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Kinetics of reprogramming in cell fusion hybrids

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ABSTRACT Somatic cells can obtain pluripotency by fusion with pluripotent stem cells. The resulting fusion hybrids display pluripotential characteristics, such as inactivation of tissue-specific genes, differentiation potential to all three germ layers, and a specific epigenetic state corresponding to the pluripotent cells. However, the fusion hybrid cells are not identical to the pluripotent fusion partner cells although they are similar to the pluripotent cells. Recently, we showed that fusion-induced reprogramming was not a solely unidirectional process. In this review, we address how much somatic cells "remember and lose" their original characteristics after fusion with pluripotent cells.

KEY WORDS: *reprogramming, stem cell, cell fusion, hybrid, pluripotency*

Introduction

Pluripotent stem cells can give rise to all cell types of an organism, while maintaining self-renewing ability for an expanded period of time. This capability permits pluripotent stem cells to be used both for research and clinical applications. The differentiation potential of pluripotent cells becomes progressively more restricted, as they become committed to a certain lineage.

The first evidence of nuclear reprogramming of somatic cells was provided by the live birth of cloned animal by somatic cell nuclear transfer (SCNT) (Campbell *et al*., 1996). The SCNT experiment suggested that the cytoplasm of enucleated oocytes possesses unknown reprogramming factors which can reprogram nuclei of differentiated somatic cells into a totipotent state, so-called totipotential reprogramming.

Somatic cells also can acquire pluripotentiality by cell fusion with pluripotent stem cells such as embryonic stem (ES), embryonic germ (EG), and embryonic carcinoma (EC) cells (Tada *et al*., 2001; Tada *et al*., 2003; Do *et al*., 2008). The fusion hybrid cells present pluripotential characteristics, such as inactivation of tissue-specific genes, reactivation of pluripotent related genes, differentiation potential to all three germ layers, and a specific epigenetic state corresponding to the pluripotent cells (Do *et al*., 2006). The differentiated state of somatic cells could also be altered by fusion with another type of somatic cell, suggesting that cellular factors between the two different types of cells dynamically interact and might be responsible for the plasticity and reestablishment of new characteristics. However, the fusion hybrid cells are not identical to the pluripotent fusion partner cells, although they are very similar to the pluripotent cells.

Recently, Yamanaka and colleagues reported that mouse and human somatic cells can be reprogrammed into a pluripotent state through overexpression of exogenous reprogramming factors (*Oct4, Klf4, Sox2*, and *c-Myc*) (Takahashi and Yamanaka, 2006). These induced pluripotent stem (iPS) cells can differentiate into all three embryonic germ layers and form germline chimera, which is characteristics of pluripotent ES cells. In this review, we address how much somatic cells 'remember and lose' their original characteristics after fusion with pluripotent cells.

Reprogramming of somatic cells using cell-cell fusion with ES, EG or EC cells

The reestablishment of new characteristics by cell fusion between two different cell types was suggested as early as in 1965 (Harris and Watkins, 1965). Cell-cell fusion between pluripotent teratocarcinoma and differentiated thymocyte cells resulted in hybrid cells maintaining the potential for unlimited self-renewal and differentiation into a variety of cell types. The authors had hypothesized that the teratocarcinoma might lose pluripotency by fusion with differentiated somatic cells, but instead, the hybrid cells obtained pluripotency and resembled EC cell morphology without expressing a tissue specific gene such as *Thy* (Miller and Ruddle, 1976; Miller and Ruddle, 1977). Following this

Abbreviations used in this paper: EC, embryonic carcinoma cell; EG, embryonic germ cell; ES, embryonic stem cell; SCNT, somatic cell nuclear transfer.

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initial study, many studies have shown that various somatic cells can be reprogrammed by fusing with pluripotent stem cell like ES, EG, or EC cells (Fig. 1) (Cowan *et al*., 2005; Flasza *et al*., 2003; Matveeva *et al*., 1998; Shimazaki *et al*., 1993; Tada *et al*., 2003; Tada *et al*., 2001; Terada *et al*., 2002, Yu *et al*., 2006).

