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Review

The role of Krüppel-like factors in the reprogramming of somatic cells to induced pluripotent stem cells

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Summary. The potential for clinical application of pluripotent embryonic stem cells is immense but hampered by moral and ethical complications. Recent advances in the reprogramming of somatic cells by defined factors to a state that resemble embryonic stem cells have created tremendous excitement in the field. Four factors, Sox2, Oct4, Klf4 and c-Myc, when exogenously introduced into somatic cells, can lead to the formation of induced pluripotent stem (iPS) cells that have the capacity for self-renewal and differentiation into tissues of all three germ layers. In this review, we focus on the role of Krüppel-like factors (KLFs) in regulating somatic cell reprogramming. KLFs are zinc finger-containing transcription factors with diverse biological functions. We first provide an overview of the KLF family of regulatory proteins, paying special attention to the established biological and biochemical functions of KLF4 and KLF5. We then review the role of KLFs in somatic cell reprogramming and delineate the putative mechanism by which KLFs participates the establishment and self-renewal of iPS cells. Further research is likely to provide additional insight into the mechanisms of somatic cell reprogramming and refinement of the technique with which to generate clinically relevant iPS cells.

Key words: KLF, iPS cells, ES cells, Reprogramming, Somatic cells

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An overview of Krüppel-like factors

Krüppel-like factors (KLFs) belong to the relatively large family of Sp1-like transcription factors with over 20 members (Dang et al., 2000b; Black et al., 2001; Kaczynski et al., 2003). A hallmark of this protein family is the presence of a DNA-binding motif that contains several (usually three) C_2H_2 zinc fingers (Philipsen and Suske 1999; Turner and Crossley 1999). The zinc finger domains of KLFs bind to CACCC elements and/or GC-rich sequences of DNA in the regulatory sequences of target genes (McConnell et al., 2007; Pearson et al., 2008). The zinc fingers are interconnected by a highly conserved linker sequence which resembles the one present in the prototypical *Drosophila* protein, Krüppel (Schuh et al., 1986). Apart from the shared DNA binding domain, KLFs contain various other domains involved in transcriptional activation or repression and protein-protein interaction. These domains contribute to the functional specificities of KLFs (Dang et al., 2000b; Geiman et al., 2000).

The first identified and characterized member of the mammalian KLF family is the erythroid Krüppel-like factor (EKLF or KLF1) (Miller and Bieker, 1993). Other members have subsequently been identified and include several extensively characterized ones such as the lung Krüppel-like factor (LKLF or KLF2) (Anderson et al., 1995), gut-enriched Krüppel-like factor (GKLF/EZF or KLF4) (Garrett-Sinha et al., 1996; Shields et al., 1996), intestinal-enriched Krüppel-like factor (IKLF or KLF5; also called BTEB2) (Sogawa et al., 1993; Conkright et al., 1999), core promoter-binding protein (CPBP/Zf9 or KLF6) (Koritschoner et al., 1997; Ratziu et al., 1998), basic transcription element-binding protein (BTEB or KLF9) (Imataka et al., 1992), and transforming growth factor-ß-inducible early genes 1 and 2 (TIEG1 and 2 or KLF10 and 11, respectively) (Blok et al., 1995; Cook et al., 1998). Many of the KLFs have been associated with

roles in embryonic development. Two KLFs (KLF4 and KLF5) have recently been intensely scrutinized due to their involvement in embryonic stem cell development and self-renewal.

Krüppel-like factor 4 (KLF4) and Krüppel-like factor 5 (KLF5)

Krüppel-like factor 4, also known as gut-enriched Krüppel-like factor (GKLF), was initially identified through low-stringency cDNA library screen using the zinc finger portion of the immediate early transcription factor zif268 (Shields et al., 1996). The 483 amino acid (aa) of mouse KLF4 contains 3 Krüppel-type zinc fingers in its immediate carboxyl terminus, which is preceded by a 20-aa peptide rich in basic residues that serves as a nuclear localization signal (NLS) (Shields and Yang, 1997). An additional NLS was located in the zinc finger portion of KLF4. Together these two NLSs define a subfamily of closely related KLFs including KLF1, 2 and 4 (Shields and Yang, 1997). Located near the amino terminus of KLF4 is an acidic aa-rich region responsible for transactivation of target genes, which also interacts with the co-activator, p300/CBP (Geiman et al., 2000).

Earlier studies indicate that KLF4 is highly expressed in epithelial tissues including the gut and skin (Garrett-Sinha et al., 1996; Shields et al., 1996). Subsequently, KLF4 is found in various other tissues including the lung, testis, thymus, cornea, lymphocytes, vascular endothelial cells and cardiac myocytes (Garrett-Sinha et al., 1996; Shields et al., 1996; Ton-That et al., 1997; Jenkins et al., 1998; Panigada et al., 1999; Fruman et al., 2002; Chiambaretta et al., 2004; Cullingford et al., 2008). These studies imply that KLF4 is usually expressed in adult tissues that have a high rate of cell turnover. In particular, KLF4 is primarily localized to the mitotically inactive (post-mitotic) population of cells. For example, KLF4 is enriched in the post-mitotic villus epithelial cells of the intestine (Shields et al., 1996; McConnell et al., 2007), suprabasal layer of the epidermis (Garrett-Sinha et al., 1996; Segre et al., 1999), and the quiescent cortical cells of the thymus epithelium (Panigada et al., 1999). These studies indicate that KLF4 expression is temporally associated with terminal differentiation of epithelial cells.

