Reprogramming somatic nuclei to a pluripotent state with cell-free extracts

Doctoral thesis

by

Christel Taranger Freberg

Institute of Basic Medical Sciences
Department of Biochemistry
Faculty of Medicine
University of Oslo

February 2010

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 962

ISBN 978-82-8072-517-2

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS.

Produced in co-operation with Unipub.
The thesis is produced by Unipub merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

This work was performed at the Department of Biochemistry, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, and was supported by a grant from The Research Council of Norway.

First of all, I am indebted to Professor Philippe Collas for offering me a PhD student position. I would like to thank you for outstanding supervision, brilliant ideas, supportive and enthusiastic talks, for always keeping the door open to your office, and for the amount of work you have put in to help me achieve this. You make difficult challenges easy.

I would like to thank all my friends and colleagues in the lab. The biggest hug goes to Marie. If not for you I would never be where I am now. We met at NLH and worked together in the lab there. Thank you for your energy, laughter and all the lunches we have shared. I miss you big time now that you're not in the office anymore. Thank you, Anne-Mari and Kristine, for leading me into the world of extracts and reprogramming. I truly appreciate your patience when teaching a master student good practical science! Thank you, Anne-Mari, Sanna, Anita, John Arne, Agate, and Andrew for your contribution to the work in this thesis. Thank you, Thomas, for sharing your outstanding knowledge in cloning, computers and wine-tasting. I have also had the pleasure of working together with Emma and Steven in Sheffield. Thank you for the good times in Philadelphia and Oslo. I truly enjoy your company. Thank you, Marie, Sanna and Bente Marie for being morning-birds together with me in the lab. Nothing is like starting the lab-work before the sun rises. ☺. Finally, I would like to thank all you guys in the group for making Philippe Collas' group the best place to work. A special thanks to Marie, Sanna, John Arne and Lidija for making our office a great place to share both laughter and tears. Special thanks go to Sanna for being you and for having the most contagious laughter. I will miss you when you leave for Australia.

My home being 119 km from the lab has led to many hours of driving in my car. Since the start of my PhD I have spent over 2 months in my car just to get to work and back. Some might raise their eyebrows and think it must have been a waste of time. However, it really gives you time to think. And that is valuable. And what always comes back to me is the thought of how lucky I am. I have a wonderful life, an exciting job, terrific friends, the world's best parents and sister, caring family-in-law and the most wonderful family to come home to.

I would like to direct a big hug to "NV-jentene". The times we share are fantastic and I really value our friendship. And to all my friends from Kristiansand- you are the best. We have so much fun when we meet and it is good to share memories of the glorious old days. ☺ Thank you to all my friends from NLH/UMB.

Thank you Mamma and Pappa for giving me the best home I could have, for believing in me, for always being interested in my work and my life. Thank you for always pushing me a bit further and for expecting a bit more every time, challenging me to work hard and to do my best. Thank you, Benedicte, not only for being my sister, but also for being my good friend. Thank you, Ragnhild and Nils Edvard, for being wonderful parents-in-law. Thank you for taking good care of Magnus and Jakob and for always being helpful. Thank you, Anstein and Edvart, for being the best brothers-in-law. Your sense of humor is worth a million!

Last but not least I would like to thank my husband, best friend and every-day stand-up comedian Bernt for your love and never-ending support. Words cannot describe what you mean to me. Now, because the two of us fell in love, we have two wonderful boys at home giving us a completely different dimension of life. Magnus and Jakob- thank you for being the best results of these years! I love you.

“Aerodynamically, the bumble bee shouldn't be able to fly.

But the bumble bee doesn't know it, so it goes on flying anyway.”

Mary Kay Ash

LIST OF PUBLICATIONS

I. Taranger, C.K., A.Noer, A.L.Sørensen, A.M.Håkelien, A.C.Boquest, and P.Collas. 2005. Induction of dedifferentiation, genome-wide transcriptional programming, and epigenetic reprogramming by extracts of carcinoma and embryonic stem cells. Mol. Biol. Cell. 16, 5719-5735

II. Freberg, C.T., Dahl, J.A., Timoskainen, S., and Collas, P. 2007. Epigenetic reprogramming of *OCT4* and *NANOG* by embryonal carcinoma cell extract. Mol. Biol. Cell. 18, 1543-1553.

III. Pewsey, E., Bruce, C., Georgiou, A.S., Jones, M., Baker, D., Ow, S.Y., Wright, P.C., Freberg, C.T., Collas, P. and Fazeli, A. Proteomic analysis of epithelial cells reprogramming in cell-free extract. Mol. Cell Proteomics. 8, 1401-1412.

