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Epigenetic Reprogramming of Nuclei Using Cell Extracts

Philippe Collas and Christel K. Taranger*

Institute of Basic Medical Sciences, Department of Biochemistry,
University of Oslo, PO Box 1112 Blindern, 0317 Oslo, Norway

Abstract

Recent evidence indicates that nuclear and cytoplasmic extracts from undifferentiated cells can reprogram gene expression and promote pluripotency in otherwise more developmentally restricted cell types. Notably, extracts of embryonal carcinoma cells or embryonic stem cells have been shown to elicit a shift in the transcriptional program of target cells to upregulate embryonic stem cell genes, downregulate somatic cell-specific markers, and epigenetically modify histones. Reprogrammed kidney epithelial cells acquire a potential for differentiation toward ectodermal and mesodermal lineages. Cell extract-mediated nuclear reprogramming may constitute an attractive alternative to reprogramming somatic cells by cell fusion or nuclear transfer. This review highlights recent observations leading to the concept that extracts derived from pluripotent cells contain regulatory components capable of reprogramming somatic nuclear function. Limitations of current extract-based reprogramming approaches are also addressed.

Index Entries: Cell extract; chromatin; differentiation; embryonic stem cells; epigenetics; nuclear reprogramming; pluripotentiality.

***Correspondence and reprint requests to:**

Philippe Collas
Institute of Basic Medical Sciences,
Department of Biochemistry,
University of Oslo,
PO Box 1112 Blindern, 0317 Oslo,
Norway.
E-mail:
philippe.collas@medisin.uio.no

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Introduction

The fate of a differentiated cell is generally thought to be stable; however, increasing evidence indicates that nuclear reprogramming events leading to cellular dedifferentiation can occur. Nuclear reprogramming can take place naturally or experimentally. One paradigm is the replacement of a limb after amputation in teleost fish and urodele amphibians. Regrowth of the new limb is believed to involve migration, dedifferentiation, proliferation, and redifferentiation of epithelial cells in the wounded area. Another classical example of nuclear reprogramming is the derivation of pluripotent embryonic stem (ES) cells *(1)* and the birth of cloned offspring *(2)* from differentiated somatic cell nuclei transplanted into an unfertilized oocyte (a process referred to as somatic-cell nuclear transfer). A third set of evidence of nuclear reprogramming has been provided by cell fusion approaches, whereby a differentiated somatic cell is epigenetically reprogrammed by fusion with a pluripotent stem cell to express stem cell genes and functions *(3–5)*. A last series of observation provides emerging evidence that nuclear and cytoplasmic extracts derived from differentiated or undifferentiated cells can be applied onto cells of other types to promote some nuclear reprogramming. Extract-based reprogramming approaches have shown that differentiated cells may be induced to "transdifferentiate" into other differentiated cell types *(6)*, or "dedifferentiate" toward a more pluripotent cell type *(7)*. Differentiation of ES or somatic adult stem cells with extracts of specific "target" cell types has also been reported *(8)*. This review critically highlights the current state of research involving cell extracts to reprogram cells to pluripotency.

Heterokaryon Experiments Provide a Rationale for Cell Extract-Based Nuclear Reprogramming

Experiments involving fusion of mouse thymocytes with mouse embryonal germ (EG) cells or ES cells have shown that epigenetic reprogramming could be triggered in the thymocyte nuclei (3,5). EG cells are derived from primordial gem cells (PGCs) and contain an epigenome similar to that of PGCs. During development, PGCs undergo complete epigenetic reprogramming through a process of genome-wide DNA demethylation. Interestingly, EG–thymocyte hybrids are characterized by stable demethylation of both nonimprinted and imprinted genes (3), suggesting that the epigenetic reprogramming property of PGCs is maintained in EG cells. Epigenetic changes in the thymocyte nucleus are consistent with induction of pluripotency markers such as ability to differentiate into the three germ layers in chimeric fetuses (3).

ES cells can also induce pluripotency when fused to somatic cells such as thymocytes (5), neuronal progenitor cells (9,10) or bone marrow-derived cells (11), and support the contribution of ES–somatic cell hybrids to all germ layers in chimeric mice (5,11) and in teratomas (9). The cell hybridization strategy for reprogramming nuclei has been recently applied to human primary fibroblasts using human ES cells (4). The hybrids display morphological and surface-molecule expression patterns resembling that of human ES cells, and genome-wide gene-expression analysis shows reprogramming of the fibroblast genome toward an embryonic state to near completion (4). Undifferentiated pluripotent embryonal carcinoma (EC) cells fused with T-lymphoma cells also promote the formation of colonies expressing pluripotent cell transcripts from the lymphoma cell genome (12). Chromatin immunoprecipitation analyses histone modifications in somatic cells after fusion with ES cells have validated the ability of ES cells to elicit epigenetic reprogramming (13).

Collectively, these observations indicate that factors from undifferentiated cells can elicit epigenetic reprogramming in more differentiated cell types when the cell contents are mixed in a heterokaryon. Of note, unlike EG cell–thymocyte hybrids, ES cell–thymocyte hybrids do not support DNA demethylation of imprinted genes (5). Thus, although ES cells can reset aspects of epigenetic reprogramming in differentiated nuclei, the ability to fully reprogram the epigenome (including DNA methylation marks underlying imprinting) in differentiated nuclei seems to be restricted to oocytes and EG cells.

Induction of Transdifferentiation With Extracts of Differentiated Cells

Reprogramming of nuclei by nuclear transplantation and cell hybridization has provided a rationale for the emergence of cell-free strategies for triggering a new differentiation program in stem cells or in differentiated cells. These approaches rely on the use of an extract from a chosen "target" cell type (the cell type one wishes to reprogram a cell into). The extract is expected to contain regulatory components necessary for driving the fate of one cell type into that of the target cell type (6). Cell extract-based systems for reprogramming cell fate have been developed with the aim of eliciting somatic cell transdifferentiation (14), stem cell differentiation (8,15), or somatic cell dedifferentiation (7,16,17).

