Chapter 9
Cell-Fusion-Mediated Reprogramming: Pluripotency or Transdifferentiation? Implications for Regenerative Medicine

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Abstract Cell–cell fusion is a natural process that occurs not only during development, but as has emerged over the last few years, also with an important role in tissue regeneration. Interestingly, in-vitro studies have revealed that after fusion of two different cell types, the developmental potential of these cells can change. This suggests that the mechanisms by which cells differentiate during development to acquire their identities is not irreversible, as was considered until a few years ago. To date, it is well established that the fate of a cell can be changed by a process known as reprogramming. This mainly occurs in two different ways: the differentiated state of a cell can be reversed back into a pluripotent state (pluripotent reprogramming), or it can be switched directly to a different differentiated state (lineage reprogramming). In both cases, these possibilities of obtaining sources of autologous somatic cells to maintain, replace or rescue different tissues has provided new and fundamental insights in the stem-cell-therapy field. Most interestingly, the concept that cell reprogramming can also occur in vivo by spontaneous cell fusion events is also emerging, which suggests that this mechanism can be implicated not only in cellular plasticity, but also in tissue regeneration. In this chapter, we will summarize the present knowledge of the molecular mechanisms that mediate the restoration of pluripotency in vitro through cell fusion, as well as the studies carried out over the last 3 decades on lineage reprogramming, both in vitro and in vivo. How the outcome of these studies relate to regenerative medicine applications will also be discussed.

9.1 Cell–Cell Fusion Methodologies

Membrane fusion is fundamental to the life of eukaryotic cells. Cellular trafficking and compartmentalization, intercellular communication, cell division, and many other physiological events are all dependent on this basic process. Fusion between two cells also occurs in a wide range of developmental and pathological processes [1].

In 1975, a biophysical discussion agreed upon the definition of the fusion process as the mixing of entrapped contents between two membrane-enclosed aqueous compartments that involves the mixing of the membrane contents, but with little escape of the entrapped contents [2].

Even though the process of cell–cell fusion is a physiological process during mammalian development [3], artificial fusion has also been used to merge together two cell types to generate a third

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cell type that would display hybrid characteristics different from both of the original cells. The development of monoclonal antibodies by Kohler and Milstein [4], for example, relied on the formation of "hybridomas" that were created by fusing antibody-producing cells with cancer cells. Following this idea, induced fusion has also been used to study other processes, such as the plasticity of cells of different origins. In this case, fusion has been induced mainly by two different methods, as now described.

9.1.1 PEG-Mediated Fusion

Polyethylene glycol (PEG) is an oligomer or polymer of ethylene oxide. PEG has been used to fuse cells from a long time [5]. However, in the 1970s, very little was known about the mechanisms by which PEG induced fusion. It was shown that the action of PEG in promoting cell–cell fusion was not due to effects such as surface absorption, crosslinking or solubilization, but that the major effect of PEG for membrane merging was due to volume exclusion, which induces an osmotic force that brings the membranes into close contact, resulting in the membrane dehydration necessary to induce fusion [6].

9.1.2 Electrofusion

One key aspect of membrane surfaces is their surface charge. Electrofusion consists of the application of pulsed electric fields [7] that allow membrane permeabilization (a reversible process without any dramatic membrane rupturing if controlled parameters are used). Thus, electropermeabilized cells brought into contact are fusogenic [8]. In subsequent experiments, when cells were first brought in contact and then an electric pulse was applied, this resulted in an increase in hybrid formation, suggesting that fusion takes place when the two cell surfaces in contact are electropermeabilized [9].

9.2 Somatic Cell Reprogramming

Cell–cell fusion has been extensively used in more recent years to study the plasticity of differentiated cells, a concept that can be strictly related to the capacity of adult cells to undergo reprogramming.

Somatic cell reprogramming can be referred to as the transition from one cell type into another. There are two major types of reprogramming: (i) reprogramming of differentiated cells into pluripotent cells; and (ii) lineage reprogramming of differentiated cells into different differentiated cells.

Reprogrammed pluripotent cells show:

(1) demethylation and reactivation of genes that are essential for pluripotency, such as *Oct4, Nanog* and *Sox2*;
(2) silencing of somatic markers;
(3) reactivation of the silent X chromosome;
(4) potential to form teratomas and to differentiate in tissues that are derived from the three germ layers after injection into nude mice;
(5) potential to generate chimeras, meaning that the reprogrammed cells can give rise to different tissues of the body.

In the lineage reprogramming that consists of the transition between specialized cellular identities, the new reprogrammed cells will acquire the features of the cells to which they convert to. In another

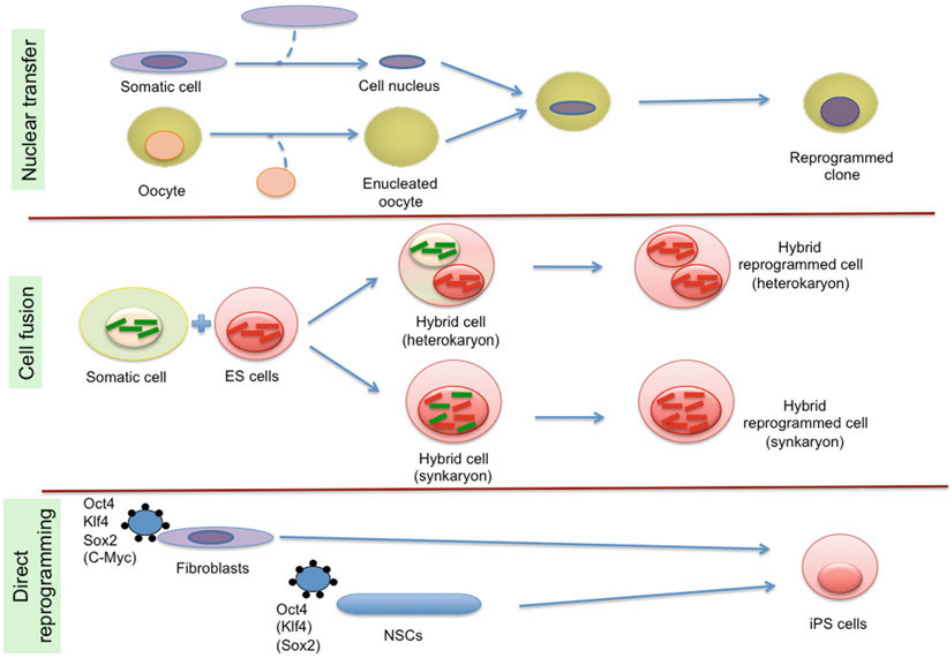

Fig. 9.1 Strategies to induce pluripotent reprogramming. There are several strategies used to induce reprogramming. In nuclear transfer, the somatic nucleus is transferred into an enucleated oocyte to yield a totipotent cell with the genetic material from the somatic cell (cloning). Cell fusion between ESCs and somatic cells results in the generation of hybrid cells where the somatic nucleus is reprogrammed into a pluripotent state. Generation of heterokaryons with two separated nuclei or synkaryons with one nucleus is possible. Finally, in the direct reprogramming strategy, the transduction of three transcription factors, Oct4, Klf4, Sox2 (with or without c-Myc) in a somatic cell induces the formation of iPSCs, which are pluripotent cells. It is also possible to generate iPSCs by transducing NSCs with only Oct4

words, if fibroblasts are to become muscle cells, they will need to express muscle cell genes and silence the expression of their fibroblast-specific genes.

To date, there are several strategies that have been used to generate pluripotent cells from somatic cells (Fig. 9.1).

9.2.1 Somatic Cell Nuclear Transfer

The process of somatic cell nuclear transfer (SCNT) involves the transfer of the nucleus of a somatic cell into an enucleated oocyte, with the goal of generating a totipotent cell. Then, under the correct conditions, an adult organism can develop. This technique is also known as cloning.