Krüppel-like factor 5, also known as Intestinal-enriched Krüppel-like factor (IKLF), was originally identified as basic transcription element-binding protein 2 (BTEB2) from a human placenta cDNA library (Sogawa et al., 1993). This BTEB2 protein was reported to be 219 aa long with three contiguous zinc finger-binding domains in the carboxyl terminus. However, subsequent studies indicate that the initial size of KLF5 was incorrect due to a sequencing error as the mouse, and subsequently human, KLF5 was shown to be at least twice the size (446 aa for mouse and 457 aa for human KLF5) (Conkright et al., 1999; Shi et al., 1999). Similar to KLF4, KLF5 is also highly expressed in epithelial tissues (Conkright et al., 1999; Ohnishi et al., 2000). However, in contrast to the growth arrest-associated nature of KLF4, KLF5 is primarily expressed in the proliferating compartments such as the intestinal crypts (McConnell et al., 2007).

In addition to the intestine, KLF5 expression is high in other tissues including the stomach, skin, lung, testis, uterus and testis (Conkright et al., 1999). The expression pattern of BTEB2 is somewhat less restrictive and is found in the intestine, bladder, uterus, lung, liver, heart and kidney (Watanabe et al., 1999). BTEB2 protein shows a marked increase in the heart neointimal smooth muscle cells after balloon injury (Watanabe et al., 1999). KLF5 expression is rapidly induced by the phorbol ester PMA and basic fibroblast growth factor (bFGF) (Kawai-Kowase et al., 1999). An early growth response gene-1 (Egr-1) response element is present in the KLF5 promoter, which mediates the inductive effect of PMA on KLF5 expression (Kawai-Kowase et al., 1999).

KLF5 has been shown to be post-transcriptionally regulated. For example, KLF5 is phosphorylated at its amino terminal region, which facilitates its interaction with the transcription co-activator CBP (Zhang and Teng 2003). An oncogenic regulator, SET, interacts with and represses KLF5's activity by inhibiting acetylation of the DNA-binding domain of KLF5 (Matsumura et al., 2005). Similarly, histone deacytelase 1 (HDAC1) inhibits KLF5 both by binding to the promoter of its gene and to its DNA-binding domain (Matsumura et al., 2005). In contrast, p300 is bound to the acetylated KLF5 and increases its expression and activity (Miyamoto et al., 2003). KLF5 interacts with the E3 ubiquitin ligase WWP1, which targets KLF5 for degradation in epithelial cells (Chen et al., 2005). Using a mouse embryonic cDNA library, protein inhibitor of activated Stat1 (PIAS1), a small ubiquitin-like modifier (SUMO) ligase, was identified as a co-activator of KLF5 (Du et al., 2007). In addition, KLF5 is directly SUMOylated at two sites that target the protein to the nucleus (Du et al., 2008). Finally, post-translational modifications of KLF5 such as acetylation and SUMOylation have been reported to serve as molecular switches of KLF5's biological activities (Guo et al., 2008; Oishi et al., 2008).

Despite the close homology in the zinc finger domains of KLF4 and KLF5, the two proteins often behave in an opposite manner to each other. For example, their cellular distributions in epithelial tissues are quite different - in the small intestine, KLF4 is expressed in the terminally differentiated villus cells and KLF5 in the proliferating crypt compartment (McConnell et al., 2007). While KLF4 has been shown to be potent inhibitor of cell proliferation (Shields et al., 1996; Chen et al., 2001, 2003b; Ghaleb et al., 2005; McConnell et al., 2007), KLF5 is pro-proliferative in epithelial cells or fibroblasts (Sun et al., 2001; Bateman et al., 2004; Chanchevalap et al., 2004). Moreover, KLF4 and KLF5 both act on the KLF4 promoter but in opposing fashion - KLF4 activates its own promoter and KLF5 represses the KLF4 promoter (Dang et al., 2002).

Finally, both overlapping and mutually exclusive patterns of expression of KLF4 and KLF5 have been reported during development (Ohnishi et al., 2000).