Another rationale for developing reprogramming extracts comes from extensive studies which have relied on cytoplasmic extracts from *Xenopus* eggs, sea urchin eggs and *Drosophila* embryos to investigate the processes of sperm chromatin decondensation and male pronuclear formation (18–23). Although at that time not qualified as "reprogramming," the functions involved in turning the transcriptionally silent sperm nucleus into a large pronucleus with decondensed chromatin and a new nuclear envelope, and capable of replicating DNA, transcribing genes, and undergoing mitotic-like chromatin condensation/decondensation events, might well today fall under the terminology "reprogramming." Postchromosomal or post-nuclear supernatants from mitotic or interphase mammalian cells have also proven to be powerful for unraveling mechanisms of mitotic chromosome condensation (24,25), decondensation (26), and nuclear envelope assembly (27,28). Because somatic cell extracts can be used to manipulate nuclear architecture, it is reasonable to speculate that similar cellular preparations could be prepared to alter nuclear function.

We have in recent years developed 15,000*g* supernatants of nuclear and cytoplasmic (whole cell) lysates from several model target cell types, which can alter cell fate to various degrees (Fig. 1; 14). The approach involves reversible permeabilization of recipient cells (e.g., kidney epithelial 293T cells) with the pore-forming toxin Streptolysin O, transient bathing of the permeabilized cells in the extract and resealing of the cells with $CaCl_2$ in serum-containing culture medium. Methods developed in our laboratory have been described in detail (29).

Using this approach, we have shown that 293T cells treated with an extract of Jurkat or primary T cells can take on T-cell properties (14,30). Within days of extract treatment, the cells display expression of T cell-specific genes encoding, for example, surface receptors and a panel of interleukins (IL), cytokines, chemokines, and various receptors, on the basis of macroarray analysis of cytokine markers, Northern blotting and Affymetrix-based microarray analyses (14,30). As importantly, genes expressed in 293T cells (and not detected in the transcriptome of Jurkat cells) are downregulated. The most prominent class of downregulated genes includes those encoding cell-adhesion molecules, an observation which correlates with changes in adhesion properties of cells treated with Jurkat or primary T cell extract (14,30). As a result of changes in gene expression, extract-treated cells display T cell-specific intracellular signaling pathways such as assembly of IL-2α and -2β receptor chains on calcium-mediated stimulation of the T-cell receptor/CD3 signaling pathway (albeit downstream of the T-cell receptor/CD3 complex) with phorbolmyristylacetate (14). In addition, 293T cells exposed to an extract of peripheral blood T cells secrete IL-2 in culture (30). Some of the new phenotypes appear to be stable for several months in culture. There is however to date no indication of how stable the changes are on long-term culture, or after transplantation into host tissue.

A similar study also attempted to promote the induction of an insulin-producing β-cell phenotype by treatment of cultured rat primary fetal fibroblasts with an extract of rat insulinoma INS1E cells (31). The pancreas-specific genes *Pdx1* and *Insulin* were transiently expressed with onset detected within days or weeks after extract treatment. However, expression of

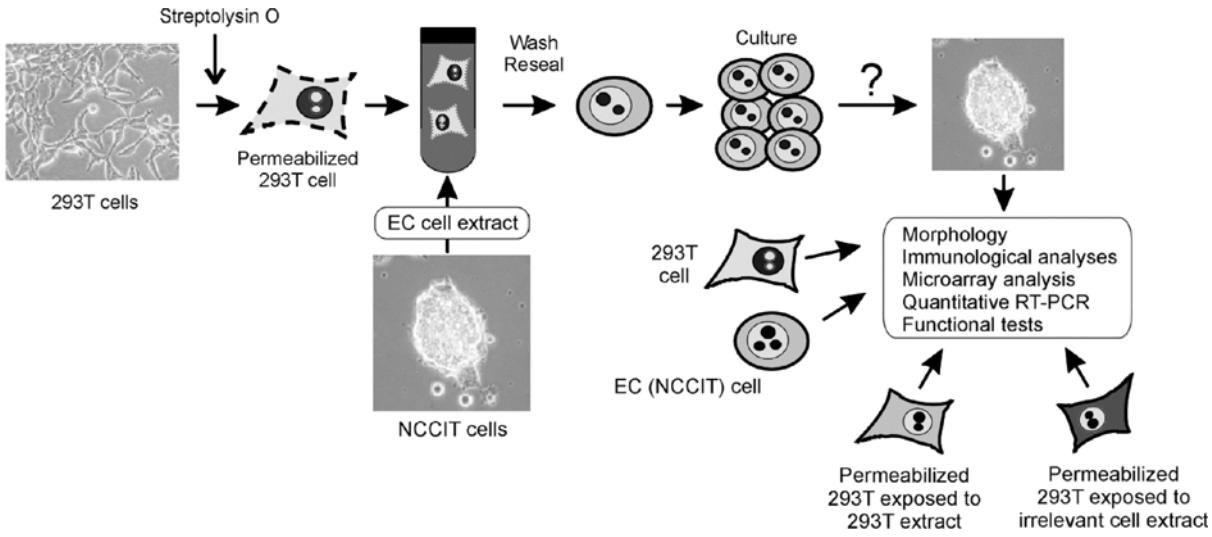

Fig. 1. Approach to cell extract-mediated nuclear reprogramming adopted by Taranger et al. *(7)*. Transformed kidney epithelial 293T cells were reversibly permeabilized with the bacterial toxin Streptolysin O and incubated for one hour in a nuclear and cytoplasmic 15,000*g* supernatant of from EC (here NCCIT) cells. After dilution of the extract, cells are resealed and cultured for analysis as shown. Several controls were included in the study, as indicated.

these genes was invariably transient, ranging from days to, at the most, 2 wk. Although insulin was immunologically detected in extract-treated cells, C-peptide was not assessed. Thus, whether true reprogramming of nuclear function occurred, as opposed to a transient alteration of cell fate remains uncertain. Nevertheless, there is some indication that insulinoma extracts can elicit histone acetylation and methylation events in exogenous chromatin (our unpublished data), so these extracts seem to have at least some chromatin remodeling capacity.