The cloning of animal cells was first performed when Briggs and King created frog embryo clones in 1952 [10]. They took fertilized frog eggs, removed the chromosomes/spindle apparatus, and replaced these with nuclei from older embryos. The net result was clones of adult frogs that were identical to the originating frog cell nuclei.

More recently, the nuclear reprogramming capacity of oocytes has been demonstrated in mammals by the production of cloned sheep, cows, mice and pigs [11–14]. However, all of the epigenetic marks that have been examined in cloned embryos show abnormalities [15] the success rate of this cloning

is very low, and there are high levels of early and late embryonic lethality. This has been suggested to be due to inaccurate and incomplete reprogramming, including incorrect DNA methylation of the somatic donor nucleus [15–19].

However, this process of cloning, and especially if carried out in humans, is fraught with fears that such events could lead to reproductive cloning: the formation of an embryo that can become an adult after implantation in the uterus.

9.2.2 Nuclear Reprogramming of Somatic Cells by Cell–Cell Fusion

Similar to the production of cloned animals from somatic nuclear transfer, the state of a somatic nucleus can be reprogrammed to that of a pluripotent stem cell by hybridization with pluripotent cells. This is due to the observation that in the majority of hybrids, the phenotype of the less-differentiated cell-fusion partner is dominant over the phenotype of the more-differentiated cell-fusion partner.

As early as the 1970s, cell–cell fusion was used to study nuclear reprogramming. In one study, mouse embryonic carcinoma cells (ECCs) were fused with primary thymocytes, and the resultant hybrid cells showed properties of pluripotent carcinoma cells [20]. More recently, different types of somatic cells have been fused with embryonic stem cells (ESCs), and both mouse (mESCs) and human (hESCs) ESCs have been shown to have the ability to reprogram somatic cells [21, 22]. Moreover, fusion between specialized cells can lead to the formation of hybrids that acquire one of the two distinct phenotypes, as was seen, for example, by fusing murine muscle cells with human primary diploid cells derived from many different embryonic lineages [23–25].

As the aim of this chapter is to summarize the information relating to cell-fusion-mediated reprogramming, the knowledge in this field is detailed below.

9.2.3 Direct Reprogramming of Somatic Cells

The first evidence that adult somatic cells can be reprogrammed into cells with ESC-like characteristics was reported by Takahaski and Yamanaka [26], when they transduced the four genes encoding for the Oct4, Klf4, Sox2 and c-Myc factors into fetal and adult mouse fibroblasts, using retrovirus infection. The overexpression of the transduced trangenes produced some cells with an ESC-like phenotype, which are known as induced pluripotent stem cells (iPSCs). Soon after, it was shown that it was possible to reprogram human fibroblasts with these same four factors, or with the combination of the Oct4, Sox2, Nanog and LIN28 factors [27]. Subsequently, other cells, such as B lymphocytes, hepatocytes, gastric epithelial cells and others, have been successfully reprogrammed by the same combinations of factors, and also by a subset of these factors, or with new "blends" of different factors [28, 29].

c-Myc is a potent oncogene that is implicated in cell proliferation, DNA replication, cell growth and metastasis formation [30–32]. When iPSCs were generated using c-Myc, Oct4, Sox2 and Klf4, about 15% of the mice derived from these iPSCs developed tumors within 4 months. Subsequent studies have reported that both human and mouse adult fibroblasts can be reprogrammed using only three genes, with c-Myc being omitted here [33, 34]. This observation has stimulated many studies towards the identification of the "essential" reprogramming factors.

Klf4 is an abundant transcript in ESCs, although Klf4 knock-down does not lead to an obvious phenotype; this is probably due to functional redundancy with other Klf family members in ESCs [35, 36]. It has been shown that Klf4 can be replaced by the orphan nuclear receptor Esrrb, which can act in conjunction with Oct4 and Sox2 to mediate reprogramming. Esrrb-reprogrammed cells share similar marker expression and epigenetic signatures with respect to ESCs [37].

Oct4 and Sox2 are probably the most important factors for the induction of reprogramming; however, even these can be replaced. Both of these factors are required for the maintenance of ESC pluripotency and for self-renewal [38, 39]. A lack of Oct4 in embryos impairs their ability to develop the inner cell mass [40], while Sox2 loss-of-function results in defective epiblasts and differentiation into trophoblast cells [41]. To date, it has been shown that for the reprogramming of somatic cells, Sox2 can be replaced with other members of its family, such as Sox1 and Sox3, albeit with reduced efficiencies [33]. In an interesting recent report, it was shown that Oct4 can be replaced by the orphan nuclear receptor Nr5a2 (also known as Lrh-1) in the production of iPSCs from mouse somatic cells [42], casting some doubts on the fundamental role of Oct4 in iPSC generation. However, NSCs have been shown to be reprogrammed to pluripotency after overexpression of only Oct4 [43], confirming that Oct4 does indeed have a critical role in reprogramming.

9.3 Induced Pluripotency Through Cell-Fusion-Mediated Reprogramming

Dolly the sheep was generated by nuclear transfer in 1997, demonstrating that fully differentiated mammalian somatic cells can be reprogrammed to a state of totipotency [11]. Most recently, by modifying existing SCNT protocols, the successful nuclear reprogramming of adult rhesus macaque somatic cells into pluripotent ESCs was achieved. These ESCs showed normal ESC morphology, expressed ESC-specific markers, and differentiated into multiple cell types in vivo and in vitro [44]. These studies have confirmed the feasibility of SCNT in mammalian cloning. SCNT has also been used to show that oocytes have the ability to reprogramme somatic cells, as does the sperm genome.

The success rate of mammalian cloning by nuclear transfer is, however, very low (3–5%), and the majority of clones die in utero or neonatally. This can also often occur in conjunction with developmental problems, such as large offspring syndrome [45]. A surviving cloned animal is a highly rigorous operational assay for effective reprogramming, but such successes at the organism level unfortunately provide little insight into the underlying molecular mechanisms that are involved in the reprogramming processes themselves. For this reason, different methods for studying reprogramming have been used, such as fusion between somatic cells and pluripotent stem cells. The stem cells that have been used in these experiments are ESCs, embryonic germ cells (EGCs), and ECCs (Fig. 9.2).

The use of pluripotent cells in vitro has been possible only in the last few years. Only in 1981 were ESCs established from normal mouse blastocysts [46, 47]. Subsequently, primordial germ cells (PGCs) were generated and EGCs were derived and called “embryonic germ” to denote their origin [48]. EGCs can retain many properties of pluripotency and can be cultured for long times.

ECCs are the stem cells of teratocarcinomas, and studies have shown that they are closely related to ESCs [46, 47, 49, 50]. This conclusion has been confirmed in humans, with the demonstration that ECCs derived from human testicular teratocarcinomas and ESCs isolated from early human embryos produced by in-vitro fertilization share common features [51, 52].