Biological and biochemical functions of KLF4

KLF4 is known to play important roles in myriads of physiological processes such as cell cycle control, transcriptional regulation, DNA repair, apoptosis, differentiation, and determination of cell fate (Adam et al., 2000; Chen et al., 2001, 2003b, 2005; Yoon et al., 2003, 2005; Yoon and Yang 2004; Feinberg et al., 2007; McConnell et al., 2007; Alder et al., 2008; Birsoy et al., 2008; Ghaleb et al., 2008; Yoshida et al., 2008a; Yusuf et al., 2008). A major mechanism by which KLF4 regulates these diverse processes is by its capacity to act as a sequence-specific transcription factor. A consensus DNA sequence of 5'-$^{G}/_{A}$ $^{G}/_{A}$GG $^{C}/_{T}$G $^{C}/_{T}$-3' was empirically determined to bind to KLF4 (Shields and Yang, 1998). In addition, KLF4 interacts with the basic transcription element (BTE) and the CACCC element (Jenkins et al., 1998; Yet et al., 1998; Zhang et al., 1998). KLF4 also interacts with other transcription factors to coordinate gene expression. For example, KLF4 and KLF6 cooperatively enhance expression of the human keratin 4 gene (Okano et al., 2000). Also, KLF4 and Sp1 co-activate the human keratin 19 gene in pancreatic acinar cells (Brembeck and Rustgi, 2000). Depending on the context, KLF4 functions as a transcription activator or repressor. Examples of targets activated by KLF4 include its own gene (Mahatan et al., 1999), the cell cycle inhibitor $p21^{WAF1/CIP1}$ (Zhang et al., 2000), and many differentiation-specific genes such as keratin and alkaline phosphatase (Chen et al., 2003b; Hinnebusch et al., 2004). Examples of genes repressed by KLF4 include the cytochrome p450 1A1 (CYP1A1) (Zhang et al., 1998) and several cyclin genes such as cyclin D (Shie et al., 2000), cyclin B1 (Yoon and Yang, 2004) and cyclin E (Yoon et al., 2005). Furthermore, transcriptional profiling of KLF4's targets revealed that KLF4 has a global inhibitory effect on macromolecular biosynthesis (Whitney et al., 2006).

Mice with targeted deletion of *Klf4* were previously established. *Klf4*$^{+/-}$ mice were phenotypically and histologically normal (Segre et al., 1999; Katz et al., 2002). *Klf4*$^{-/-}$ mice were born at the expected Mendelian ratio but die shortly after birth due to a combination of inability to feed and defective barrier function of the skin, as measured by penetration of external dye and rapid loss of body fluid (Segre et al., 1999). In addition, newborn *Klf4*$^{-/-}$ mice had a selective loss of goblet cells from the colon, suggesting that KLF4 is a specific goblet cell differentiation factor (Katz et al., 2002). Mice with conditional deletion of *Klf4* have also been established. Depending on the promoter used to drive the conditional deletion of *Klf4*, mutant mice had altered proliferation and differentiation of the gastric epithelium (Katz et al., 2005), corneal epithelial fragility, stromal edema and loss of conjunctiva goblet cells (Swamynathan et al., 2007), and altered proliferation and differentiation of vascular smooth muscle cells (Yoshida et al., 2008b). These observations indicate that KLF4 is an important regulator of many physiological functions.

In agreement with its association with a growth arrest state, overexpression of KLF4 inhibits cell proliferation in culture (Shields et al., 1996). It has a potent checkpoint activity at the G_1/S and G_2/M transitions in the cell cycle, particularly following DNA damage (Zhang et al., 2000; Yoon et al., 2003, 2005; Yoon and Yang 2004). A mechanism by which KLF4 exerts this effect is by coordinately activating transcription of genes encoding cell cycle inhibitors while repressing transcription of genes encoding promoters of the cell cycle (Chen et al., 2003b). Consistent with its inhibitory effect on cell proliferation, Klf4 expression is reduced in intestinal tissues derived from *Apc*$^{Min/+}$ mice, a model of intestinal tumorigenesis (Ton-That et al., 1997). Moreover, expression of KLF4 is lower in intestinal adenomas from *Apc*$^{Min/+}$ mice when compared to the surrounding normal mucosa as well as in colonic adenomas derived from patients with familial adenomatous polyposis when compared to normal colonic tissues (Dang et al., 2000a). In addition, expression of KLF4 is reduced in human colorectal cancer specimens compared to normal tissues and its loss of expression is due to a combination of allelic loss of the KLF4 locus and promoter hypermethylation in a subset of tumor specimens (Zhao et al., 2004). A recent study also implied a trend toward better overall survival in KLF4-positive colorectal cancer patients with lymph node metastasis than in KLF4-negative cancer patients with lymph node metastasis (Xu et al., 2008). Thus, KLF4 behaves as a tumor suppressor in colorectal cancer. This notion was further substantiated by the recent finding that haploinsufficiency of the mouse *Klf4* gene led to an increase in the number of intestinal adenomas in *Apc*$^{Min/+}$ mice as early as 10 weeks of age (Ghaleb et al., 2007b). Consistent with the fact that APC is a major component of the Wnt/ß-catenin pathway of tumorigenesis (Gregorieff and Clevers, 2005; Reya and Clevers, 2005), KLF4 interacts with ß-catenin in the nucleus and suppresses transformation of cancer cells both *in vitro* and *in vivo* (Zhang et al., 2006). Lastly, KLF4 was recently shown to be inhibited by the Notch signaling pathway that is often up-regulated in intestinal tumors and colorectal cancer cells (Ghaleb et al., 2008). Importantly, inhibition of Notch in mice enhances Klf4 expression and goblet cell differentiation in the intestines of wild type and *Apc*$^{Min/+}$ mice as well as reduces proliferation and tumor formation in the intestines of *Apc*$^{Min/+}$ mice (Ghaleb et al., 2008). KLF4 is therefore an intestinal tumor suppressor that counters the oncogenic effect of Wnt/ß-catenin and Notch.