Directed Stem Cell Differentiation With Extracts of Differentiated Target Cells

Two published reports support the view that extracts of differentiated cells can elicit signs of differentiation in somatic or ES cells. We have prepared extracts of freshly isolated cardiomyocytes from juvenile rats and shown the expression of cardiomyocyte proteins and functions in cultured mesenchymal stem cells purified from human adipose tissue *(15)*. Adipose tissue stem cells (ATSCs) display a propensity to differentiate into primarily mesodermal cell types in vitro and in vivo, but had not been shown to be responsive to extract stimulation. Within a few days of extract treatment, ATSCs expressed cardiac-specific proteins including sarcomeric α-actinin, troponin I, and desmin. In addition, markers of gap junction assembly appeared in extract-treated cells, as judged by targeting of connexin 43 and phosphorylated connexin 43 to the plasma membrane. Evidence for functional nuclear reprogramming was shown by the beating of a low proportion of the cells in culture *(15)*.

More recently, a pneumocyte extract was shown to induce differentiation of mouse ES cells toward a pneumocyte phenotype *(8)*. Permeabilized ES cells exposed to mouse type II pneumocyte (marine lung epithelial [MLE]-12 cells) extract displayed increased expression of a *surfactant protein C-green fluorescent protein* transgene. Immunodetection of cytoplasmic surfactant protein C and nuclear thyroid transcription factor-1, and detection of lamellar bodies, organelles specific to type II pneumocytes supported differentiation. Subsequent differentiation to a type I pneumocyte phenotype was demonstrated by expression of aquaporin 5. Pneumocyte formation also occurred faster than with growth factor-induced differentiation *(8)*. This experimental model provides a tool for analysis of the key factors involved in the differentiation of ESCs to type II pneumocytes.

Published studies on extract-based nuclear reprogramming have solely relied on the in vitro characterization of extract-treated cells; thus, the extent to which the novel phenotype—or the new program—is stable is uncertain. Most studies indicate that the changes elicited are transient, with the duration of expression of a target cell-specific gene varying with the type of cell to be reprogrammed and the source of extract. A microarray analysis of gene expression in 293T cells exposed to a Jurkat extract indicates that among Jurkat cell-specific genes upregulated within 1 wk of extract treatment, less than 20% remain expressed after three months *(30)*. A proportion of genes downregulated by the extract also gradually becomes reactivated over time. These variations may reflect incomplete transcriptional and epigenetic reprogramming in these systems. By analogy to nuclear reprogramming events characterizing somatic cell nuclear transfer, an improvement of reprogramming efficiency may involve a "resetting" of the somatic cell program, possibly through a dedifferentiation step.

Reprogramming Using Extracts of Blastema and Eggs

The creation of ES cells and cloned offspring by nuclear transplantation *(1,2,32)*, together with the DNA demethylation and activation of embryonic genes in somatic–EG or somatic–ES cell hybrids *(3,5)* suggest that undifferentiated cells contain regulatory factors necessary for reprogramming cells to pluripotency. Experimental evidence collected in the last five years suggests that extracts derived from undifferentiated cells may do the job.

A first illustration is based on the induction of dedifferentiation with extracts of regenerating new limbs (16). When continuously exposed to cultured postmitotic mouse C2C12 myotubes, regenerating limb extracts promote cell cycle reentry in 18% of the myotubes, on the basis of bromodeoxyuridine incorporation into replicating DNA (16). This is accompanied by a downregulation of muscle-specific markers in approx 15% of the myotubes. Interestingly, mitosis is also detected in approx 10% of the myotubes and approximately half of these continue proliferating as mononucleated cells (16). The dedifferentiated phenotype seems to be maintained even after removal of the extract, suggesting that some heritable reprogramming events have taken place. The response of other terminally differentiated cell types to extract of regenerating limbs or blastema has not been reported but would be informative to provide insights on the generalization and species-specificity of the mechanisms involved in nuclear reprogramming.

As expected from nuclear transplantation experiments in amphibians (33,34), extracts of *Xenopus* eggs were recently shown to induce expression of pluripotency markers in 293T cells and in primary leukocytes (17). The cells form expanding clusters resembling ES cell colonies and upregulate expression of *OCT4* and *germ cell alkaline phosphatase* whereas downregulating differentiation markers (17). However, reprogrammed leukocytes have been shown to have a limited life-span and do not express ES-cell surface markers, indicating that reprogramming under these conditions may also be only partial.

Nuclear Reprogramming Using Extracts of Undifferentiated Carcinoma Cells

We have recently extended these studies to show that nuclear and cytoplasmic extracts of undifferentiated human EC cells can reprogram 293T cells to take on properties of undifferentiated cells with a potential for pluripotency (Fig. 1; 7). In countries where current legislation does not allow derivation or use of human ES cells, EC cells constitute an attractive alternative to ES cells for producing reprogramming extracts because they express a gene-expression profile and stem cell functions similar (but not identical) to ES cells (35,36). Undifferentiated EC cells form malignant teratocarcinomas when transplanted into ectopic sites. Interestingly however, some EC cell lines can also contribute to tissues of a developing fetus when introduced into a blastocyst (37).

A first result of treatment with EC cell extract is a change in cell morphology (7). Colonies of 293T cells with defined edges develop and are maintained for many passages in culture (Fig. 2A). Cells exposed to their own extract or to extract of Jurkat T cells do not form colonies or form clearly morphologically distinct aggregates (7). A second line of evidence of reprogramming by EC extract is the induction of *Oct4* gene and protein expression (Fig. 2B). Approximately 60% of extract-treated cells exhibit persisting intranuclear *Oct4* protein labeling. Concomitantly, nuclear lamin A/C, a differentiated cell marker is strongly downregulated—a clear sign of dedifferentiation (Fig. 2B). Induction of *OCT4* (*POU5F1*) transcription and loss of lamin A (*LMNA*) gene expression over time are also evident. Furthermore, *OCT4* expression is accompanied by expression of additional markers of pluripotency including several Oct4-responsive genes (7).