9.3.1 Fusion of Somatic Cells with ECCs

In 1976, Miller and Ruddle first reported that pluripotent teratocarcinoma–thymus somatic cell hybrids can differentiate into a wide variety of tissues, which indicated that pluripotency is not abolished by the presence of the differentiated cells in the hybrids [20]. Reprogramming was evident since the hybrid cells resembled an ECC morphologically, and they showed reactivation of specific genes

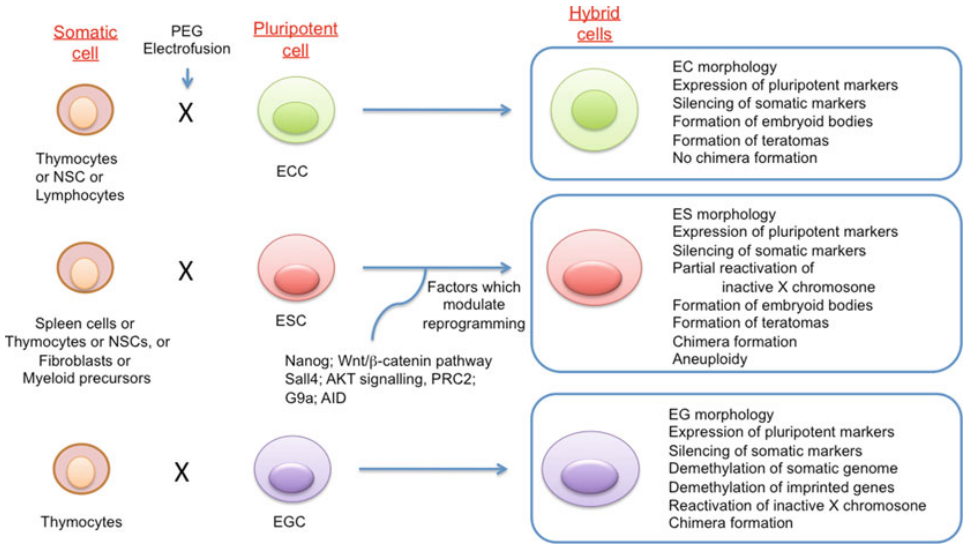

Fig. 9.2 Cell-cell fusion between somatic cells and pluripotent cells. Somatic cells can be fused using PEG or electrofusion, with the generation of pluripotent cells (ECCs, EGCs and ESCs) and hybrid cells. Features of the different hybrids obtained are indicated

and/or activation of the inactive X chromosome derived from the somatic partner [20, 53, 54]. Cross-species fusion has also been carried out: hybrids formed between murine ECCs and cells from a human T-lymphoma resulted in formation of an inter-species hybrid colony where the expression of the Oct4 and Sox2 human transcription factors was detected, as characteristic of undifferentiated pluripotent stem cells. This thus demonstrated activation of endogenous human markers of pluripotency.

One question that has been raised regarding fusion-induced reprogramming was whether somatic cells can retain the memory of their origin after being reprogrammed, as reprogramming itself does not necessarily imply that the somatic cells have completely lost this memory. To answer this question, ECCs were fused with mouse neurosphere cells. By examining the changes in gene expression and DNA methylation of the hybrid cells during re-differentiation, it was found that not only did the ECCs reprogram these neural stem cells (NSCs), but they also caused the NSCs to lose their epigenetic memory [55]. This thus confirmed the conclusion that hybrid cells can lose the memory of their somatic origin and adopt an identical differentiation potential to that of their pluripotent fusion partner.

Furthermore, hybrid ECC–somatic cells can give rise to true carcinomas that contain derivatives of all three of the embryonic germ layers [53, 54, 56, 57] or that form embryoid bodies in suspension culture [57]. The presence of embryonic antigens has also been seen for fusion between ECCs and lymphocytes and thymocytes [58]. However, the hybrid cells produced by the fusion of ECCs with fibroblasts resembled fibroblasts with respect to their morphology [59], suggesting that ECCs cannot completely reprogramme all kinds of somatic nuclei.

Even if an important amount of our knowledge derives from studies with hybrids of ECC–somatic cells, it is clear that ECCs have some limitations. While they have the features of pluripotent embryonic cells, ECCs retain a low level of developmental potential as compared to ESCs and EGCs. ECCs rarely generate chimeras, and moreover, they cannot contribute to germ lines (Fig. 9.2) [54].

The view that is emerging from these studies is that hybrid cells generated by ESCs or EGCs with somatic cell fusion provide better systems for studying somatic-cell reprogramming.

9.3.2 Fusion of Somatic Cells with ESCs

A large number of recent studies have demonstrated the potent reprogramming activities of ESCs. One of the earliest studies of fusion-mediated reprogramming was carried out by fusing male mESCs deficient for hypoxanthine phosphoribosyl-transferase ($HPRT^-$) with mouse female spleen cells, using PEG. The hybrids were then selected for HAT-resistance, and four clones were isolated. These were positive for the embryonic ECMA-7 antigen and negative for the TROMA-1 somatic antigen, showed alkaline phosphatase activity, had both X chromosomes synchronously replicating (indicating that they were both in an active state), were able to form embryoid bodies containing derivatives of all three germinal layers, and, most importantly, were able to generate chimeric mice when injected into blastocysts. Of note, three of the four isolated clones contained 41–43 chromosomes, and one clone was nearly tetraploid. However, this last proved to be unstable: when grown under non-selective conditions, the cells of this clone rapidly lost chromosomes, retaining only 40–43 chromosomes after 5–7 passages [60, 61]. Nevertheless, it was not clear if the chromosomes from the ESCs or those from the spleen cells segregated into the hybrids.

In a subsequent study, a more efficient method for somatic reprogramming selection was used. Somatic thymocytes harboring a silent GFP transgene under the control of the Oct4 promoter were fused with ESCs [22]. When the somatic genome was reprogrammed, Oct4-GFP was expressed and hybrid reprogrammed clones were successfully generated. The thymocyte/ ESC hybrids contained reactivated X chromosomes of thymocyte origin, as judged by fluorescent *in-situ* hybridization for Xist RNA. In this study, Xist RNA was seen to bind unstably to all three X chromosomes, suggesting a state of partial X chromosome inactivation. The Oct4-promoter-driven GFP transgene was observed approximately 48 h after fusion. Interestingly, ESCs were not able to reprogram parental imprints, given that the methylation status of the imprinted H19 and Igf2r genes was not altered by the fusion with ESCs. Furthermore, ESC/thymocyte hybrids contributed to all three germ layers in mouse chimeras at day E7.5 of development (Fig. 9.2).

To analyze the differentiation potential of the hybrid cells, fusion of mouse $Hprt^-$ ESCs and thymocytes containing a selectable ROSA 26βgeo transgene (which ubiquitously expressed neomycin drug resistance and β-galactosidase activity) were carried out. These hybrids retained chromosomes from both fusion partners, as judged by the presence of specific polymorphisms, and they produced teratomas in SCID mice [62]. Furthermore, they differentiated into neural lineages in culture, and were shown to be immunoreactive for the post-mitotic neuron-specific factor TuJ and for the dopaminergic neuronal marker PitX3. When neuronally differentiated hybrids were implanted into the striatum of mouse brains, the cells expressed tyrosine hydroxylase, providing evidence that hybrid cells can differentiate into neural cells with dopaminergic characteristics. The survival of cells in the grafts was confirmed by X-gal staining 15 days after injection.

It has also been shown that human somatic cells can be reprogrammed by fusion with hESCs. Like mESCs, hESCs can self-renew indefinitely and can differentiate into all cell types of the body [52]. Hygromycin-resistant hESCs were fused with puromycin-resistant human fibroblasts using PEG, and double drug selection was used for the formation of hybrids [21]. The resulting hybrids were formed of cells predominantly containing 92 chromosomes, and the Oct4 promoter was demethylated, a necessary step for reinitiating its expression. Other pluripotent markers were expressed in these hybrids, while the expression of fibroblast-specific genes was repressed. Hybrids formed embryoid bodies and teratomas after injection into nude mice [21]. Similar results were obtained when myeloid precursors were fused with hESCs [63].

Fusion of ESCs with somatic cells in all the mentioned studies produced highly proliferative hybrids with nuclear fusion, called synkaryons. This system unfortunately does not allow the unequivocal assertion that the somatic cell nucleus is reprogrammed, as it coexists with the original chromosomes from the ESCs in the hybrids. Thus a different approach has been used recently to analyze reprogramming: the heterokaryon system. Heterokaryons are hybrids containing two

different nuclei in a common cytoplasm, whereby all of the genetic material remains intact within each independent nucleus. This type of short-term, non-dividing fusion product makes it possible to assess the influence of two nuclear components on gene expression [64]. Two research groups have generated heterokaryons between mESCs and human somatic cells (B lymphocytes and fibroblasts). This method allowed the reprogramming of the human nuclei to be followed. Interestingly, the reprogramming was very fast, and the human nuclei were shown to start expressing pluripotent markers (Oct4 and Nanog) 24 h after fusion [65, 66]. Furthermore, the reprogrammed human cells expressed a profile of transcripts seen in hESCs that were not expressed in mESCs, suggesting that the human nuclei was reprogrammed through trans-acting factors from the mouse nuclei. Later, however, the reprogramming process was finalized and established by reactivated cis-acting factors from the human nuclei [66].