Despite the unambiguous tumor suppressor nature of KLF4 in the intestine, there have been conflicting reports on its status as a tumor suppressor in other cancers. For example, high nuclear and low cytoplasmic expression of KLF4 in infiltrative ductal breast

carcinomas correlates with poor prognosis in early stage cancers (Pandya et al., 2004). Similarly, KLF4 mRNA and protein levels are increased in ductal carcinomas in situ prior to invasion, suggesting that KLF4 may be causal to malignancy of breast cancer (Foster et al., 2000). Similar observations have been made connecting nuclear expression of KLF4 in squamous epithelial dysplasia and squamous cell carcinoma of the skin (Foster et al., 2005; Huang et al., 2005; Chen et al., 2008). These studies suggest that KLF4 may in fact have an oncogenic activity in certain types of cancers.

How can one reconcile the findings that KLF4 is both tumor suppressive and oncogenic? Several recent studies highlighted a context-dependent nature by which KLF4 performs these opposite functions. One showed that KLF4 is growth suppressive in primary cells, but could help bypass Ras^{V12}-dependent cell senescence by inhibiting p53 expression (Rowland et al., 2005). Moreover, Ras^{V12} targets cyclin D1, which then neutralizes the cyclin inhibitor $p21^{WAF1/CIP1}$, conferring KLF4 the ability to transform cells (Rowland and Peeper, 2006). Similar oncogenic effect of KLF4 was reported in adenovirus E1A-immortlaized rat kidney epithelial cells (Foster et al., 1999). A recent study supported these findings by demonstrating an anti-apoptotic effect of KLF4 following DNA damage due to its ability to inhibit p53-mediated activation of the pro-apoptotic gene, *BAX* (Ghaleb et al., 2007a). Thus, in the absence of $p21^{WAF1/CIP1}$, KLF4's anti-apoptotic effect dominates its cytostatic effect, rendering KLF4 oncogenic. These studies suggest that KLF4 and $p21^{WAF1/CIP}$ may be important nodal points in cell fate determination.

Biological and biochemical functions of KLF5

Similar to KLF4, KLF5 has many roles in regulating cell cycle, development, proliferation, apoptosis and tumorigenesis. In the intestine, KLF5 is expressed predominantly in the proliferating epithelial cells in the crypt compartment (Conkright et al., 1999; McConnell et al., 2007). The pro-proliferative role of KLF5 is supported by its induction in response to mitogenic agents including phorbol ester (Kawai-Kowase et al., 1999; Sun et al., 2001; Sur et al., 2002). KLF5 is also induced upon supplementation of fetal bovine serum (FBS) to serum-starved cultured mouse fibroblasts (Sun et al., 2001). Moreover, overexpression of KLF5 in mouse fibroblasts causes anchorage-independent transformation and increased colony formation similar to those caused by oncogenic H-Ras (Sun et al., 2001). Consistent with these findings, the Wnt signaling pathway activates KLF5 in a non-canonical, ß-catenin-independent fashion (Ziemer et al., 2001). Wnt1 overexpression, both *in vivo* and *in vitro*, increases KLF5 expression that is partially controlled by protein kinase C (PKC) signaling (Ziemer et al., 2001). KLF5 also interacts with NF-κB with which to activate downstream targets including a keratinocyte-specific, PMA-responsive VLTRE enhancer element (Sur et al., 2002).

We previously reported a role for KLF5 in mediating the transforming activity of oncogenic H-Ras and K-Ras (Nandan et al., 2004, 2005, 2008). KLF5 is increased in mouse fibroblasts and intestinal epithelial cells that contain oncogenic H-Ras and K-Ras, respectively (Nandan et al., 2008). In these cells, oncogenic Ras activates KLF5 through the mitogen activated protein kinase (MAPK) pathway via Egr-1 (Nandan et al., 2004). KLF5 then activates a number of cell cycle-promoting genes including cyclin D1, cyclin B1 and Cdc2 (Nandan et al., 2004, 2005). KLF5 also has a role in regulating proliferation of colon cancer cells as its reduction by all-*trans* retinoic acid (ATRA) results in reduced proliferation (Chanchevalap et al., 2004). Consistent with these findings, KLF5 is increased in human colorectal cancer cell lines and primary tumors that possess oncogenic mutations of K-Ras (Nandan et al., 2008). Moreover, intestinal-specific overexpression of oncogenic K-Ras in mice display increased KLF5 expression in intestinal tumors (Nandan et al., 2008). Together, these findings support a pro-proliferative and perhaps oncogenic function for KLF5 in at least a subset of intestinal and colorectal tumors.

Despite the evidence, the oncogenic function of KLF5 has not been ascertained. For example, down-regulation or deletion of KLF5 has been reported for several cancers including prostate and breast cancers (Dong 2001; Chen et al., 2002, 2003a). Another report showed that KLF5 expression is high in early gastric cancers and this high expression corresponds to increased survival after surgery (Kwak et al., 2008). On the other hand, breast cancer patients with higher KLF5 expression had shorter disease-free and overall survival compared to patients with lower KLF5 expression (Tong et al., 2006). Amplification of the KLF5 locus has also been reported in salivary gland tumors (Giefing et al., 2008). These studies suggest that the spatio-temporal expression pattern of KLF5 in specific tissues may be important in the initiation and maintenance of tumors. A recent study provides some insight into the bipotential of KLF5 in controlling proliferation by demonstrating a switch from pro-proliferation to anti-proliferation as a result of transforming growth factor-ß (TGF-ß)-mediated acetylation (Guo et al., 2008).