Cell fusion also was suggested as a mechanism for somatic cell plasticity. Phenotype and potency of somatic cells (bone marrow cells and brain cell) were changed by spontaneous cell fusion with ES cells after co-culture with ES cells (Terada *et al*., 2002, Ying *et al*., 2002). The hybrid cells that showed over-diploid DNA content expressed pluripotent related genes and could differentiate into all three embryonic germ layer *in vivo* and *in vitro*.

EC cells are another source of pluripotent cells that can reprogram somatic cells by fusion. Since EC cells share many of the key pluripotent characteristics with ES and EC cells can provide a readily amenable alternative source for reprogramming (Do *et al*., 2009a; Flasza *et al*., 2003; Mise *et al*., 1996). Moreover, mouse EC cells can reprogram human somatic cells into pluripotent state, indicating that reprogramming factors can cross-act through another species (Flasza *et al*., 2003).

Key reprogramming factors reside in the nucleus

The ooplasm of an enucleated oocyte of metaphase II stage has the capacity to recondition or reset the epigenetic program of a fully differentiated somatic cell nucleus to the totipotent stage. However, enuleated ES cells do not assume the ability to reprogram somatic cells (Do and Scholer, 2004). To investigate whether the cytoplasm of ES cells has reprogramming factors and can reprogram somatic cells, we fused cytoplasts of ES cells (cyESCs) with somatic cells from the OG2 mouse, which contain *Oct4* promoter driven *green fluorescence protein* (*GFP*) transgene (*Oct4-GFP*). An *Oct4-GFP* positive colony was not observed in cyESC-somatic cell hybrids. By contrast, the karyoplasts of ES cells were able to reactivate *Oct4-GFP* of somatic cells at day 2 post-fusion. Karyoplast fusion hybrid cells formed ES cell-like colonies and expressed pluripotency markers, *Oct4*, *Rex*-1, and *Nanog*. This result suggested that nuclei of ES cell but not cytoplasts contain reprogramming factors that are required to reprogram somatic cells. This result was confirmed by Yamanaka's experiment; direct reprogramming of somatic cells to the pluripotent state can be induced by only four transcription factors, or nuclear factors.

Differentiation potential and erasure of cellular memory by cell fusion

Pluripotent cells reprogram somatic cells and induce erasure of somatic cell memory by fusion. Recently, we demonstrated that the hybrid cells were not preferentially committed to the lineage of the somatic cells that had been fused with the pluripotent cells under differentiation-inducing conditions. To investigate whether somatic cells lose the memory of their origin, the differentiation potential of two different types of EC cell fusion hybrids was compared. F9 EC cells do not differentiate into neural lineage, but P19 EC cells preferentially differentiate into neural cells after exposure to retinoic acid. By showing the inability for neural differentiation of the F9-neural stem cells (NSC) fusion hybrid cells, we demonstrated that NSCs lose their memory and adopt the similar differentiation potential of their pluripotent fusion partner, F9 EC cells. Therefore, the differentiation potential of fusion hybrid cells is contingent on the type of pluripotent fusion partner cells, and the resulting hybrid cells have the same potential as the pluripotent fusion partner cells. This phenomenon is also observed when two types of pluripotent cells are fused. After fusion of ES and F9 EC cells, EC-like and ES-like hybrid cells were generated and the EC-like cells rarely differentiated into neural cells, otherwise, ES-like hybrid cells easily differentiate into neural cells (Do *et al*., 2009a).