9.3.3 Fusion of Somatic Cells with EGCs

EGCs were established from PGCs [67, 68]. PGCs can be identified by the expression of an Oct4-*GFP* transgene, which allows their purification by fluorescence-activated cell sorting (FACS). Analysis of DNA from PGCs revealed demethylation at multiple genetic loci, including for both imprinted and non-imprinted genes [69, 70]. This might explain why EGC-derived chimeras show phenotypic abnormalities, which include fetal overgrowth and skeletal malformations, even if they can contribute to many tissues in chimeric embryos [71]. However, EGCs maintain important pluripotent characteristics, which make them attractive candidates for induced fusion with somatic cells to study reprogramming.

With fusion of female EGCs carrying the Rosa 26βgeo transgene and female somatic thymic lymphocytes, the hybrids obtained showed phenotypic properties that were similar to those of EGCs, including both pluripotency and repression of expression of the somatic cell genome (Fig. 9.2). Extensive demethylation of the thymocyte genome was also detected, which was similar to that seen in the EGC nuclei; this demonstrated that the demethylation activity from EGCs was dominant over the somatic genome. Futhermore, the X chromosome derived from the somatic nuclei was reactivated. Interestingly, unlike ESCs, EGCs can erase the parental imprints of *H19* and *Igf2r*. Finally, when the hybrids were injected into host blastocysts and implanted into pseudopregnant mothers, β-galactosidase expression was seen in chimeric embryos at days E9.5 and E10.5 [72].

9.3.4 Mechanisms Controlling Somatic Cell Reprogramming

Somatic cell reprogramming is an inefficient process. Furthermore, for many years it was not clear if the factors that induce reprogramming are in the nucleus or in the cytoplasm. The nuclear transfer experiments told us that the cytoplasm of an enucleated mammalian oocyte had the ability to reset the genetic program of a fully differentiated somatic cell nucleus [44, 73–76]. In addition, extracts from *Xenopus* eggs and ECCs were shown to activate expression of Oct4 in the somatic cells [77, 78], further suggesting that cytoplasmic elements are the reprogramming factors responsible.

However, in an interesting report, karyoplasts (cellular nuclei) and cytoplasts (intact cytoplasm without nuclei) of ESCs were separated and fused with NSCs. Fascinatingly, Oct4-*GFP* was activated in NSCs only after fusion with ESC karyoplasts, and hence not with ESC cytoplasts. These data were the first evidence that indicated that ESC nuclei contain factors that are sufficient to reactivate Oct4-GFP in somatic cells and to initiate reprogramming [79].

In addition, Do and Scholer [79] demonstrated the importance of the cell-fusion-induced reprogramming approach for identification of nuclear factors that regulate and increase reprogramming

efficiency. Below we summarize the reprogramming factors that have been identified to date by cell-fusion-mediated reprogramming.

9.3.4.1 Nanog

Nanog is a homeodomain-bearing protein that acts as a transcriptional factor, and is itself transcribed specifically in mouse pluripotent cells, mESCs and mEGCs [80, 81]. The loss of epiblasts soon after implantation in Nanog-null embryos, and the clonal expansion of ESCs over-expressing Nanog via bypassing of the regulation by LIF-STAT3 signals, indicate that Nanog is an important regulator for maintaining pluripotency and self-renewal of ESCs [82, 83]. These observations identified Nanog as a good candidate to increase reprogramming. Indeed, a 200-fold increase in the number of reprogrammed colonies was seen after fusions of ESCs overexpressing Nanog with NSCs, as compared with controls [84]. Nanog also improved the yield of reprogrammed hybrids when thymocytes and fibroblasts were fused with ESCs.

However, Nanog is not a part of the minimal combinations of exogenous factors that can convert mouse somatic cells into iPSCs [26]. To solve this apparent controversy, using Nanog-deficient cells, it has been shown that Nanog is fully dispensable for the initial steps of reprogramming, which consist of the loss of differentiated features and the creation of a pre-pluripotent state. Instead, Nanog mediated the acquisition of pluripotency by inducing the completion of dedifferentiation of partially reprogrammed cells [82].

9.3.4.2 The Wnt/β-Catenin Pathway

Wnt/β-catenin signaling controls ESC self-renewal and maintenance of "stemness" [85], and regulates expression of the ESC genes. The stability of β-catenin is essential to the signaling activity of the canonical Wnt pathway. In the absence of Wnt binding to its receptor, GSK-3β kinase phosphorylates β-catenin and targets it for ubiquitin-mediated destruction. Activation of the pathway by Wnt inhibits GSK-3β activity and results in the accumulation of β-catenin. Stable β-catenin then translocates into the nucleus, where it interacts with different Tcf DNA-binding factors; this complex in turn activates the transcription of target genes [86, 87]. Periodic activation of the Wnt/β-catenin signaling pathway strikingly enhances cell-fusion-mediated reprogramming. Specifically, by treating ESCs for a limited and specific time with Wnt3a or with an inhibitor of GSK-3 activity, which both lead to nuclear accumulation of β-catenin, these cells became "super-able" to reprogram somatic cells (NSCs, thymocytes and mouse embryonic fibroblasts [MEFs]) after fusion [88].

It would be interesting to identify the important downstream effectors of Wnt that participate in this process. For example, c-Myc is a prominent downstream regulator of the Wnt pathway [86, 89]. However, enhancement of reprogramming efficiencies by Wnt3a-conditioned medium was not accompanied by up-regulation of c-Myc [88]. Tcf3 is another candidate effector of the Wnt signaling pathway. Tcf3 co-localizes with ESC core regulators, such as Oct4, Sox2 and Nanog, to regulate the balance between ESC pluripotency and differentiation [86, 90, 91].

Interestingly, Nanog and the Wnt pathway can cooperate; in a system where Nanog is overexpressed and the Wnt pathway is activated, the reprogramming of NSCs is strikingly enhanced [92].

9.3.4.3 AKT Signaling

Phosphoinositide 3-kinase (PI3K) has a decisive role in a broad range of cellular functions relating to responses to extracellular signals. The serine-threonine kinase Akt is a key downstream effector of PI3K, and in response to PI3K activation, Akt phosphorylates and regulates the activities of a number of targets, including kinases, transcription factors, and other regulatory molecules. This PI3K/Akt signaling regulates both tumorigenic potential and pluripotency of stem cells. This pathway promotes

the de-differentiation of primordial germ cells into EGCs, and it is sufficient to maintain the pluripotency of mouse and primate ESCs cultured in the absence of LIF and feeder cells [93, 94]. These features defined PI3K/Akt as a strong candidate signaling pathway that can increase reprogramming. Indeed, activation of Akt signaling enhanced the yield of pluripotent hybrid colonies after cell fusions between ESCs and somatic cells [93]. However, activation of Akt signaling significantly reduced the efficiency of nuclear reprogramming by nuclear transfer. This controversy into the effects of Akt signaling might be due to transcriptional activation of different sets of target genes in each of these methods.

9.3.4.4 Sall4

Sall4 is a member of the Spalt family of transcription factors, and it was originally identified in *Drosophila* as a homeotic gene that is required for head and tail development [95]. Sall4 is also essential for maintenance of pluripotency and self-renewal of ESCs, and for their derivation from blastocysts [96]. Although Sall4 can act as a transcription factor that regulates numerous genes, one of its few known target genes is Oct4 [97].