Targeted deletion of *Klf5* by homologous recombination in transgenic mice has been developed. Mice null for the *Klf5* alleles ($Klf5^{-/-}$) are embryonic lethal and die before embryonic day (E) 8.5 (Shindo et al., 2002). In contrast, $Klf5^{+/-}$ mice grow to adulthood but show abnormal thinning of the medial and adventitial aortic walls, consistent with an earlier identified role for KLF5 in activating the transcription of SMemb/non-muscle myosin heavy chain-B, a marker of smooth muscle cells (Watanabe et al., 1999). In addition, neointimal aortic wall formation following balloon injury is significantly reduced in $Klf5^{+/-}$ mice (Shindo et al., 2002). A reason for this effect is due to the ability of

KLF5 to activate the platelet-derived growth factor A (PDGF-A) promoter activity in response to angiotensin II activation (Shindo et al., 2002).

In addition to the vascular manifestation of *Klf5* deficiency, *Klf5*$^{+/-}$ mice exhibit defects in other organs or tissues. For example, adipocyte differentiation is reduced in the *Klf5*$^{+/-}$ mice due to decreased Klf5 levels that in turn reduce peroxisome proliferator-activated receptorγ (PPARγ) expression (Oishi et al., 2005). *Klf5*$^{+/-}$ mice are also more tolerant than wild type mice to hypercholesterolemia, glucose intolerance and high fat-induced obesity due to increased energy expenditure (Oishi et al., 2008). The architecture of the intestinal epithelium in *Klf5*$^{+/-}$ mice is distorted by misshapen villi and reduced extracellular matrix and mesenchymal cells in a manner that is similar to those in *Pdgfa*$^{-/-}$ mice (Shindo et al., 2002). When infected with a bacterial pathogen, *Citrobacter rodentium*, *Klf5*$^{+/-}$ mice exhibit an attenuated hyperproliferative response of the intestinal epithelium compared to wild type littermates (McConnell et al., 2008). These findings support a crucial role of KLF5 in mediating physiological responses to external stressors (McConnell et al., 2007).

The reprogramming of somatic cells to embryonic stem cells

Embryonic stem (ES) cells have the capacity to expand indefinitely (self-renewal) and retain the ability to differentiate into the all of the cells in the three germ layers (pluripotency). This potential of ES cells to mature into different cell types ordains a great deal of scientific interest and fascination in the use of ES cells as a therapeutic for various disease conditions. However, ethical and moral issues complicate the routine use of ES cells in regenerative medicine. Reprogramming of somatic cells to pluripotent stem cells appears to be an attractive and efficacious alternative to circumvent some of the problems arisen from ES cell research.

Reprogramming of adult somatic cells was first reported by Ian Wilmut and colleagues, using a technique called somatic cell nuclear transplantation (SCNT) (Wilmut et al., 1997). SCNT, the injection of terminally differentiated somatic cell nuclei into enucleated unfertilized oocytes, can lead to the development to term of viable offsprings (Wilmut et al., 1997; Wakayama et al., 1998). However, not all terminally differentiated somatic cells can be used in SCNT and the rate of development of embryos to full term is extremely low (Shi et al., 2003). The majority of failures in development to term is due to incomplete or incompetent activation of the embryonic genes and repression of somatic genes (Alberio et al., 2006). Thus, an important event during embryogenesis is the establishment and maintenance of pluripotency genes including *Oct4, Sox2 and Nanog* (Alberio et al., 2006). Another important event in the establishment and growth of an embryo to full term is the formation of the trophoectoderm. The establishment of this trophoectoderm occurs very early in embryogenesis, starting around the two-cell stage and requires the expression of maternal and embryonic *Cdx2* gene (Alberio et al., 2006; Deb et al., 2006). This can be viewed as the start of the differentiation process during embryogenesis, which then gives rise to extra-embryonic structures. Since the onset of pluripotency involving Oct4 is the earliest event in the establishment of the embryo, deletion of Cdx2 in this pathway gave rise to the formation of just the inner cell mass (ICM) filled with ES cells (Meissner and Jaenisch, 2006).

This method of generating pluripotent ES cells is also marred by moral issues as it involves the destruction of embryos. A system of deriving ES cells from a single blastomere instead of destroying a complete embryo has been developed (Chung et al., 2006). Using single-cell biopsy techniques used in pre-implantation diagnostics, single blastomeres were isolated to produce ES lines that were viable and remained pluripotent up to 50 passages in cell culture. However, somatic cell nuclear transfer in humans using oocytes runs into the same ethical issues discussed earlier (Evans, 2005). Egli et al. have recently shown that it is possible to use fertilized zygotes as recipients instead of oocytes (Egli et al., 2007). They derived ES cells and chimeric mice from zygotes that had been mitotically arrested and their chromosomes replaced. These studies provide new avenues towards generation of personalized human ES cells for treatment and therapy.