Gene-expression profiling of EC extract-treated cells showed that about 1800 and about 1700 genes were up- and downregulated, respectively, relative to 293T cells (7). Of these, approx 70 and 34%, respectively, were shared with EC cells (EC genes) (Fig. 3A,B). Treatment of 293T cells with 293T cell extract altered expression of only a handful of EC genes (~5% of total genes altered), as did an extract of Jurkat T cells. This argues for target cell type-specificity of the changes observed. Virtually all EC genes affected by 293T or Jurkat extract were the same and these genes proved to be altered by chance rather than by extract treatment. When the consistency of EC-gene expression changes in EC extract-treated cells was examined, we found that approx 700 genes immediately upregulated remained expressed for more than two months, whereas more than 160 genes remained consistently downregulated (Fig. 3B). Most annotated upregulated genes encoded proteins involved in transcription, cytoskeletal organization, metabolism, signaling, and chromatin remodeling, whereas downregulated genes were more evenly distributed across functional classes (Fig. 3C).

These observations suggest that at least some of the transcriptional alterations are stable. Nevertheless, probably not all changes are heritable. Genes with unstable expression pattern may include passive bystanders causing "transcriptional noise" as a result of more specific alterations in the transcriptional network. Perturbation in the network would be predicted to induce changes trickling down the network until transcriptional equilibrium is reached (38). Accordingly, expression of these genes would be expected to return to a background level, but we have not noticed such stabilization, suggesting that in addition to target cell type-specific changes long-lasting perturbations in the transcriptome also exist. Fluctuations in the gene-expression profile may result from incomplete reprogramming and from heterogeneity in the transcriptional response to extracts. Nonetheless, the dynamics of gene expression may also illustrate a temporal compartmentalization of gene activity required to establish a heritable transcriptional program.

Induction of Potential for Pluripotency and Multilineage Priming

Downregulation of Expression of LMNA, a Marker of Differentiated Cells

The gene-expression profile elicited by EC-cell extract in 293T cells suggests the establishment of a potential for multilineage differentiation (7). An indicator of dedifferentiation is the repression of *LMNA*. *LMNA* encodes nuclear lamins A and C, which are expressed only in differentiated or committed progenitor cells. In agreement with an induction of dedifferentiation, we noted the strong downregulation of *LMNA* expression specifically in EC (or ES) extract-treated 293T cells (*see* Fig. 2B; 7). In contrast, cardiomyocyte extracts upregulate *LMNA* expression in human ATSCs, an event, which correlates with differentiation toward a cardiomyocyte phenotype (15), and *LMNA* is reactivated on retinoic acid-mediated differentiation of EC extract-treated cells (7). Thus, the state of *LMNA* transcription provides a direct assessment of (de)differentiation transitions mediated by cell extracts. The mechanism of gene inactivation mediated by extracts is not known. An

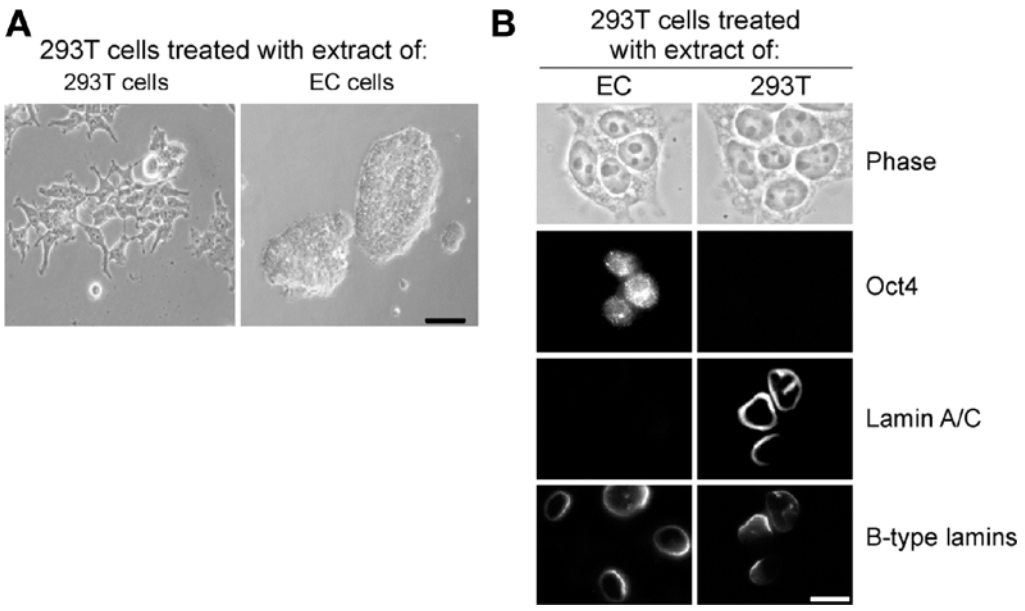

Fig. 2. Kidney epithelial 293T cells undergo phenotypic transformations after treatment with extract of EC cells. **(A)** Morphological changes of 293T cells 12 wk after control 293T (left panel) or EC (right panel) extract treatment and in vitro culture. Cells were passaged every week. EC extract-treated cells form distinct colonies. Bar = 20 μm. **(B)** Induction of Oct4 expression and downregulation of lamin A/C in 293T cells reprogrammed in EC extract. B-type lamins, constitutively expressed, remain apparently unaltered. Bar = 10 μm. Reproduced with modifications from ref. 7 with permission.

attractive hypothesis involves small interfering RNAs *(39)*, perhaps through a control of DNA methylation *(40)*, but this remains to be tested.

Transcriptional Upregulation and Activation of ES Cell-Specific Genes

An indicator of induction of pluripotency in EC extract-treated cells is the upregulation of genes characteristic of undifferentiated EC or ES cells (Table 1; Fig. 4). As result of treatment with EC extract, several embryonic, germ cell, and stem cell genes are activated to levels matching those of EC cells *(7)*. The *Oct4* gene is expressed in ES cells to maintain pluripotency, and the Oct4 protein acts by binding to a subset of target genes including *SOX2*, *UTF1*, and *REX1/DRN3*. We found all these genes were to be upregulated by the EC extract. As *UTF1* expression requires synergistic activities of Oct4 and Sox2 *(41)*, it is possible that Oct4-dependent functions are established in the extract-treated cells. Other pluripotency markers induced include telomerase (*TERT*), alkaline phosphatase (*APL1*), leukemia inhibitory factor (*LIF*), stem cell growth factor-β (*SCGF*), and germ cell nuclear factor (*GCNF*). Activation of these genes occurs for at least two months in culture, but how stable these changes are in the long run remains to be determined. The extent to which activation of these genes conveys stem cell functions in reprogrammed cells is also an open question.