MEFs carrying the Oct4-*GFP* transgene and overexpressing each of Oct4, Nanog, Sox2 and Sall4 have been fused with ESCs, with the number of GFP-positive cells after fusion monitored. Unexpectedly, after fusion with ESCs, MEFs that overexpressed Oct4, Nanog or Sox2 did not show significant increases in Oct4-*GFP* expression relative to the controls. In contrast, the relative numbers of GFP-positive cells in MEFs overexpressing Sall4 increased sevenfold with respect to the controls.

However, in another experimental system, double drug selection was used to measure reprogrammed colony formation after fusion. In this setting, after fusion with ESCs, MEFs overexpressing Nanog, Sox2 or Sall4 showed significant increases in the number of reprogrammed colonies, relative to the controls. In contrast, the overexpression of Oct4 in MEFs did not promote formation of reprogrammed colonies [98]. These data showed that Nanog, Sox2 and Sall4 can induce reprogramming even if they are overexpressed in the somatic genome. However, the duration of the reprogramming process and the re-expression of the Oct4 promoter can vary across different systems, and so the reactivation of Oct4-GFP appears not necessarily to be indicative of successful reprogramming.

9.3.4.5 Epigenetic Modulation: Roles of PRC2, AID and G9a

Using the method of direct reprogramming, most infected cells are trapped in a partially reprogrammed state, due to their inability to overcome major reprogramming barriers. When the DNA-methylase inhibitor 5-aza-cytidine was applied to these pre-iPSC clones, conversion of the pre-iPSC state into the complete iPSC state was shown [99]. This thus demonstrated that DNA methylation is an important epigenetic barrier that partially reprogrammed cells can encounter and can fail to overcome.

With heterokaryon formation between mESCs and human fibroblasts, the DNA demethylase AID was identified as an important player in the reprogramming process. After fusion, rapid demethylation of the Oct4 and Nanog promoters in the somatic heterokaryon genome was seen, which was also followed by the expression of these genes. This suggested that demethylase activity is important for the reprogramming process. DNA demethylation is essential to overcome gene silencing and to induce temporally and spatially controlled expression of mammalian genes, although no consensus mammalian DNA demethylase has been identified, despite years of efforts [100]. AID was a candidate factor, as it has a role in mammalian DNA demethylation of pluripotent germ cells and DNA demethylation in zebra fish during post-fertilization events [101, 102]. AID belongs to a family

of cytosine deaminases, and the deamination of cytosine followed by DNA repair leads to DNA demethylation [103]. In heterokaryons, the knock-down of AID prevented DNA demethylation of the human Oct4 and Nanog promoters and the expression of these pluripotency factors by fibroblast nuclei. Furthermore, initiation of nuclear reprogramming towards pluripotency was inhibited in human somatic fibroblasts when AID-dependent DNA demethylation was reduced. Interestingly, AID binding was seen at silent methylated Oct4 and Nanog promoters in fibroblasts, but not in active unmethylated Oct4 and Nanog promoters in ESCs [65].

As well as DNA demethylation, histone modifications are important for the enhancement of reprogramming efficiency.

Polycomb-group (PcG) proteins were originally identified in *Drosophila melanogaster*, where they form multiprotein complexes that are required for maintaining transcriptional silencing of a subset of repressed genes [104]. Two main repressor complexes, polycomb repressive complexes 1 and 2 (PRC1, 2) have been identified. These have different catalytic properties and core components. PRC2 consists of three core components: embryonic ectoderm development (Eed), suppressor of Zeste 12 (Suz12), and the SET-domain-containing protein enhancer of Zeste homolog 2 (Ezh2). The catalytic subunit, Ezh2 is a SET domain-containing methyltransferase that catalyzes the formation of the H3K27me3 marker, which forms the docking site for recruitment of PRC1 [104].

The involvement of the PcG proteins in the maintenance of ESC identity and pluripotency was first suggested by genome-wide studies that showed that PcG targets are highly enriched in genes involved in developmental patterning, morphogenesis, and organogenesis [38]. Loss of the EED gene in ESCs leads to genome-wide and almost total loss of H3K27me3, and consequently, to derepression of the PcG targets [105]. Despite this dramatic reduction in H3K27Me3, ESCs can be derived in the absence of EED. Embryos lacking individual components of the PRC2 complex, such as Eed, Ezh2 and Suz12, can survive post-implantation but die from gastrulation defects from 7 to 9 days post-fertilization [106–108].

In mESCs and hESCs, PRC1 and PRC2 localize to the promoters of a subset of repressed genes that encode transcription factors that are required for specification during later development. These genes contain overlapping binding sites for the pluripotent genes Oct4, Sox2, Nanog and Sall4 within their promoters [109–111], and are enriched in both H3K4me3 and H3K27me3 histones [112–114]. This explains why ESCs require PcG proteins for maintenance of the self-renewing state; EED-/- ESCs tend to differentiation in culture as lineage development genes are derepressed [105, 115]. However, EED–/– ESCs cannot give rise to all cell types after in-vitro differentiation, and the chimeras show developmental defects that are similar to knock-out embryos [105]. Thus PcG complexes are also required for the full differentiation potential of ESCs.

Interestingly, it has been shown recently that deletion of individual PRC1 and PRC2 members (Eed, Suz12, Ezh2 and Ring1a/B) in ESCs abolished the ability of these ESCs to induce reprogramming when they were fused with human lymphocytes to form heterokaryons. Importantly, given that Eed-deficient mESCs can themselves self-renew, and are pluripotent and can contribute to the three germ layers in vivo but can not reprogram the somatic genome, this finding clearly showed that pluripotency and reprogramming function can be dissociated, and represent two different pathways [116].

As part of H3K27 methylation, H3K9 methylation has also been shown to be important as a marker for reprogramming. It has been showed that knock-down of the histone H3 lysine 9 (H3K9) methyltransferase G9a, and overexpression of the jumonji-domain-containing H3K9 demethylase Jhdm2a, can enhance Oct4-EGFP reactivation from adult NSCs after ESC-fusion-mediated reprogramming. In addition, coexpression of Nanog and Jhdm2a enhanced the reprogramming even further. After overexpression of Jhdm2a or inhibition of G9a, a reduction in DNA methylation in the Oct4 promoter was seen, demonstrating that H3K9 and DNA methylation restricts somatic cell reprogramming by cell fusion with ESCs [117].

9.4 Lineage Reprogramming by Cell Fusion

Although differentiated cells normally retain cell-type-specific gene expression patterns throughout their lifetime, cell identity can sometimes be changed both in vitro and in vivo through different mechanisms. Besides the strategies described above that are based on de-differentiation of somatic cells to a pluripotent fate, several studies have been performed to determine whether, and especially how, the cellular identity can be modified, rather then reversed back along the developmental cascade, to achieve pluripotency.

The ability to impose changes in gene expression and to transfer epigenetic markers associated with a different cell fate to more specialized cells after cell fusion has been considered a unique characteristic of ESCs [21, 22, 84]. However, in the 1980s, it was already thought that differentiated cells also have the capacity to change the epigenetic state of other nuclei. Following fusion of two distinct somatic cell types to form proliferating hybrids (synkaryons) or post-mitotic hybrids (heterokaryons), somatic nuclei were reprogrammed towards specific differentiated fates [23, 25, 118]. These findings surprisingly highlighted the possibility to switch directly between different cell fates; that is, in other word, the re-programming of cell identity.

The first studies on phenotype modulation induced by synkaryon formation in specialized cells revealed that gene activation specific for a different cell is seen. Fusion of murine hepatoma cells, which secrete mouse serum albumin, with human leukocytes, which did not produce albumin, resulted in the formation of hybrids that secreted both mouse and human serum albumin, indicating that the albumin gene in human leukocytes was re-activated. These data are consistent with the hypothesis that the murine genome contributes with activators to the human genome [119].