Epigenetic modification during fusion-induced reprogramming

During nuclear reprogramming it is expected that the final structure of chromatin, which is believed to function in establishing cell-type-specific gene expression pattern, should be significantly modified by two major events of epigenetics, histone modification and DNA methylation. DNA methylation, acetylation and methylation of histone H3 and H4 amino terminal tail are crucial epigenetic modifications involved in regulating gene activity (Lachner *et al*., 2003). Following hybridization of ES cells with thymocytes, the somatic cells undergo chromatin remodeling which is induced by reprogramming factors residing in ES cells (Kimura *et al*., 2004). Therefore, the erasure of somatic cell-specific histone modifications is a crucial step in the induction of successful nuclear reprogramming. DNA methylation is also a crucial remodeler of chromatin structure and gene expression regulation, which control

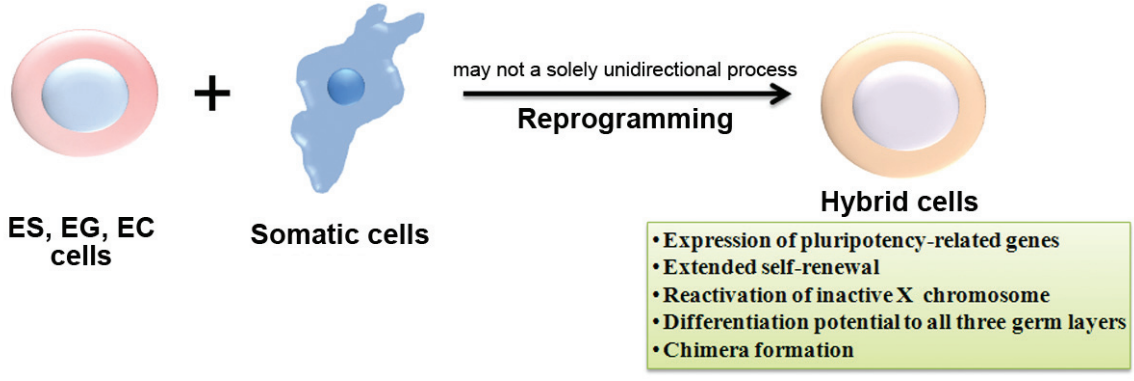


Fig. 1. Fusion-induced reprogramming reestablishes pluripotency of somatic cells. *The genome of somatic cells acquires pluripotent characteristics after fusion with ES, EG, or EC cells. Reprogrammed hybrid cells show a gene expression pattern and differentiation potential similar to ES cells.*

differentiation, cell cycle progression, and early embryonic development. However, tissue-specific genes of somatic cells become methylated and pluripotency-related genes become demethylated after being reprogrammed to the pluripotent state by fusion-induced reprogramming. For example, the *Oct4* promoter region of NSCs become demethylated during reprogramming; partially methylated patterns of the *Oct4* proximal enhancer (55.0%) and the promoter region (46.0%) in NSCs were completely demethylated just day 2 post-fusion with ES or EC cells (Do *et al*., 2007).

DNA demethylation is essential to overcome gene silencing and induce temporally and spatially controlled expression of mammalian genes. Activation-induced cytidine deaminase (AID, also known as AICDA) has been suggested as a potential DNA demethylase in zebrafish—(Rai *et al*., 2008). In mammals, AID is expressed in primordial germ cells where DNA demethylation occurs and in pluripotent cells (Morgan *et al*., 2004). Bhutani *et al*. demonstrated a new role for AID in active DNA demethylation and reprogramming of mammalian somatic cells towards pluripotency by fusion (Bhutani *et al*., 2010). They fused mouse pluripotent stem cell with human somatic cells, and inhibited DNA replication and cell division. Surprisingly, human somatic cells could be reprogramming without replication and cell division only one day post-fusion. These heterokaryons expressed ES cell-specific and self-renewal related genes. They found that demethylation of the pluripotency related gene was independent of cell division and DNA replication. To investigate the role of AID in these hybrid cells, they inhibited AID by using siRNA transfection. In parallel with the reduction in demethylation of the *OCT4* and *NANOG* promoters following AID knockdown using siRNA transfection, expression of *OCT4* and *NANOG* regions was reduced by at least 80%. They showed that AID binds to the heavily methylated promoter regions of human *OCT4* and *NANOG* in fibroblasts and demethylates these regions during reprogramming. In summary, AID is involved in demethylate *OCT4* and *NONOG* promoter regions of the somatic genome during reprogramming of differentiated cells toward a pluripotent state. This result provides further support for our previous observation that reprogramming of differentiated cells requires neither DNA replication nor cell division (Do and Schöler, 2004), only when the somatic cells were pre-treated with 5-Aza-c, a DNA methyltransferase inhibitor. Taken together, these two independent experiments demonstrate that DNA demethylation is a precedent event for successful cellular reprogramming.