Transcriptional Activation of Genes Indicative of Multilineage Priming

EC cell extract-treated cells also express genes suggestive of a potential for multiple lineage differentiation (Table 1; 7). These include markers of osteogenic, endothelial, myogenic, neurogenic, adipogenic, and chondrogenic lineages *(42)*. Interestingly, multilineage priming is a hallmark of hematopoietic stem cells *(43)*, mesenchymal stem cells from bone marrow *(44)*, and adipose tissue *(42)*. Multilineage priming may reflect the ability of these stem cells to differentiate into multiple cell types in the tissue in which they reside. Expression of multilineage priming genes in EC cell extract-treated cells suggests that the cells may be able to "survey" environmental cues. It seems, therefore that the transcriptional signature of EC cell extract-treated cells crosses germ layer boundaries.

Differentiation Plasticity of Cells Exposed to EC Extract

In vitro observations suggest that the differentiation plasticity of EC extract-treated cells is enhanced *(7)*. Exposure of reprogrammed cells to retinoic acid stimulates the emergence of neuronal progenitor cells. These exhibit neurite outgrowths, express neuron-specific genes and neuronal markers such as cytoplasmic NF-200 and intranuclear NeuN. EC extract-treated cells can also be induced to acquire characteristics of adipocytes, osteoblasts, or endothelial cells. Logically, expression of *OCT4* is downregulated. These observations strongly suggest that extracts of EC cells promote dedifferentiation as well as multilineage differentiation potential in an otherwise more restricted cell type. However, in vivo evidence of pluripotency is still lacking, and it would be valuable to demonstrate whether reprogrammed cells can contribute to chimerism or form teratomas.

Can Cellular Extracts Promote Epigenetic Reprogramming?

Maintenance and persistence of *de novo* transcriptional activity in extract-treated cells require the establishment of an

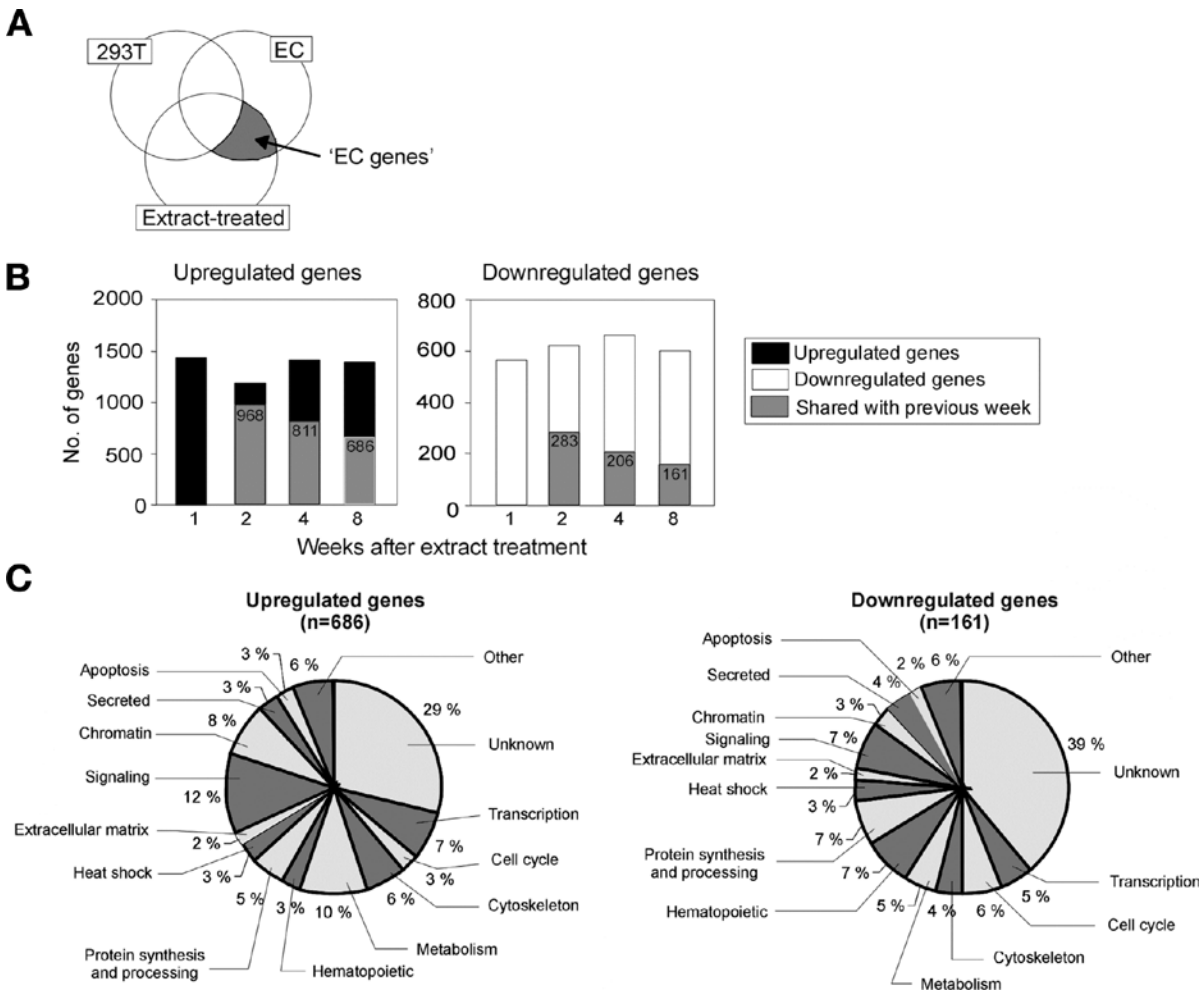

Fig. 3. Microarray analysis of gene expression in EC extract-treated 293T cells. **(A)** Venn diagram identifying EC-specific genes that are up- or downregulated as a result of EC extract-treatment. **(B)** Number of EC genes specifically up- or downregulated by treatment with EC extract, over time. Genes consistently up- or downregulated during consecutive weeks are shown in gray. **(C)** Functional class distribution of genes consistently up- or downregulated more than eight weeks after treatment with EC extract. These genes are those included in the gray bars, week 8 in (B). A complete list of these genes has been published *(7)*. Modified from ref. 7 with permission.

epigenetic code characteristic of, ideally, the chosen target cell type. Emerging evidence indicates that whole cell extracts may impose target cell type-specific remodeling activity onto exogenous chromatin. A chromatin immunoprecipitation analysis using an antibody against pan-acetylated histone H4 showed that the *IL2* promoter undergoes histone H4 hyperacetylation following treatment of 293T cells in T cell extract *(14)*. This modification is specifically elicited by extract of activated T cells (an extract of resting T cells had no effect) and is accompanied by transcriptional activation of the *IL2* gene. This argues for the physiological relevance of chromatin remodeling activities triggered by the extract at the *IL2* promoter.

