et al., 2009; Menendez et al., 2010; Utikal et al., 2009).

In a recent report by Lee K.H. et al., *p53* preferentially targets the Wnt signaling pathway in the murine ESC differentiation program (Lee et al., 2010). Evidently, the crosstalk between *p53* and Wnt signaling pathway plays an integrated role in stemness acquisition. A *p53* downstream phosphatase, *Wip1*, which shows high expression in the intestinal cells, was reported to associate with *p53*-dependent apoptosis of stem cells in the mouse intestine (Demidov et al., 2007). Removal of *Wip1* reduced the polyp formation in the APC^{Min} mice. The $APC^{Min/+}$ mice contain a nonsense mutation in the *APC* gene. Constitutively activated Wnt signaling pathway increased the apoptosis events of intestinal stem cells in the *Wip1*-deficient mice (Demidov et al., 2007). Low level of *Wip1* reduced the threshold of *p53*-dependent apoptosis of stem cells. However, *Wip1* deficiency does not affect the activities of β-catenin in terms of its nuclear localization level. A high level of β-catenin can be observed in the nuclei of polyp cells and this contributes to up-regulation of *c-Myc* and *Cyclin D1* in the *Wip1* null/$Apc^{Min/+}$mice. The β-catenin signaling pathway activation and attenuation of *p53* resulted in increasing efficiency of intestinal stem cell apoptosis (Demidov et al., 2007).

Recently, researchers demonstrated that the p53-miR34-Wnt network is a determinant factor of dichotomy between stem cell properties and tumor progression. miR34, one of the direct downstream targets of *p53*, is found to interact with Wnt and EMT genes, including *β-catenin*, *AXIN2*, *LEF1* and *Snail*. With the loss of *p53* due to miR34, the Wnt pathway is activated, which further induced the transformation of EMT (Liu et al., 2011). Therefore, the *p53* gene plays an important role in the controlling EMT.

Chang et al suggested that *p53* induced transcriptional activation of microRNA, miR-200c, through direct binding to its promoter region. The miR-200c was reported to regulate the EMT process through inhibition of transcriptional suppressors of an epithelial marker, E-cadherin (Chang et al., 2011). The miR-200c can target to and suppress ZEB1/2 (Wellner et al., 2009), which is a well-studied E-cadherin transcriptional suppressor and thus, regulates the EMT process. The knockdown of *p53* in MCF12A cells resulted in loss of epithelial phenotype and shows a significant elevation of the $CD24^{-}CD44^{+}$ population. Re-expression of *p53* in TGF-β-treated MCF12A showed inhibition of TGF-β-induced increase of the stem cell population.

p53 is not a sole player in deciding the cell fate determination. In fact, *p53* works as an integrated network, interplaying with other important pathways, depending on the external stimuli and microenviroment. However, there is a great need to further elucidate the roles of the *p53* network in reprogramming, dedifferentiation, self-renewal, and pluripotency.

2. Signaling pathways involved in the reprogramming and stemness transition

2.1. TGF–β signaling pathway

TGF-β signaling pathways play multiple roles in regulating tumorigenesis and other cellular processes, including reprogramming, stemness transition, and EMT events. Many components in this signaling pathway were defined to participate in both oncogenic and tumor suppressive pathways in various tumors. This provides a complicated story for researchers to study the

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function of TGF-β signaling pathways in stem cells or reprogrammed cells. The ligands of the TGF-β family have multiple functions and can cause opposite effects in different cell types. The TGF-β can regulate cell proliferation, growth arrest, differentiation, survival, cell migration, and also the pluripotency of cells. In cancer, over-expression of TGFβ1 and deregulation of the TGF-β receptor type II (TGFBRII) were reported to associate with skin cancer tumorigenesis and invasiveness (Cui et al., 1996). However, the role of TGF-β signaling in regulating reprogramming is still not well-defined. In a previous report, TGF-β family ligands play an important role in reprogramming of somatic cells into iPS cells, regulating ESCs self-renewal, pluripotency maintenance, and controlling differentiation.

TGF-β signalling may have the ability to induce reprogramming of somatic cells into iPS cells. Treatment of TGF-β/activin inhibitor in partially reprogrammed iPS cells can induce Nanog expression (Ichida et al., 2009; Maherali and Hochedlinger, 2009). Furthermore, the functional role of TGF-β in regulating the reprogramming was defined by utilizing chemical TGF-β inhibitors. Interestingly, inhibition of TGF-β signaling can enhance the mouse fibroblast reprogramming efficiency. A substitute of Sox2 (E-616452) and TGFBR1 kinase (SB-431542) inhibitor, were reported to replace the function of Sox2 in MEFs with Oct4, Klf4, and c-Myc expression (Ichida et al., 2009; Maherali and Hochedlinger, 2009). These results suggest the important roles TGF-β plays in the controlling reprogramming process.