The reprogramming of the imprinted genes of somatic cells is dependent on the pluripotent fusion partners. Previous studies suggest that the resulting fusion hybrid cells display an identical potential to their respective pluripotent fusion partners. For example, the methylation pattern of the *Igf2r* region of thymocytes was not changed after fusion with ES cells (methylated on the maternal allele as in thymocytes), but was changed after fusion with EG cells (not methylated on both alleles) (Tada *et al*., 2001).

Fusion-induced reprogramming is not a solely unidirectional process

Somatic cells acquired pluripotency through cell-cell fusion with pluripotent stem cells. The reprogrammed hybrid cells expressed pluripotency-related genes but did not express tissue-specific genes. The 'memory' of somatic cells is-almost like a dogma-considered to be erased by fusion with pluripotent cells during fusion-induced pluripotential reprogramming. Silva *et al*., for example, mentioned that fusion-induced reprogramming is a unidirectional process resulting in an ES cell phenotype without other viable cell states (Silva *et al*., 2006). However, we have demonstrated that pluripotent stem cells also could acquire some characteristics of differentiated cells. As a first step to investigate bidirectional reprogramming in fusion hybrid, we fused ES and F9 EC cells, because both cell types have a potential to reprogram other types of cells. ES and F9 EC cell fusion resulted in both ES and EC cell like fusion hybrids. F9-like hybrid, which were expected to display F9 gene expression profiles, showing ES cell gene expression patterns of *Stra8* and *Vasa*; the hybrid cells are morphologically F9 cells (reprogramming of ES cells to F9 EC cells), but *Stra8* and *Vasa* genes were reprogrammed to the ES cell state (reprogramming of F9 EC cells to ES cells) — bidirectional (Do *et al*., 2009a).

In pluripotent hybrids of ES (or EC) cells and male neural stem cells, the *Xist* gene is reprogrammed to the somatic state, while pluripotency and tissue-specific markers were reprogrammed to the pluripotent state; *Xist* could be oppositely reprogrammed to the pluripotent state in hybrid cells. These findings indicate that the reprogramming direction in pluripotent hybrid cells is not solely unidirectional, and some genes could be reprogrammed opposite to that of the pluripotent fusion partner. However, the mechanism underlying bidirectional reprogramming induced by the fusion of pluripotent cells with somatic cells remains to be elucidated.