Reprogramming of somatic cells to pluripotency and its maintenance has been achieved with a number of methods. One of the earlier methods utilized the ability of pluripotent cells to change the state of somatic cells upon cell fusion (Tada et al., 1997; Cowan et al., 2005; Egli et al., 2007). Another method took advantage of ES and embryonic carcinoma cell extracts which when added to differentiated cell lines could be changed into a pluripotent state (Taranger et al., 2005; Freberg et al., 2007; Bru et al., 2008). Other groups have used synthetic heterocyclic molecules to induce cellular reprogramming in cultured cells (Chen et al., 2004; Ding and Schultz, 2004). Leukemia inhibitory factor (LIF) is sufficient for ES cell self-renewal upon binding to its receptor and Gp130 (Smith et al., 1988; Williams et al., 1988). The complex then causes activation of the intracellular Jak/Stat pathway (Ernst et al., 1996). Stat3 was also characterized as the main downstream regulator of ES cell renewal (Niwa et al., 1998), although other studies showed that the LIF/Gp130/Stat3 pathway is required but not essential for pluripotency (Stewart et al., 1992; Yoshida et al., 1996; Dani et al., 1998; Reubinoff et al., 2000). Another study reported the sustenance of ES cell self-renewal *in vitro*, with the addition of LIF and bone morphogenesis proteins (BMP) in the absence of serum (Ying et al., 2003). They reported that BMP could induce Id proteins through Smads, which are crucial in ES cell self-renewal. Upon withdrawal of LIF, the ES cells undergo differentiation. In addition, Nanog could bypass the need for BMP or serum (Ying et al., 2003).

Both Oct3/4 and Nanog are specifically expressed in ES cells, epiblasts and germ cells (Okamoto et al., 1990; Scholer et al., 1990; Chambers et al., 2003; Mitsui et al., 2003). Lastly, Nanog is a critical factor in maintaining pluripotency of ES cells independent of the LIF/Gp130/Stat3 pathway (Mitsui et al., 2003).

The generation of induced pluripotent stem cells by somatic cell reprogramming

A breakthrough in the field of somatic cell reprogramming came from the laboratory of Yamanaka who reported the induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors (Takahashi and Yamanaka, 2006). The group had earlier reported specific gene expression patterns in ES cells (Mitsui et al., 2003; Maruyama et al., 2005). Starting with 24 candidate factors for pluripotency, they identified 4 that are necessary and sufficient for somatic reprogramming to pluripotency (Takahashi and Yamanaka, 2006). To achieve this, a mouse embryonic fibroblast (MEF) cell line containing the *Fbx15* gene attached to a ß-Geo cassette was used (Tokuzawa et al., 2003). The *Fbx15* gene is specifically expressed in ES cells, but dispensable for pluripotency. The factors hypothesized to induce pluripotency were introduced via retroviral transduction into $Fbx15^{\beta Geo/\beta Geo}$ MEFs individually or in combination. No colony formation was noted when the factors were introduced individually, but multiple colonies with pluripotency were generated when all 24 factors were introduced together. Analysis upon serial withdrawal of factors led to the conclusion that Oct3/4 (i.e. Oct4), Sox2, Klf4 and c-Myc are indispensible for pluripotency. The same four factors also induced ES-cell like fates upon transducing adult mouse tail-tip fibroblasts. Removal of Oct3/4 and Klf4 from the transduction was detrimental and resulted in no colony formation. Sox2 removal resulted in a very few number of colonies, while c-Myc withdrawal resulted in multiple colonies but with altered morphologies (i.e. non-ES-cell-like) (Takahashi and Yamanaka, 2006). The authors also report that the majority, but not all, of the clones, named induced pluripotent stem (iPS) cells, derived from the transduced cultures actually attained pluripotency *in vivo*, when transplanted into nude mice. In addition, when gene expression patterns were analyzed between iPS and ES cells, some differences were noted, suggesting the necessity for the involvement of additional factors in somatic reprogramming.

In a follow-up study, a Nanog-driven iPS cell selection instead of the *Fbx15* system was used (Okita et al., 2007). Okita et al. created chimeric mice and MEF cells stably expressing a Nanog-green fluorescence protein (GFP) reporter (Okita et al., 2007). They could derive specific iPS cell clones with ES cell morphology when the Nanog-GFP MEFs were transduced with the four factors (Oct3/4, Klf4, Sox2 and c-Myc). However, they did not derive any clones when transducing with any three of the four factors alone. They also characterized the Nanog iPS cell clones and found that they had similar (but not identical) expression pattern to ES cells, but more so than the *Fbx15* iPS cell clones. The Nanog iPS cell clones were able to form chimeras when injected into blastocysts that were then transplanted. The same study with the Nanog iPS cell clones were also carried out by other groups that yielded similar results (Maherali et al., 2007; Wernig et al., 2007). Maherali et al. additionally reported similar global methylation patterns and X-linked inactivation in the iPS cell clones as the ES cells. They also observed that the iPS cell clones when injected into blastocysts differentiated into numerous cell types including germ cells. Wernig et al., using both Oct4 and Nanog iPS cell clones, induced teratomas in SCID mice upon injection of the iPS cell clones and the teratomas differentiated into the three germ line layers. However, Okita et al. also discovered the presence of tumors in a significant percent of the Nanog iPS chimeric mice (Okita et al., 2007). They attributed the presence of tumors to the reactivation of c-Myc in the mice. None of the other three factors (Oct3/4, Klf4 or Sox2) was reactivated in the chimeric mice.