A second line of evidence of epigenetic reprogramming comes from recent unpublished data from our laboratory. 293T cells exposed to EC extract exhibit hyperacetylation of H3 lysine (K)9 in the *OCT4* distal enhancer several weeks after extract treatment. However, no indication exists to date on how early histone modifications take place, or on how stable these alterations are. Yet, because H3K9 acetylation of *OCT4* was detected weeks after extract exposure, and because extract-treated cells divide every 24 h, there is indication that at least this modification is heritable. A panel of six histone modifications is currently being examined in EC extract-treated cells in correlation with transcriptional up- or downregulation of specific genes, to provide additional evidence that cell extracts can elicit locus-specific chromatin remodeling in exogenous substrates.

Although histone modifications are heritable, they are more labile than DNA methylation changes and thus may not provide a comprehensive indication of heritable chromatin remodeling in extract-treated cells. Recent preliminary published evidence suggests that the EC (NCCIT) cell extract retains the ability to reprogram DNA methylation at the *OCT4* locus in 293T cells *(7)*. Perhaps the most accurate way of assessing DNA methylation changes is the bisulfite sequencing method. Bisulfite sequencing enables identification of individual methylated cytosines in CpG dinucleotides in single DNA molecules *(45,46)*. Our data illustrate the clear demethylation of six out of eight cytosines between conserved regions CR2 and CR3 in

Table 1
Undifferentiated Stem Cell and Multilineage Priming Genes Induced in Kidney Epithelial Cells After Treatment With Extract of EC Cells

ES cell genes		*Fold upregulation[a] in reprogrammed cells (EC)*	*Lineage-specific genes*		*Fold upregulation[a] in reprogrammed cells (EC)*
OCT4 and Oct4-responsive genes			Adipogenic		
POU5F1	POU domain, class 5, TF1 (Oct4)	36 (36)	*APOA2*	Apolipoprotein A-II	9 (13)
SOX2	Sex determining region Y-box 2 (Sox2)	121 (119)	*APOD*	Apolipoprotein D	12 (11)
			APOE	Apolipoprotein E	2 (21)
UTF1	Undifferentiated embryonic cell transcription factor 1	318 (317)	*APOC1*	Apolipoprotein C1	4 (4)
			PPARG2	Peroxisome proliferator activated receptor γ2	22 (80)
REX1	Deoxyribonuclease III (Drn3)	9 (5)			
FOXD3	Forkhead box D3 (FoxD3)	254 (847)	Osteogenic		
Telomerase and associated factors			*BMP1*	Bone morphogenic protein 1	470 (870)
TERT	Telomerase reverse transcriptase	89 (102)	*BMP2*	Bone morphogenic protein 2	663 (655)
TERF1	Telomerase-associated factor 1	3 (3)	*OGN*	Osteoglycin	17 (12)
TERF2	Telomerase-associated factor 2	0.6 (0.7)	*CTSK*	Cathepsin K	6 (8)
Others			*TNFRSF11B*	Osteoprotegerin	65 (82)
POU3F1	POU domain, class 3, TF1 (Oct6)	84 (164)	Neurogenic		
ALP1	Placental alkaline phosphatase 1	927 (1311)	*NTS*	Neurotensin	48 (73)
CD44	CD44 antigen (homing function)	663 (1028)	*NRG1*	Neuregulin 1 isoform-γ	86 (857)
LIF	Leukemia inhibitory factor	271 (221)	*MBP*	Myelin basic protein	14 (21)
SCGF	Stem cell growth factor-β	906 (934)	*MOBP*	Myelin-associated oligodend. basic protein	22 (43)
GCNF	Germ cell nuclear factor	696 (1105)			
SPINK2	Serine protease inhibitor, Kazal type, 2	555 (662)	*NCAM1*	Neural cell adhesion molecule 1	14 (21)
			CD56	Neural cell adhesion molecule CD56	120 (16)
DKK2	Dickkopf (*Xenopus laevis*) homolog 2	476 (1122)	Endothelial		
			VWF	Von Willebrand factor	
			NOS3	Nitric oxide synthase 3 (endothelial cell)	
			Myogenic		
			MYF5	Myogenic factor 5	
			TMP1	Tropomyosin 1α	
			MYH11	Myosin, heavy polypeptide 11	
			Chondrogenic		
			COL4A3	Collagen type IVα3	
			COL5A2	Collagen, type V, α2	
			COL8A1	Collagen, type VIII, α1	
			COL11A1	Collagen, type X1, α1	
			CSPG2	Chondroitin sulfate proteoglycan 2	
			AGC1	Aggrecan 1	
			FN1	Fibronectin 1	
			DSPG3	Dermatan sulfate proteoglycan 3	

[a]Microarray analysis (Affymetrix U133A arrays). EC: NCCIT human EC cells.