Maintenance of the pluripotencies and self renewal properties are important for both ESCs and iPS cells.The canonical TGF-β signaling pathway may play important regulatory roles in ESCs maintenance and generation of pluripotency. BMP4 together with the LIF protein can induce Oct4 expression (Ying et al., 2003). The BMP activated Smad signaling to support self-renewal properties of stem cells. The inhibition of Smad activities by the Smad6 and Smad7 in the ES cells induced smaller and fewer ES cell colon formation (Ying et al., 2003). Secretion of BMP4 by the feeder cells is necessary for ES cell self-renewal (Ying et al., 2003). Inhibition of the Erk and p38 MAPK pathways can further enhance the BMP4-associated effect on self-renewal of mouse ESCs (Qi et al., 2004). Besides this, bFGF (basic fibroblast growth factor) and activin are also important to maintain the pluripotency in human ESCs (Greber et al., 2010; James et al., 2005). The TGF-β signalling may play multifunctional roles in regulating pluripotency of cells. Smad1 was reported to suppress the expression of Nanog by inhibiting its promoter activities (Jiang and Ng, 2008; Xu et al., 2008). The Smad proteins were reported to bind directly to Nanog promoter (Xu et al., 2008) and this is the major mechanism for Smad proteins to regulate Nanog expression. These results suggest the multiple roles of TGF-β signaling in the regulation of stem cell renewal.

Furthermore, TGF-β also plays a role to control the differentiation of ESCs. One of the TGF-β family members, BMP4, was reported to associate with induction of inhibitor of differentiation (*Id)* gene via interaction with the LIF/Jak-Stat3 and Smad pathways. The *Id* gene is an important factor to block ESC differentiation. The undifferentiated ES cells expressed BMP signaling ligands (Ying et al., 2003) and regulated downstream molecules, the Smads, to control the cell differentiation process (Ying et al., 2003).

Collaborating with Wnt signaling, TGF-β signaling is also involved in the EMT program and both pathways are regarded as the axis of EMT in breast cells (Scheel et al., 2011). The hy-

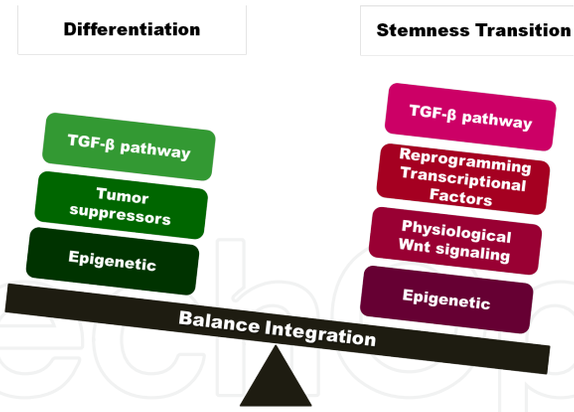

Figure 2. The cell fate determination is delicately controlled by positive and negative forces. Cellular activity balance regulated by both core stem gene-mediated pathways and TSGs is the key determinant in reprogramming process.

pothesis of these two pathways linked to the stem cell networks and TSG pathways is presented in Figure 2.

2.2.Wnt pathway

Cellular reprogramming can be achieved by overexpression of defined transcription factors in somatic cells (Ichida et al., 2009; Takahashi et al., 2007). However, the underlying mechanism of signaling activities that regulated these factors are not fully understood now. Overexpression for certain genes may not be suitable for all pathways, such as *β-catenin*, a mediator of Wnt signaling, because discrete levels of expressed genes are usually needed for maintaining the pluripotent status or direct programming through this pathway (Gu et al., 2010; Lluis et al., 2008; Marson et al., 2008; Merrill, 2008). It still remains unclear the gene-dosage effects of critical factors on somatic cell reprogramming and stem cell renewal. Recent studies revealed that activation of Wnt/β-catenin signaling may directly control reprogramming of fused somatic cells. For example, Wnt stimulators, Wnt3a and BIO, strikingly enhanced reprogramming ability after cell fusion (Lluis et al., 2008; Merrill, 2008). The fusion clones derived from both ESCs and somatic cells had an obvious β-catenin accumulation with increased expression of *Axin2*, a *β-catenin*-dependent gene, suggesting that basic or lower levels of stabilized β-catenin might drive somatic cell reprogramming.

The lower levels of Wnt signaling play a critical role in the control of development of several types of tissues through a dosage-dependent manner, as reported in crypt progenitor cells (Batlle et al., 2002; Korinek et al., 1998), hair follicles (Lowry et al., 2005), and hematopoietic stem cells (Luis et al., 2011). Taken together, observations from both *in vitro* and *in vivo* studies indicated that Wnt/β-catenin signaling was a single dominant force in the control of cell fate determinations in some of tissues, which suggests that basic or physiological levels of Wnt signaling may be required for many cellular activities.