Introduction of c-Myc, as one of the factors, to induce pluripotency in somatic cells poses the potential problem of uncontrolled cell proliferation and cancer. Nakagawa et al. recently showed that it was possible to generate mouse and human iPS cells without activation of the c-Myc oncogene (Nakagawa et al., 2008). They found that ES cell-like colonies developed upon transduction of Nanog-GFP MEF cells with only three factors (Oct3/4, Klf4, and Sox2), when selection pressure was applied later than 14 days, but not earlier. The number of non-specific colonies was also reduced upon delayed selection. They could also generate specific iPS cell clones without any selection. When chimeric mice were produced, they did not develop tumors. They concluded that c-Myc was not essential but could enhance somatic cell reprogramming into iPS cells. Similar results were also reported in another study working with Oct4 and Nanog-reporter MEFs (Wernig et al., 2008b). Another group has also developed iPS cells from IMR90 human fetal fibroblasts through induction of Oct4, Sox2, Nanog and Lin28 without using Klf4 and c-Myc (Yu et al., 2007).

Since the initial reports outlining the generation of induced pluripotent stem cells from human and mouse somatic cells, many groups have further refined the process and identified numerous potential therapeutic implications. Two groups have accounted for the generation and systematic differentiation of cardiac myocytes from mouse iPS cells (Mauritz et al., 2008; Narazaki et al., 2008). Similarly other groups have reported establishment of iPS cells derived from pancreatic cells (Stadtfeld et al., 2008a), dermal fibroblasts (Lowry et al., 2008; Park et al., 2008b), mature B lymphocytes (Hanna et al., 2008), liver and stomach cells (Aoi et al., 2008; Wernig et al., 2008a).

Differentiation of iPS cells into neuronal precursors efficiently developed into neurons and glial cells and could improve behavior in rat models of Parkinson's disease (Wernig et al., 2008c). Another breakthrough study described the ability to generate iPS cell-derived motor neurons from amyotrophic lateral sclerosis (ALS) patients (Dimos et al., 2008). Park et al. also showed that iPS cells could be generated from patients with several genetic diseases (Park et al., 2008a). Two studies showed that adult mouse neural stem cells could be reprogrammed into iPS cells using only two (Oct4 and Klf4 or c-Myc) (Kim et al., 2008b) or three factors (Oct4, Klf4 and c-Myc) (Eminli et al., 2008). Recently, several groups have generated iPS cells without viral vectors or integration (Carey et al., 2008; Okita et al., 2008; Stadtfeld et al., 2008b).

The role of Krüppel-Like factors in the generation of induced pluripotent stem cells

The mechanism by which KLF4 regulates ES cell self-renewal was first revealed by its identification as a highly up-regulated target gene of LIF signaling in ES cells (Li et al., 2005). ES cells overexpressing KLF4 had a great propensity for self-renewal based on secondary embryoid body (EB) formation. KLF4-transduced EBs expressed higher levels of Oct4, consistent with the notion that KLF4 regulates ES cell self-renewal (Li et al., 2005). The role of KLF4 in regulating pluripotency of ES cells is further revealed by global analysis of promoter occupancy by the four somatic cell reprogramming factors (Klf4, Oct4, Sox2 and c-Myc) (Kim et al., 2008a). The results identified a transcriptional hierarchy within the four reprogramming factors with both auto-regulatory and feed-forward regulation. In addition, the study indicated that KLF4 is an upstream regulator of a large feed-forward loop that contains *Oct4, Sox2, and c-Myc*, as well as other common downstream factors including Nanog (Kim et al., 2008a). Combining the results of these studies, it appears that KLF4 exerts a crucial role in somatic cell reprogramming and maintenance of ES cell self-renewal.

As seen earlier in this review, KLF4 exhibits both cytostatic and anti-apoptotic effect that is context-dependent. The ability of KLF4 in maintaining immortality of iPS cells maybe explained in part by the requirement of c-Myc as a member of reprogramming factor. Thus, in a manner similar to the cooperation between KLF4 and Ras to affect transformation (Rowland and Peeper, 2006), KLF4 and c-Myc cooperate to affect iPS cell self-renewal. Thus, KLF4 may suppress apoptosis induced by c-Myc and c-Myc neutralizes KLF4's cytostatic effect by suppressing $p21^{WAF1/CIP1}$ (Yamanaka 2007). In this manner, the balance between KLF4 and c-Myc might play a critical role in the establishment of an immortalized state of iPS cells.

In an attempt to identify additional factors that can generate iPS cells, Nakagawa et al. substituted the different factors used in somatic reprogramming with their respective homolog (Nakagawa et al., 2008). They reported the successful generation of iPS clones upon substitution of Klf2 for Klf4 or L-Myc for c-Myc. Other substitutions including Klf5 for Klf4, Sox1 for Sox2 and N-Myc for c-Myc also resulted in positive clones, but fewer in number. No positive clones developed when only three factors, devoid of Klf4, were used for reprogramming. The essential requirement of Oct4 and Klf4 for reprogramming of somatic cells has subsequently been substantiated (Di Stefano et al., 2008; Shi et al., 2008).