Reactivation of inactive X chromosome might be the last reprogramming marker

One of the distinct differences of pluripotent cells vs. somatic cells (including multipotent somatic stem cells), regardless of sex, is the absence of an inactive X chromosome (Xi) (Do *et al*., 2009a; Lee, 2005). Therefore, pluripotent cells only contain active X chromosome (Xa); male cells have one and female cells have two Xa. The Xa of pluripotent cells is different from that of somatic cells in X inactive specific transcript (*Xist*) expression level (Do *et al*., 2009a). The *Xist* expression levels of female ES and F9 EC cells are about 500-1000 times lower than that of female NSCs (fNSCs). Therefore Xa of pluripotent cells can be detected as a pinpoint signal by *Xist* RNA fluorescence *in situ* hybridization (FISH), but Xa of somatic cells do not express *Xist*, and thus are not detected by *Xist* RNA FISH. When the somatic cells are reprogrammed to a pluripotent state, Xi of somatic cells is changed into Xa. We showed that F9 EC cells could completely reactivate the *Oct4* (as well as other pluripotency markers) of somatic cells within 49 h after fusion, whereas reactivation of Xi was a much slower process, which required 9 days (Do *et al*., 2008). Therefore, reactivation of Xi is an event that is completed at the late stage of reprogramming. The slower reprogramming process of reactivation of Xi could be accelerated by upregulation of *Tsix* and *Dnmt3a. Tsix* is a key gene that represses *Xist* expression by mediating the formation of a repressive chromatin structure through histone modifications and DNA methylation (Sado *et al*., 2005) and *Dnmt3a* is involved in *de novo* methylation of *Xist* (Chen *et al*., 2003), which is associated with inhibition of *Xist* expression, following fusion with pluripotent stem cells. In addition, there is another change in the X chromosome; induction of

partial inactivation of Xa of somatic genomes in the hybrid cells. Xa of somatic cells, which do not express *Xist*, become express low levels of *Xist* similar to pluripotent cells.

Interestingly, not all pluripotent cell lines appear to have the potential to reactivate somatic Xi. Mise *et al.* observed that several EC cell lines could not reactivate somatic Xi by cell fusion (Mise *et al.*, 1996). EC cell sub-lines containing the fully methylated 5' region of *Xist*, which originated from P19 (XY), C86S1A1 (XO) and B242g (XO), did not have the ability to reactivate the Xi of somatic fusion partners. These reactivating-incompetent EC cell lines are fully methylated in the 5' region of *Xist*, indicating that only partial methylation of the 5' region of *Xist* is correlated with the reactivation potential of the EC cells (Mise *et al.*, 1996). The authors suggested that partial methylation of the 5' region of *Xist* is related to *de novo* methylation potential, enabling EC cells to reactivate somatic Xi.

Direct *vs.* cell fusion-induced reprogramming

Fusion-induced reprogramming is comparable to direct reprogramming by transduction of reprogramming factors (Fig. 2). Differentiated somatic cells can be directly reprogrammed by transduction of four transcription factors, *Oct4, Sox2, Klf4*, and *c-Myc* (Takahashi and Yamanaka, 2006) and subsequently use of *Oct4, Sox2, Nanog*, and *Sall4* (Yu *et al.*, 2007). iPS cells are very similar to ES cells in their gene expression pattern and developmental potential (Huang *et al.*, 2009; Okita *et al.*, 2007; Wernig *et al.*, 2007; Zhao *et al.*, 2009). Therefore, iPS cells become invaluable tools for studying differentiation and tissue formation *in vitro* and represent a promising resource for tissue replacement therapy (Chan *et al.*, 2009; Do *et al.*, 2009b).

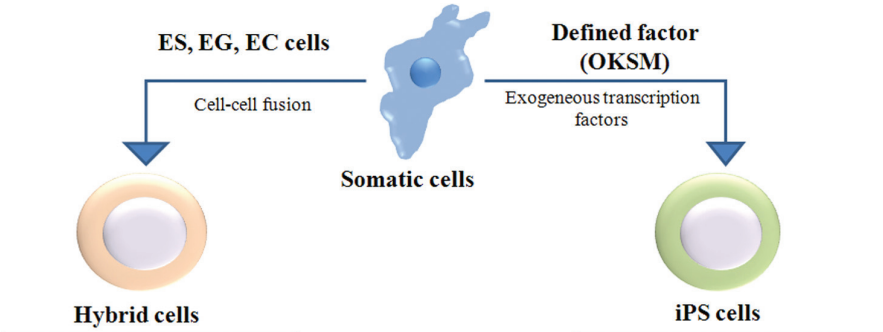
However, the low efficiency of iPS cell generation is the major problem of the direct reprogramming system. The reactivation of endogenous *Oct4* in somatic cells requires only one to two days of fusion induced reprogramming (Han *et al.*, 2008), but requires at least one week in generating iPS cells. The reprogramming efficiency could be enhanced by treatment with an epigenetic modifier or by additional factors involved in fusion-induced and direct reprogramming. Somatic *Oct4* gene can be reactivated within 2 days after fusion with pluripotent stem cells, whereas reprogramming of *Xist* took about 9 days. *Xist* reprogramming could be enhanced by histone deacetylase inhibitor (trichostatin A) treatment (Do *et al.*, 2008). Several small molecules also were reported to enhance reprogramming efficiencies in direct reprogramming. Histone deacetylase inhibitor (valproic acid) and G9a histone methyltranferase inhibitor (BIX-01294) significantly enhanced the efficiencies of iPS cell generation (Huangfu *et al.*, 2008; Shi *et al.*, 2008a; Shi *et al.*, 2008b).