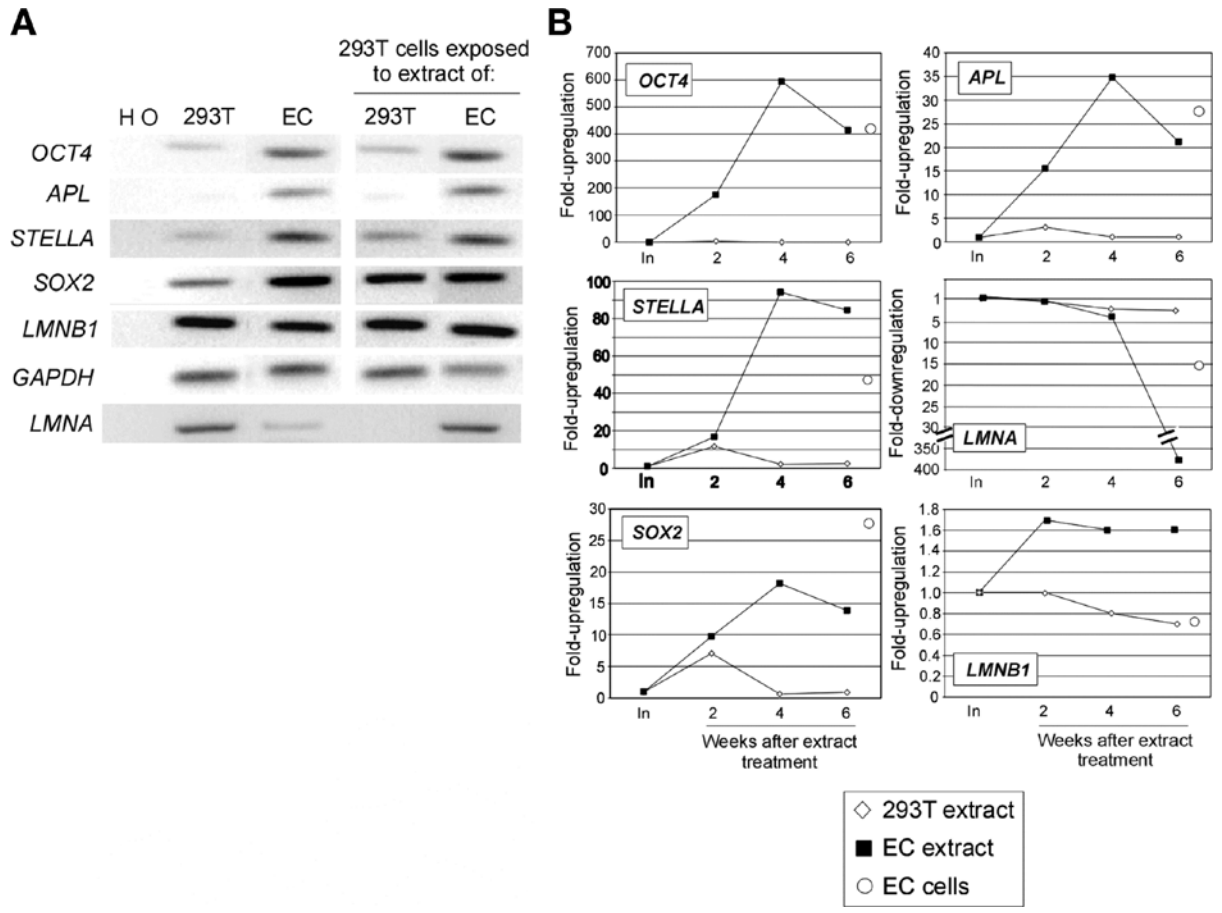

Fig. 4. Expression of ES cell genes in 293T cells exposed to EC extract. **(A)** End point RT-PCR analysis of expression indicated genes in 293T cells exposed to 293T or EC extract and cultured for two weeks. **(B)** Time-course real-time RT-PCR analysis of expression of indicated genes in cells treated as in (A). Expression values in EC (NCCIT) cells relative to 293T cells are also shown ( ). Expression levels are adjusted to those of *GAPDH*.

the *OCT4* promoter *(47)*. In mouse nuclei transplanted into Xenopus oocytes, *Oct4* demethylation is required for *Oct4* transcription *(34)*, thus our observations are consistent with long-term *OCT4* expression in extract-treated cells. A important remaining issue is how early *OCT4* promoter demethylation takes place after extract treatment, particularly in light of the early induction of *Oct4* transcription in 3T3 fibroblasts exposed to mouse ES cell extract *(7)*. The process driving *OCT4* DNA demethylation remains unclear but seems to require deproteinization *(34)*, and may involve cleavage of methyl groups *(48)* or cytosine deamination *(49)*. The ability to induce DNA demethylation in bulk nuclei after extract treatment may make it technically possible to characterize and possibly identify the DNA demethylation activity involved.

In addition to locus-specific modifications, reprogramming of global gene expression in extract-treated cells is likely to involve a genome-wide remodeling of chromatin. Remodeling of mammalian chromatin by *Xenopus* egg extract has been shown to require the ATPase activity of the SWI/SNF chromatin remodeling complex *(50)*. This activity is presumably used to slide nucleosomes along DNA to facilitate transcription of nucleosomal genes. In a similar system, the SWI/SNF ATPase BRG1 was shown to be involved in the transcriptional activation of *OCT4* by *Xenopus* egg extract *(17)*. Controlled manipulations of epigenetic alterations may enhance the heritability of gene expression in reprogrammed cells and may prove beneficial for stable reprogramming of nuclear function.

Challenges Ahead

A number of significant developments are required to provide a full validation of extract-based nuclear reprogramming strategies.

1. The genotype of the reprogrammed cells needs to be characterized to ensure that no genetic perturbations result from extensive manipulations. Because undifferentiated ES tend to develop aneuploidy and EC cells are aneuploid, ploidy of the extract-treated cells should also be assessed particularly if cells are to be cultured for many passages.
2. One needs to assess the long-term stability of the phenotypic and transcriptional changes elicited in the reprogrammed cells. Only results extending to several months of culture have been reported to date, and the (consistent) trend observed whereby transcriptional changes may be perturbed by noise and/or prove to be unstable

(at least for some of them) raises concerns on the long-term maintenance of a new gene-expression profile.

3. To our knowledge no effect has been reported to date on the in vivo function of the reprogrammed cells. Teratoma formation in immunosuppressed mouse hosts, or contribution to all germ layers in chimeric animals are required tests to convincingly demonstrate induction of pluripotency by nuclear reprogramming. Similarly, the in vivo fate of either transdifferentiated cells or reprogrammed pluripotent cells redirected toward a new differentiation pathway should be addressed.
4. More efforts are required to map the epigenetic status of reprogrammed cells. Only a few reports provide evidence for changes in DNA methylation *(3,4)* and histone modifications *(13)* after reprogramming somatic cells by fusion with ES cells; such evidence is only emerging for extract-treated cells. Ongoing work in our laboratory aims at unraveling epigenetic marks characterizing EC or ES extract-treated cells. Investigating these issues is anticipated to identify new variables susceptible to affect the efficiency of extract-based nuclear reprogramming.