Many reports have shown that ectopic expression of single transcription factors that are known to have a key role in specification of a certain cell identity during development can be sufficient to convert the cell fate to that of a different somatic cell. For example, expression of individual muscle regulatory factors of the MyoD family has been shown to be sufficient to convert a range of non-muscle cell types into muscle cells (Fig. 9.3). Surprisingly, the pattern of gene activation resembles the expression of the muscle regulatory transcription factors during normal muscle differentiation [120–124].

Similarly, high levels of the transcription factors C/EBPalpha and C/EBPbeta can directly reprogram committed mature B lymphocytes to become macrophages [125, 126]. More recently, fibroblasts were converted into functional neurons by expression of three factors (Ascl1, Brn2 [also known as Pou3f2] and Myt1l) [127] (Fig. 9.3).

Unexpectedly, the liver-derived BNL cell line was directly converted to muscle cells by induction of overexpression of the transcription factor MyoD, whereas other similar cell types, such as the human HepG2 hepatocyte cell line, were not [121, 128]. One explanation of such differences could be that the BNL cells are less differentiated than HepG2 cells and primary hepatocytes. Thus cell type, cell-cycle phase, differentiation state, and age of the nuclei might all influence the efficiency of nuclear reprogramming; however, the effects of these differences need to be investigated.

Interestingly, the apparently refractory HepG2 cell type starts to express specific muscle genes when forced to fuse with myotubes, to form heterokaryons [129]. These data demonstrated that heterokaryon formation induces human muscle gene expression in non-muscle nuclei.

The phenotypic changes observed in MyoD-overexpressing HepG2 hepatocytes differ from those in hepatocyte-derived heterokaryons: the response of a somatic cell to a single regulator depends on the cellular developmental state and the specific regulatory gene expression. In contrast, the phenotype of hybrid cells obtained by fusion results from a complex interaction of the regulatory factors that are contributed by each former cell type. Based on these differences, the stable differentiated state of a cell has been defined as the product of dynamic interactions among different sets of regulators [130].

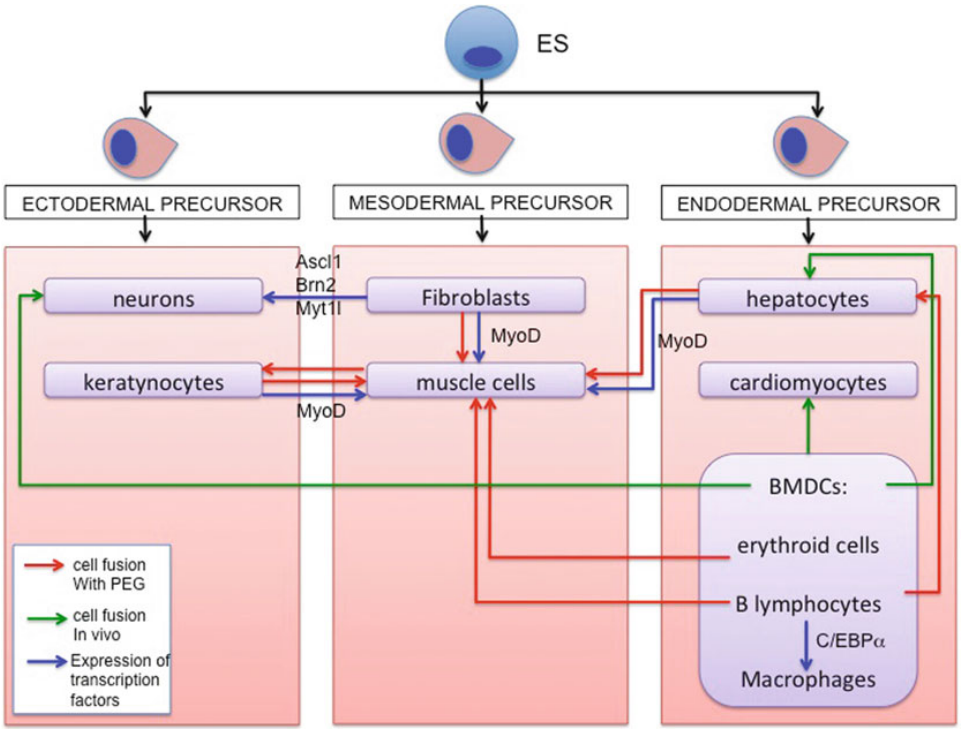

Fig. 9.3 Overview of lineage reprogramming induced in vitro and in vivo. Scheme summarizing the different transitions from one cell fate to another that have been observed experimentally after ectopic expression of key transcription factors (*blue lines*), in vitro cell fusion forced with PEG (*red lines*), and spontaneous cell fusion observed in vivo (*green lines*), starting from cells derived from different developmental precursors, such as ectoderm, mesoderm and endoderm. The bi-directionality of the process has been investigated experimentally only for the muscle–keratinocyte transition. Interestingly, the kinetic of the transition appears to be more rapid for the same developmental lineage transitions (i.e. fibroblast–muscle) with respect to different developmental lineage transitions (i.e. hepatocyte–muscle)

9.4.1 Synkaryon Versus Heterokaryon Strategies to Investigate the Mechanisms of Cell-Fusion-Mediated Lineage Reprogramming

Muscle cells are not unique in their potential to directly reprogram a differentiated cell by cell fusion. For instance, other studies have demonstrated that the adult human globin genes can be reactivated in stable hybrid cell lines produced by PEG-mediated cell fusion of adult mouse erythroleukemia cells or human hematopoietic cells, and fibroblasts [131–133]. However, it was difficult to draw conclusions about trans-acting regulatory mechanisms in these studies that came from stable synkaryon lines. Many generations were required before the hybrids could be isolated and analyzed; furthermore, the gene activation program was generally transient after the formation of synkaryons, because cell division during the passages in culture led to the loss of the human chromosomes.

To overcome these problems, other investigations have fused human fetal or mouse adult erythroid cells with non-erythroid cells, to form transient heterokaryons, with isolation and analysis of the total RNA 24 h later. The conclusion here was that previously inactive globin gene expression can be activated in a variety of non-erythroid cell types, and thus the globin genes appeared not to

be irreversibly inactivated in non-erythroid cells: in conclusion, the erythroid cells should contain developmental-stage-specific factors that act in *trans* to regulate globin gene expression [25].

However, the synkaroyon strategy used in these studies had some limitations. For instance, in synkaryons from the same species, it was not possible to determine the timing or extent of reprogramming, as after many passages the nuclear components of one parental cell type were physically mixed with those of the other; thus the contributions of each of the two nuclei could not be distinguished. Therefore, the heterokaryon strategy appears to be the most appropriate to circumvent these problems. In contrast to synkaryons, heterokaryons do not undergo mitosis, and they stably retain all of the nuclear components of the parental cell types in their distinct intact nuclei. In addition, in studies of nuclear reprogramming towards a differentiated fate in non-dividing interspecific heterokaryons, the species-specific transcriptome can be analyzed to profile the gene expression changes from the nucleus of interest in the fused cells, throughout the time course of the reprogramming.

In one of the first studies on heterokaryons, Ringertz showed that fusion of rat myoblasts and chick erythrocytes resulted in the swelling of the erytrocyte nuclei and diffusion of the chromatin, in anticipation of the reprogramming events [134]. Later, primary mouse muscle cells were fused in tissue culture with human primary cells derived from all of the three embryonic lineages: endoderm (hepatocytes), ectoderm (keratinocytes) and mesoderm (fibroblasts) (Fig. 9.3). Of note, nuclei from each of these cell types were capable of activating a number of previously silent muscle genes, indicating that the differentiated state can be altered, even in different specialized human cells [23, 25, 135, 136].