Even if resulting iPS cells derived from many different cell types have similar potencies, the reprogramming efficiency is different depending on the somatic cell types (Giorgetti *et al.*, 2009). ES cell-like colonies expressing an *Oct4-GFP* reporter transgene appeared 3 week after viral transduction for four factors (Meissner *et al.*, 2007), but somatic stem cells, such as NSCs, were reprogrammed on day 5 after infection (Silva *et al.*, 2008). Moreover, recent studies reported that exogenous expression of only one factor, *Oct4*, is sufficient to generate iPS cells from mouse and human NSCs (Kim *et al.*, 2009b; Kim *et al.*, 2009c); as NSCs endogenously express *Sox2, c-Myc*, and *Klf4.*

However, when it comes to reprogramming timing in fusion-induced reprogramming, there was no difference between somatic stem cells and differentiated somatic cells; both acquired pluripotency 2 days after fusion with pluripotent stem cells (Do and Scholer, 2005). However, somatic cell type is an important factor affecting reprogramming efficiency in direct reprogramming. These results indicate that many other pluripotency inducing factors from pluripotent stem cells, in addition to *Oct4, Klf4, Sox2*, and *c-Myc*, may interact with the genome of somatic cells when pluripotent cells are fused with somatic cells.

In developmental potential, iPS cells have advantage over pluripotent fusion hybrids, as iPS cells meet all the criteria for pluripotency, including germline transmission and live pups after tetraploid complementation (Kang *et al.*, 2009; Meissner *et al.*, 2007; Okita *et al.*, 2007; Wernig *et al.*, 2007; Zhao *et al.*, 2009). Fusion hybrid cells, however, may not develop into the germ cells after blastocyst injection, due to the tetraploidy of the cells.

Fusion-induced and direct reprogramming



Fusion hybrids		iPS cells
No	**Ethical issue**	No
1 ~ 2 day	**Onset of endogenous Oct4**	1 ~ 5 week
Chimera (no germline contribution)	**Pluripotency**	Chimera formation Germline transmission Tetraploid complementation
Polyploid karyotype	**Defects**	Low efficiency Integration of exogenous genes (should be induced by proteins or small molecules)

Fig. 2. Comparison between cell fusion-induced and direct reprogramming. *Cell fusion induces pluripotency much faster and more efficiently than direct reprogramming but has a major defect in polyploidy. iPS cells have an advantage over pluripotent fusion hybrids in developmental potential* in vivo*, including formation of germline chimera and the live pups after tetraploid complementation.*

of somatic cells definitely overcome ethical issue concerning use of egg. When considering future clinical applications of these two technologies, the ES cell genome in fusion-induced pluripotent hybrid cells need to be removed to prevent induction of immune rejection, and the directly reprogrammed iPS cells need to be generated without exogenous gene integration in the host genome. Accordingly, iPS cells generated by proteins (Kim *et al*., 2009a; Zhou *et al*., 2009) or small molecules will be the preferred option for clinical applications.

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