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References

1. Cibelli JB, Stice SL, Golueke PJ, et al. Nat Biotechnol 1998;16:642–646.
2. Gurdon JB, Byrne JA. Proc Natl Acad Sci USA 2003;100:8048–8052.
3. Tada M, Tada T, Lefebvre L, Barton SC, Surani MA. EMBO J 1997;16:6510–6520.
4. Cowan CA, Atienza J, Melton DA, Eggan K. Science 2005; 309:1369–1373.
5. Tada M, Takahama Y, Abe K, Nakatsuji N, Tada T. Curr Biol 2001;11:1553–1558.
6. Collas P, Håkelien AM. Trends Biotechnol 2003;21:354–361.
7. Taranger CK, Noer A, Sørensen AL, Håkelien AM, Boquest AC, Collas P. Mol Biol Cell 2005;16:5719–5735.
8. Qin M, Tai G, Collas P, Polak JM, Bishop AE. Stem Cells 2005; 23:712–718.
9. Ying QL, Nichols J, Evans EP, Smith AG. Nature 2002;416: 545–548.
10. Pells S, Di Domenico AI, Callagher EJ, McWhir J. Cloning Stem Cells 2002;4:331–338.
11. Terada N, Hamazaki T, Oka M, et al. Nature 2002;416:542–545.
12. Flasza M, Shering AF, Smith K, Andrews PW, Talley P, Johnson PA. Cloning Stem Cells 2003;5:339–354.
13. Kimura H, Tada M, Nakatsuji N, Tada T. Mol Cell Biol 2004;24: 5710–5720.
14. Håkelien AM, Landsverk HB, Robl JM, Skålhegg BS, Collas P. Nat Biotechnol 2002;20:460–466.
15. Gaustad KG, Boquest AC, Anderson BE, Gerdes AM, Collas P. Biochem Biophys Res Commun 2004;314:420–427.
16. McGann CJ, Odelberg SJ, Keating MT. Proc Natl Acad Sci USA 2001;98:13,699–13,704.
17. Hansis C, Barreto G, Maltry N, Niehrs C. Curr Biol 2004;14: 1475–1480.
18. Collas P, Pinto-Correia C, Poccia DL. Exp Cell Res 1995;219: 687–698.
19. Collas P, Poccia DL. J Cell Sci 1996;109:1275–1283.
20. Lohka MJ, Masui Y. Science 1983;220:719–721.
21. Lohka MJ, Masui Y. J Cell Biol 1984;98:1222–1230.
22. Ulitzur N, Gruenbaum Y. FEBS Lett 1989;259:113–116.
23. Wilson KL, Newport JW. J Cell Biol 1988;107:57–68.
24. Collas P, Le Guellec K, Taskén K. J Cell Biol 1999;147:1167–1180.
25. Eide T, Carlson C, Taskén KA, Hirano T, Taskén K, Collas P. EMBO Rep 2002;3:426–432.
26. Landsverk HB, Kirkhus M, Bollen M, Kuntziger T, Collas P. Biochem J 2005;390:709–717.
27. Martins SB, Eide T, Steen RL, Jahnsen T, Skålhegg BS, Collas P. J Cell Sci 2000;113:3703–3713.
28. Steen RL, Martins SB, Taskén K, Collas P. J Cell Biol 2000;150:1251–1262.
29. Håkelien AM, Gaustad KG, Collas P. Meth Cell Biol 2005; 325:99–114.
30. Håkelien AM, Gaustad KG, Taranger CK, Skålhegg BS, Kuntziger T, Collas P. Exp Cell Res 2005;309:32–47.
31. Håkelien AM, Gaustad KG, Collas P. Biochem Biophys Res Commun 2004;316:834–841.
32. Wilmut I, Beaujean N, De Sousa PA, et al. Nature 2002; 419:583–586.
33. Byrne JA, Simonsson S, Western PS, Gurdon JB. Curr Biol 2003;13:1206–1213.
34. Simonsson S, Gurdon J. Nat Cell Biol 2004;6:984–990.
35. Liu Y, Shin S, Zeng X, et al. BMC Dev Biol 2006;6:20.
36. Sperger JM, Chen X, Draper JS, et al. Proc Natl Acad Sci USA 2003;100:13,350–13,355.
37. Blelloch RH, Hochedlinger K, Yamada Y, et al. Proc Natl Acad Sci USA 2004;101:13,985–13,990.
38. Miklos GL, Maleszka R. Nat Biotechnol 2004;22:615–621.
39. Mathieu O, Bender J. J Cell Sci 2004;117:4881–4888.
40. Matzke MA, Birchler JA. Nat Rev Genet 2005;6:24–35.
41. Nishimoto M, Fukushima A, Okuda A, Muramatsu M. Mol Cell Biol 1999;19:5453–5465.
42. Boquest AC, Shahdadfar A, Fronsdal K, et al. Mol Biol Cell 2005;16:1131–1141.
43. Akashi K, He X, Chen J, et al. Blood 2003;101:383–389.
44. Woodbury D, Reynolds K, Black IB. J Neurosci Res 2002;69: 908–917.
45. Grunau C, Clark SJ, Rosenthal A. Nucleic Acids Res 2001;29:E65.
46. Warnecke PM, Stirzaker C, Song J, Grunau C, Melki JR, Clark SJ. Methods 2002;27:101–107.
47. Nordhoff V, Hübner K, Bauer A, Orlova I, Malapetsa A, Scholer HR. Mamm Genome 2001;12:309–317.
48. Ramchandani S, Bhattacharya SK, Cervoni N, Szyf M. Proc Natl Acad Sci USA 1999;96:6107–6112.
49. Morgan HD, Dean W, Coker HA, Reik W, Petersen-Mahrt SK. J Biol Chem 2004;279:52,353–52,360.
50. Kikyo N, Wade PA, Guschin D, Ge H, Wolffe AP. Science 2000;289:2360–2362.