More and more evidence revealed that Wnt signaling plays important roles in maintenance of pluripotency in ESCs and cell self-renewal (Cole et al., 2008; Lluis et al., 2008; Macarthur et al., 2009; Marson et al., 2008; Takao et al., 2007). For example, expression of *β-catenin* was confirmed

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to associate with hemtopoietic stem cells and neural stem cell growth (Kalani et al., 2008; Reya et al., 2003). *Wnt3A* activation associated with expression of the stem cell reprogramming markers, Oct4 and Nanog (Ogawa et al., 2007) and maintenance of the pluripotency of mouse ES cells (Hao et al., 2006; Singla et al., 2006). *Wnt3A* induced generation of iPS cells in the absence of *Myc* (Marson et al., 2008). Those cells contained iPS cell properties and were able to form teratomas during subcutaneous injection into SCID mice (Marson et al., 2008). The Wnt signaling pathway is also involved in regulating pluripotency factors, *Oct4, Nanog*, and Sox2 expression (Anton et al., 2007; Sato et al., 2004). This observation was confirmed by down-regulation of the stem cell pluripotency genes in the *β-catenin* deficient mouse ES cells (Anton et al., 2007). Wnt signaling pathway was also associated with cell reprogramming through the telomerase reverse transcriptase (TERT) and Brahma-related gene 1 (BRG1) interaction (Barker et al., 2001) to modulate chromatin structure during reprogramming (Miki et al., 2011).

Interestingly, previous study demonstrated that Wnt3a can also stimulate human ES cell differentiation, rather than only regulate human ES cell proliferation. The canonical Wnt signaling levels are minimal in the undifferentiated human ES cells but greatly increase after Wnt3a treatment and induce differentiation (Dravid et al., 2005). Dramatic increase of reprogrammed cell numbers can be observed when ES cells, which have a low level of nuclear β-catenin, are fused with neural stem cells. This is mainly due to the low nuclear β-catenin level being able to protect fused cells from apoptosis (Lluis et al., 2010), suggests the importance of β-catenin levels in the regulation of stem cell reprogramming. This finding may help to explain the balance between the maintenance of pluripotency of stem cells and apoptosis, as excess β-catenin can induce p53 expression (Damalas et al., 1999), which was found to induce apoptosis in stem cells to maintain genome integrity. The p53 protein was reported to be a transcription regulator of the Wnt signaling and it bound on the promoter regions of some Wnt signaling members for a general stress response in the mouse ES cells (Lee et al., 2010), which may provide a feedback mechanism to control the deregulation of the β-catenin during the reprogramming process.

It should be noted that inappropriate activation of components of this signaling pathway have been observed in many human cancers and differentiated stem cells, in which the high levels of *β-catenin* signaling were usually detected (Dravid et al., 2005; Fodde and Brabletz, 2007; ten Berge et al., 2008a; Vermeulen et al., 2010). Except for *p53* described previously, some components of the Wnt pathway can be regarded as both oncogenes and TSGs. For example, *AXIN2, APC, DKK1,* and *WIF1* are negative regulators of this pathway, and are called TSGs. In summary, the detailed mechanism of Wnt signaling in the control of stemness transition and reprogramming of somatic cells needs to be further explored.

3. Possible mechanisms to regulate TSGs expression in reprogramming

It is well-accepted now that epigenetic regulations are important events to control gene expression in human cells. Promoter hypermethylation and histone modification are two major events to regulate gene expression in various human tumors. The *DNA methyltransferase*

(*DNMTs*), *histone deacetylases* (*HDACs*), *histone acetyl transferase* (*HATs*), and *histone methyl-transferase* are the key regulators to controlgene expression in the genome. Epigenetic changes of gene expression were reported to be important during the iPS cell reprogramming (Han and Sidhu, 2008). The epigenetic changes can also help to maintain the pluripotency by regulating the expression of the key transcription factors, Oct4, Nanog, and Sox2 (van Vlerken et al., 2012). In previous studies, mouse ES cell genomes were found to contain less methylation than the somatic cells, while human ES cells show a distinct epigenetic profile, when compared to somatic cells (Jackson et al., 2004; Lagarkova et al., 2006; Zvetkova et al., 2005). A silenced TSG, *p16*, was found to be re-expressed during the reprogramming process (Ron-Bigger et al., 2010). On the other hand, a previous study suggested that the promoter region of INK4A/ARF was found to be hypermethylated in the iPS and ES cells. Inhibition of *DNMTs* by inhibitor and siRNA increased the *INK4A* and *p21* (*CIP1/WAF1*) expression in human umbilical cord blood-derived multipotent stem cells (So et al., 2011). However, the epigenetic regulation of TSGs during the reprogramming process is still not fully understood now. It is necessary to further explore epigenetic changes of TSGs in the reprogramming process and relevant other cellular activities.