Interestingly, these heterokaryon studies revealed that tissue derivation and embryonic origin have marked effects. Fibroblasts, which are from the same embryonic lineage as muscle (mesoderm), show faster kinetics and a higher ultimate frequency of muscle gene expression, with respect to keratinocytes (ectoderm) and hepatocytes (endoderm) (Fig. 9.3). Despite this, the characteristics of cell history, or the lineage of origin, appear to influence cell reprogrammability [129].

9.4.2 The Importance of Gene Dosage in the Direction of Lineage Reprogramming by Cell Fusion

Cell-fusion-mediated lineage reprogramming can occur in either direction. In addition to keratinocytes being reprogrammed to a muscle transcriptional state, muscle nuclei can be reprogrammed towards a keratinocyte state, and these two phenotypes are mutually exclusive [137]. These two developmental states do not coexist: thus, there must be a controlling mechanism that supports and maintains the dominant transcriptional program.

Cell fusion results in the combination of two entire genomes and the cytoplasm from two cells of different functional and developmental states. Thus, the final phenotype will ultimately be determined by the dominance that arises from an excess of cytoplasmic factors or nuclear gene dosing, and not by a particular dominant phenotype or a master regulator [137]. To better examine the concentration requirements in the activation and expression of differentiation-specific genes over time, different ratios of myoblasts and keratinocytes were fused. Higher proportions of muscle cells induced an increase in reprogramming towards the muscle fate, and *vice versa*, indicating that the gene reactivation obtained in heterokaryons is dependent on the relative ratio of the nuclei that is contributed by each of the parental cell-type concentrations and stoichiometries.

9.4.3 Global Chromatin Changes in Cell-Fusion-Induced Lineage Reprogramming

The first evidence of cell-fusion-induced lineage reprogramming that was seen in heterokaryons suggested that the gene expression changes were restricted to permissive or open loci. However, many years ago, global chromatin remodeling in somatic cell heterokaryons was already predicted

by the observation of nuclear swelling and chromatin redistribution prior to gene reactivation in heterokaryons formed between rat myoblasts and nucleated chicken erythrocytes [138]. As differentiation involves a unique chromatin fingerprint configuration for each cell type, and moreover, as differentiated cells required continuous and active regulation to maintain their identity, it is reasonable to believe that lineage reprogramming is driven by chromatin remodeling at key loci, which in turn allows the expression of trans-acting regulators. Recently, a detailed study of muscle–keratinocyte hybrids revealed that the muscle gene activation in keratinocytes is not limited to a few genes, but is extensive [137]. Importantly, muscle gene re-activation was followed by a substantial silencing of the keratinocyte genes. Thus, this heterokaryon formation induced not only activation of silent genes in the non-expressing cell type, but also repression of the differentiated functions. So, the activation of muscle genes appears to be due not to genome-wide de-repression, but instead it is part of a global conversion from the expression program of a keratinocyte to that of a muscle cell [129].

In 1984, it was already postulated that differential regulation in hybrids after cell fusion resulted from the interactions of specific trans-acting factors with cis-acting genomic sequences, such as promoters or enhancers [139]. Whereas cell-type-specific gene expression programs were established through a network of transcriptional activators and repressors, epigenetic factors might also be required to maintain specification, by stabilizing the chromatin domains [140, 141].

As chromatin remodeling factors, including histone deacetylases (HDACs), were required for resetting gene expression, the role of HDAC activity in the dominant conversion of human lymphocytes to muscle cells in heterokaryons was investigated. The aim here was to elucidate the mechanisms of chromatin remodeling in cell-fusion-mediated lineage reprogramming. In heterokaryons, where chromatin replication is precluded, the lymphocyte nuclei increased in size and heterochromatin domains were redistributed to mimic the spatial rearrangement of neighboring mouse myocytes [142]. This was followed by the de-novo expression of human muscle genes in a temporal order that accurately recaptured gene expression in normal development. Moreover, activation of muscle-specific genes was associated with reduced expression of several lymphocyte genes. This confirmed that cell fusion not only induced reactivation of some specific genes, but it also resulted in global transition to a new cell identity in which silencing of pre-expressing genes also occurred. Remarkably, by inhibiting HDAC activity in the heterokaryons, the nuclei were seen to co-express two different lineage-associated gene programs. This indicated that although gene activation and silencing are mechanistically distinct, they are coordinated events in reprogramming [142].

The positive effects of treatment with an HDAC inhibitor prior to heterokaryon formation suggested that pre-fusion relaxation of chromatin at muscle regulatory regions renders the non-muscle nuclei more susceptible to muscle cytoplasmic factors after fusion.

Thus, in addition to activators and repressors of transcription, structural information, such as DNA methylation patterns, have to be transferred via the cytoplasm from the nuclei of one cell type to those of another in heterokaryons. It is now well supported that specialized skeletal muscle cells confer epigenetic information to epidermal progenitor nuclei by directing the methylation and demethylation of DNA of the genes they activate or silence [142]. These methylation changes are targeted in a tissue-specific manner, with keratinocyte-specific gene methylation accompanied by muscle-specific gene demethylation. Interestingly, DNA methylation has to occur by an active mechanism, as the cell hybrids did not undergo mitosis or DNA replication.

9.5 Cell-Fusion-Mediated Reprogramming as a Regeneration Mechanism

Generation of one adult phenotype directly from another as an alternative to reprogramming somatic cells to an intermediate stem cell state has broad implications for regenerative medicine. If cells can de-differentiate to a less differentiated state also in vivo, this implies that not only stem cells, but also the differentiated cells can show a degree of plasticity. Evidence that cell fusion between somatic

cells leads to reprogramming of differentiated nuclei had already arisen in the 1980s; however, that similar fusion events occurred in adult vertebrate organs was not predicted 3 decades ago. Thus, today, cell–cell fusion cannot be considered only as an in vitro mechanism to induce changes in cellular identity, but it is also acquiring a high impact in the field of regenerative medicine, as a possible in-vivo physiological way of regenerating damaged tissue.

9.5.1 Transdifferentiation Versus *Cell–Cell Fusion Theories to Determine Cellular Plasticity*

There is evidence that adult stem cells can fuse and change their cell identity in vivo. This is the case for bone marrow-derived cells (BMDCs), which are known to be the most plastic stem cells in the human body. These adult stem cells have the unique ability to switch their differentiation fate following bone-marrow transplantation in human and rodents, which contributes to the regeneration of hematopoietic and non-hematopoietic tissues. For instance, many studies have demonstrated that transplanted BMDCs can take on different lineages in vivo, including myocytes [143], hepatocytes [144], neurons [145], and many other cell types [146] (Fig. 9.3). This idea challenges the long-standing concept of cell-fate determination in mammalian developmental biology, and has received significant attention because it offers possibilities of expanding the therapeutic potential of adult stem cells.

The developmental fate of transplanted BMDCs during their new phenotype acquisition has been followed. It appears that after transplantation, BMDCs first acquire the characteristics of heritably diploid muscle stem cells (satellite cells), and then they later contribute to mature myofibers of muscle tissue. This thus suggests that BMDCs can undergo cell-fate changes as diploid mononucleate cells, and once reprogrammed, they can contribute to various tissues [147]. Two different theories can explain this phenomenon: the first is the trans-differentiation theory, which proposes that adult stem cells differentiate outside the tissue of origin in response to a new microenvironment by converting directly in the new cell type, while not globally changing their genetic identities. The second theory is that their change in cell identity is mediated by cell fusion events. In early 2002, spontaneous cell fusion was proposed as an alternative mechanism by which BMDCs can contribute to different tissues. The theory is that BMDCs first fuse with pre-existing differentiated cells within the target tissue, and then their nuclei are reprogrammed in response to intracellular cytoplasmic factors. Through co-culturing BMDCs with ESCs, Terada et al. showed that BMDCs can fuse spontaneously with other cells and subsequently adopt the phenotype of the recipient cells [148]. This finding was particularly significant, considering reports at the same time of transplanted BMDCs turning into unexpected cell types in vivo as a result of cell fusion events. Thus, despite the LaBarge study excluded cell fusion events and indicated that BMDCs can be transformed into satellite stem cells and then into muscle cells on the basis of cell karyotypes, several studies have now indicated that the changes in cellular identity can also result from cell-fusion events.