The importance of Krüppel-like factors in somatic reprogramming is highlighted by a recently published study, which demonstrated that depletion of Klf4, Klf2 or Klf5 in mouse ES cells failed to influence ES self-renewal (Jiang et al., 2008). Similarly, pair-wise depletion of the three factors had no effect either. In contrast, when all three factors were depleted, the ES cells underwent differentiation. Using chromatin immunoprecipitation, they showed that the three Klfs share many common targets of Nanog. When one Klf was depleted, the other two still bound to their targets. Importantly, the Klfs bound to two distinct regions in the *Nanog* gene upstream of Nanog's coding sequence, one of which has been shown to be a *Nanog*-enhancer element. *Nanog*-enhancer activity was completely abolished upon depletion of the three Klfs. These findings indicate that the three Krüppel-like factors form a core circuitry which regulates self-renewal of ES cells (Jiang et al., 2008).

As a variation to the above study, Parisi et al. found that depletion of Klf5 alone resulted in the differentiation and abolishment of self-renewal of mouse ES cells (Parisi et al., 2008). Klf5 was found in the undifferentiated ES cells and colocalized with both Oct3/4 and Nanog. Upon differentiation, starting from day 2, Klf5 levels decrease rapidly. They also found nuclear Klf5 expression, which correlates with nuclear Oct3/4 and Nanog expression patterns, in the pre-implantation embryos and blastocysts at E3.5, but not in epiblasts of E6.5 embryos. At E11.5, Klf5 is expressed only in the primordial germ cells along with Oct3/4. *In vitro* depletion of Klf5 resulted in the morphological differentiation of ES cells with a concomitant reduction in both Oct3/4 and *Nanog* mRNA levels. Interestingly, they also noted that *Klf4* or *Klf2* mRNA levels were unchanged upon *Klf5* depletion. They showed that Oct3/4 depletion also resulted in the decrease of Klf5, Nanog and Sox2 levels. Klf5 depletion also led to increased expression of mesodermal and trophoectodermal markers, but not endodermal or ectodermal markers. Klf5 was found to bind to both Oct3/4 and *Nanog* promoters. Klf5, however, bound to a site different from the Oct3/4 binding region on the *Nanog* promoter. Klf5 overexpression or knockdown in ES cells did not affect cell proliferation, but Klf5

overexpression induced Oct3/4 and Nanog expression. Klf4 knockdown did not affect Klf5 expression patterns. The authors, however, noted that Oct3/4 and Nanog suppression all decreased expression of Klf5, Klf4 and Klf2 similar to the findings of Jiang et al. The authors concluded that Klf5 was important for ES cell self-renewal and could not be completely substituted by either Klf4 or Klf2 (Parisi et al., 2008).

Another recent report also demonstrated that Klf5 is critical for derivation and self-renewal of mouse ES cells (Ema et al., 2008). They report that *Klf5*-knockout (KO) embryos failed to develop past E6.5, as reported earlier (Shindo et al., 2002), due to failure of implantation resulting from trophoectoderm defects. They show that *Klf5*-KO embryos displayed a marked decrease in Cdx2. Also Ema et al. managed to culture *Klf5*-KO ES cells, which remained undifferentiated with normal ES cell marker expression but became differentiated upon longer culture time. The *Klf5*-KO ES cells were pluripotent, but could not sustain ES cell self-renewal and displayed an increase in differentiation markers similar to those observed by Parisi et al. Also similar to the results in Parisi et al., the authors showed that overexpression of *Klf5* in ES cells suppressed the expression of differentiation-specific genes and maintained pluripotency even in the absence of LIF. However, *Klf5*-KO ES cells proliferated slower than the wild type ES cells, displaying decreased G1 cell cycle progression and increased $p21^{Waf1/Cip1}$ expression. Klf5 overexpression in ES cells led to elevated *Tcl1* expression, Akt phosphorylation and corresponding increased cell proliferation. The authors speculate that Klf5 expression could maintain ES cell self-renewal through stimulation of the Akt pathway. They further note that Klf4 overexpression could suppress differentiation of *Klf5*-KO ES cells, but caused decreased cell proliferation, suggesting that Klf4 and Klf5 function similarly to suppress differentiation but have opposing effects on cellular proliferation (Ema et al., 2008).

Conclusion

Recent advances in the reprogramming of somatic cells to cells resembling embryonic stem cells using defined factors have generated tremendous amount of enthusiasm and excitement in the field of regenerative medicine. In a short period, the practice of exogenous introduction of four factors (Oct4, Sox2, Klf4 and c-Myc) in somatic cells to generate induced pluripotent stem cells with properties similar to embryonic stem cells has become routine. However, the mechanism by which the four factors induce the reprogramming process has only been partially unraveled. It is hoped that with further research into the mechanism that regulates the establishment of iPS cells, there might be further refinement of the requirements for somatic cell reprogramming, which could lead to eventual clinical application.

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