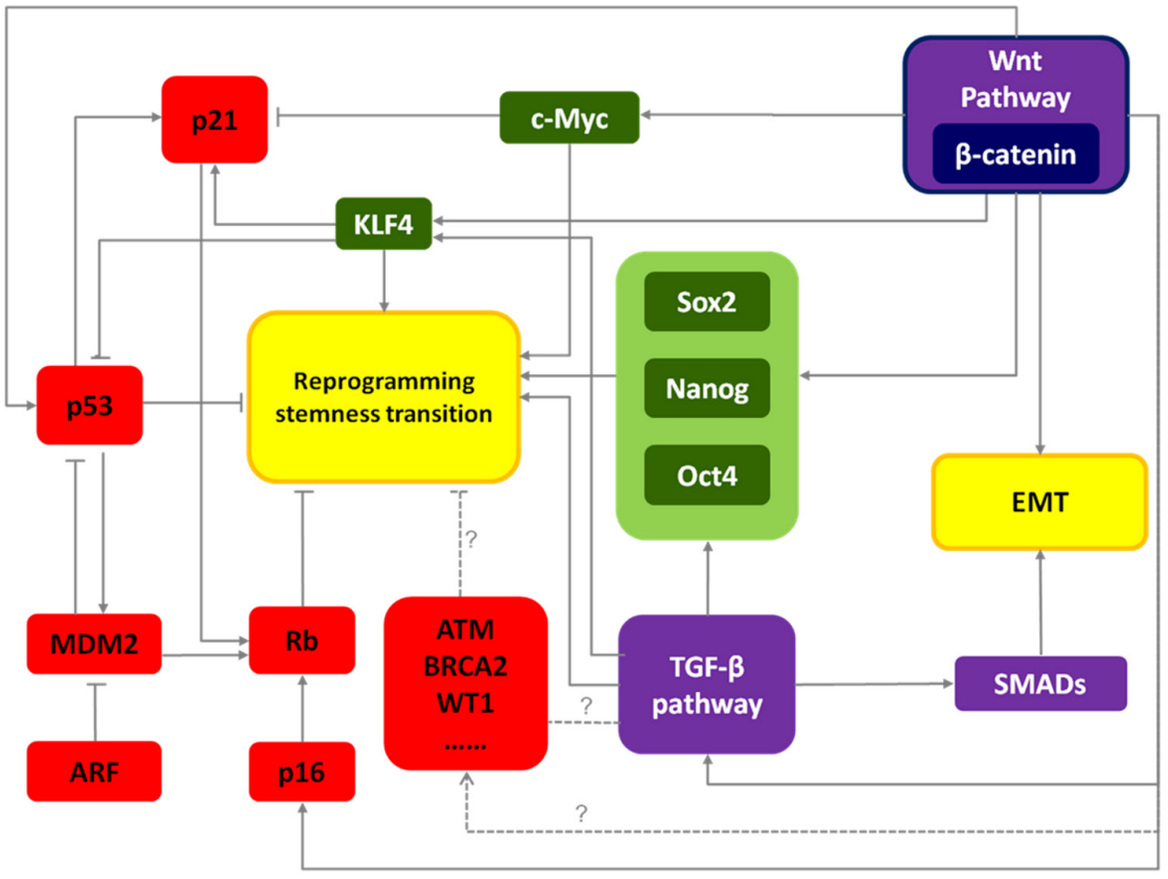

Figure 3. Hypothesis of integrated networks of TGSs, Wnt/β-catenin and TGF-β pathways in controlling reprogramming, stemness transition and EMT events. These pathways may play central roles in regulating other TSGs, transcriptional factors and other signaling pathways.

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4. Conclusions

The known and unknown TSGs are the important participators in the regulation of cell reprogramming and stemness transition. These genes are components of various signaling pathways, and play different roles in maintaining cell pluripotency, regulating cell differentiation and proliferation, cell cycle control, apoptosis, and other cell fate decisions. These genes controlling cellular activities act in a time-dependent or a dosage-dependent manner in various tissues. Although detailed underlying mechanisms are not fully clear now, more and more evidence indicates that some TSG signaling activities are determinant forces in important cellular processes, including cell reprogramming. A proposed hypothesis illustrates this in Figure 3. Understanding of the delicate control of these signaling networks in living cells will provide more insight in reprogramming studies and regenerative medicine.

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