As an example, in a central nervous system biopsy from a woman who also received bone marrow transplants from male donors, it was shown that some of her Purkinje neurons were tetraploid (XXXY) and contained chromosomes from both female and male bone-marrow donors. This indicated that the Purkinje cells had fused with hematopoietic cells from the bone-marrow donors [149].

Several in-vivo cell-fusion events were then experimentally demonstrated in mice. Evidence of cell fusion in vivo emerged from injecting GFP^+ BMDCs expressing CRE recombinase into lethally irradiated mice carrying a LacZ reporter gene that was expressed only after excision of a LoxP-flanked-STOP codon by CRE-mediated recombination after fusion. Here, BMDCs were found to have fused with hepatocytes, cardiomyocytes and Purkinje neurons, even if at low rates [150].

Already in 1997, Eglitis and Mezey demonstrated that after transplantation of NeoR expressing hematopoietic stem cells, micro and macroglia that expressed the donor-derived reporter gene (*NeoR*)

were found in the brain [151]. However, despite a large number of reports indicated that cell fusion can occur in vivo after bone-marrow transplantation and that the phenotype change of the hybrid cells suggested a global change of expression profile by the donor genome, evidence of "reprogramming" was still missing.

It was Weimann and colleagues who for the first time suggested global chromatin reorganization and reprogramming of the donor nuclei to express a Purkinje cell specific gene after cell fusion. In this study, BMDCs fused spontaneously with Purkinje neurons after transplantation to form stable, non-dividing, binucleate, chromosomally balanced heterokaryons. Initially, the bone-marrow-like nuclei were characterized by compact chromatin, while the Purkinje nuclei retained dispersed chromatin. Over time, the nuclei of the BMDC donor cells became less compact and dense, and then finally they assumed the morphology of the Purkinje nuclei. As the authors indicated, this suggested that the neuronal program was dominant over the BMDC phenotype. To support their hypothesis, they reported reactivation of a Purkinje-specific trangene, L7-GFP, within the genome of the donor transgenic BMDCs. Thus, this mechanism that involved changes in gene expression was defined as nuclear reprogramming, which provided the first evidence that the differentiated state can be altered in vivo [152].

Given that the nucleus can be reprogrammed, at least partially, by cytoplasmic factors (as demonstrated by mammalian cloning), and based on the observation of the importance of the factors ratio in heterokaryons formed in vitro, the amounts of pre-existing protein and mRNA in the cytoplasm also appear to have roles in the outcome of cell-fusion events. In this sense, the size and composition of a cell might be a factor in the determination of the phenotypic dominance. Indeed, Purkinje neurons, hepatocytes, cardiac myocytes and skeletal myotubes have relatively large cytoplasmic volumes, and thus the reprogramming of the BMDC genome is presumably achieved through the increased dose of regulatory proteins in the much larger fusion partners.

However, it appears that these changes in cell function after cell fusion in vivo are the results of rare events, rather than the result of a biological process, as the frequency of these events is very low. In contrast, in a study from Johansson et al. in 2008, they confirmed that after transplantation, BMDCs can fuse with Purkinje neurons, although more interestingly, the low incidence of cell fusion that is detected under normal conditions can be enhanced under specific condition of stress, such as chronic brain inflammation. Remarkably, following species-mismached BMDC transplantation, specific Purkinje neuron gene products (Calb1, Pcp2, Kcnc1 and Gsbs) were detected in BMDC nuclei after fusion, whereas hematopoietic gene products (CD45, CD11b, F4/80 and Iba1) were not. These data demonstrated that the nuclei of BMDCs in the Purkinje heterokaryons can activate previously silent genes that are typical of mature Purkinje neurons and can repress hematopoietic genes. This is consistent with the hypothesis that they are reprogrammed to a Purkinje neuron fate, and that this mechanism is enhanced in response to tissue injury [153].

Another groundbreaking study demonstrated that cell-fusion-mediated reprogramming will be important in regenerative medicine. Female mice deficient in the enzyme fumarylacetoacetate hydrolase (Fah–/–; a model of fatal tyrosinaemia type 1) can be rescued by transplantation of BMDCs carrying the *Fah* allele. The novel generated hepatocytes in the transplanted Fah–/– mice were polyploid and contained chromosomes from the recipient and donor cells, which indicated that the regenerating nodules were derived from the donor hematopoietic cells that fused with the host hepatocytes, and not from transdifferentiated hematopoietic stem cells. More interestingly, it was thought that these cell-fusion events led to reprogramming of donor hematopoietic cell nuclei, as indicating by the Fah expression in regenerating liver nodules. In support of this, the BMDC surface marker CD45 was shown not to be expressed in Fah+/+ hepatocytes, indicating that after fusion the hematopoietic donor genomes underwent reprogramming of both activating and silencing genes to acquire the hepatocyte expression profile [154].

Similarly, cell fusion events have been demonstrated in a lethally irradiated female mouse model of lung inflammation that lacks lung-specific surfactant protein c (Sp-c). These mice were transplanted

with male wild-type BMDCs. The heterokaryon formation that was demonstrated by the Y chromosome in newly formed binucleate pneumocytes led to lung-specific reprogramming of the transplanted BMDCs, as indicated by activation of lung-specific gene expression as well as the expression of the *Sp-C* gene in the null mice [155].

Moreover, after transplantation of dermal fibroblasts into mdx mice, which is a mouse model for Duchenne muscular dystrophy, it was shown that the dermal fibroblasts can fuse with myotubes. As a result, the heterokaryons contained nuclei from both mdx and wild-type fibroblasts, which resulted in phenotypic and functional reversion of muscular dysgenesis [156].

9.6 Concluding Remarks

As has already been reviewed, there was new evidence by 2002 that some cells, such those from the bone marrow, can change lineage to generate completely new types of cells. This generated many doubts, and various news headlines like "Cell fusion makes confusion", "Plasticity: time for a reappraisal", and "Is transdifferentiation in trouble?". Also, the science editor of the UK broadsheet The Daily Telegraph (14/03/02) opined, "Scientists are generating freak cells". However, as Wright commented, this appears to be something that happens as part of the physiology of the cell, and it is thus "not necessarily a bad thing if it has cured a potential fatal metabolic disease!" To date, the number of studies that have investigated the function of heterokaryons in vivo is not high enough to have a clear idea of the full scenario; the molecular mechanisms that regulate cellular plasticity are still poorly understood, and this could in part justify the skepticism in this field.

The dissection of the entire gene expression profile from both genomes in heterokaryons formation in vivo is necessary for us to understand whether these mechanisms of reprogramming are only partial achieved, or if the are the result of global changes in cellular identity. Moreover, the whole genome methylation and demethylation patterns in all of the system need to be evaluated. If chromatin remodeling occurs, the target accessible sequences will also change. Thus, an important question remains to be answered: What happens during the developmental cell state transition? It has been shown that the phenotypic transition is rapid, and this has hampered the evaluation of the existence of any putative unstable intermediates. However, it remains to be seen whether the transition from one cell fate to another is direct, or whether between re-expression of previously silent genes and silencing of expressed genes there exists an intermediate where the chromatin state and the gene profile are similar to an intermediate state that resembles a common, less-specialized developmental precursor. BMDCs fuse and acquire the phenotype of fusion partners that arise from all of the three embryonic lineages, and most interestingly, BMDCs can de-differentiate to pluripotency. It still remains, however, to demonstrate whether during lineage transition in vivo, cells return to a pluripotent state, which would be unstable in adult tissue, before they go down through an alternative developmental pathway.

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