LIST OF ABBREVIATIONS

2D PAGE	two dimensional polyacrylamide gel electrophoresis
5aza	5'-azacytidine
ac	acetylation
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
bp	base pair
cAMP	cyclic adenosine mono phosphate
ChIP	chromatin immunoprecipitation
CpG	cytosine-phosphate-guanine
DNA	deoxyribo nucleic acid
DNase	deoxyribonuclease
DNMT	DNA methyltransferase
DRB	5,6-dichlorobenzimidazole
EC cell	embryonal carcinoma cell
EG cell	embryonic germ cell
ES cell	embryonic stem cell
Ezh2	enhancer of zeste homologue 2
G2	gap 2
EGFP	enhanced green fluorescent protein
GSK3	glycogen synthase kinase 3
GV	germinal vescicle
H	histone

HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HCP	high CpG content promoter
HMG	high mobility group
HMT	histone methyltransferase
ICM	inner cell mass
ICP	intermediate CpG content promoter
Igf2	insulin-like growth-factor II
iMS cell	induced multipotent stem cell
iPS cell	induced pluripotent stem cell
K	lysine
LCP	low CpG content promoter
M	mitosis
mII	metaphase II
me	methylated
MEK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
miRNA	microRNA
MRL	Murphy Roths Large
MSC	mesenchymal stromal cell
PcG	polycomb group
PCR	polymerase chain reaction
PE	proximal enhancer
PG cell	primordial germ cell

Pol II	polymerase II
PP	proximal promoter
POU	Pit-Oct-Unc
PRC	polycomb repressive complex
q	quantitative
RNA	ribo nucleic acid
RNase	ribonuclease
RT	reverse transcriptase
SAHA	suberoylanilide hydroxamic acid
SAM	s-adenosylmethionine
SCNT	somatic cell nuclear transfer
SLO	Streptolysin O
SSEA	stage-specific embryonal antigen
SV40	Simian virus 40
TE	trophectoderm
TGF-	transforming growth factor beta
TRA	tumor rejection antigen
Trx	trithorax group
TSA	trichostatin A
TSS	transcription start site
VPA	valproic acid

INTRODUCTION

1. Stem cells and pluripotency

1.1. Differentiation is the process of cell specialization

Differentiation is a developmental process by which cells become more and more restricted to one particular fate. After fertilization and up to the two- to eight cell stage (depending on the species), the mammalian embryo contains cells that can in principle give rise to any cell type of the organism. These cells are said to be totipotent. The first visible sign of differentiation during development correlates with the formation of the blastocyst. The blastocyst consists of an inner cell mass (ICM), giving rise to the embryo proper, and the trophectoderm (TE), giving rise to extraembryonic tissues associated with formation of the placenta.

Massive reorganization of the embryo takes place during gastrulation when the three primary germ layers (the endoderm, mesoderm and ectoderm) are formed and organized in their proper locations. From the endodermal layer arise linings of lungs, tongue, tonsils, urethra and associated glands, bladder and digestive tract. The mesodermal layer forms muscles, bones, lymphatic tissue, spleen, blood cells, heart, lungs and reproductive and secretory systems. Skin, nails, hair, eye lens, lining of the internal and external ear, nose, sinuses, mouth, anus, tooth enamel, pituitary gland, mammary glands and all parts of the nervous system develop from the ectodermal layer. Organogenesis and tissue development occur through a series of successive differentiation events starting from stem cells, or precursor cells **(Fig. 1)**. These divide to give rise to more of themselves (self-renewal) and can differentiate into more committed progenitor cells which in turn can give rise to more specialized cell types **(Fig. 1)**. Thus as differentiation proceeds, cells become functionally distinct from each other.

A key feature of differentiation is dynamic changes in gene expression, which result in synthesis of proteins that characterize the different cell types. In particular, genes encoding markers of pluripotency are repressed while genes encoding lineage-specific markers are turned on.

Tuning of gene expression is regulated by changes in chromatin organization in specific regions of the genome. These so-called epigenetic (*epi-* meaning besides in Greek) modifications are heritable and do not affect genome sequence.

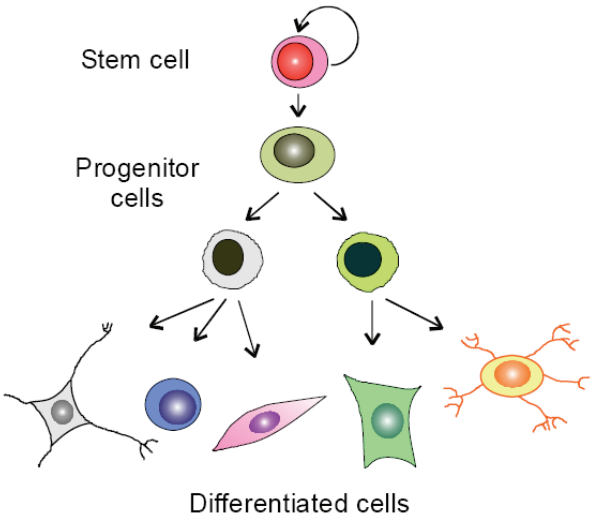


Fig. 1. A simplified view of cellular differentiation

1.2. Stem cells and their differentiation potential

Stem cells can be derived from early embryos or can be found in most tissues. To be qualified as stem cells, cells must have the ability to at least (i) self-renew by symmetric division, where daughter cells retain the characteristics of the parent, and (ii) differentiate by dividing asymmetrically to give rise to at least one more committed cell type. Stem cells may be classified according to their differentiation potential. Pluripotent stem cells (such as embryonic stem (ES) cells, embryonic germ (EG) cells and embryonal carcinoma (EC) cells) can differentiate into any cell type of the body except extra-embryonic tissues **(Fig. 2)**. Multipotent stem cells have been isolated from many tissue types and can differentiate into, primarily but not exclusively, cells of their own developmental lineage. Unipotent stem cells, in contrast, can differentiate into one cell type.

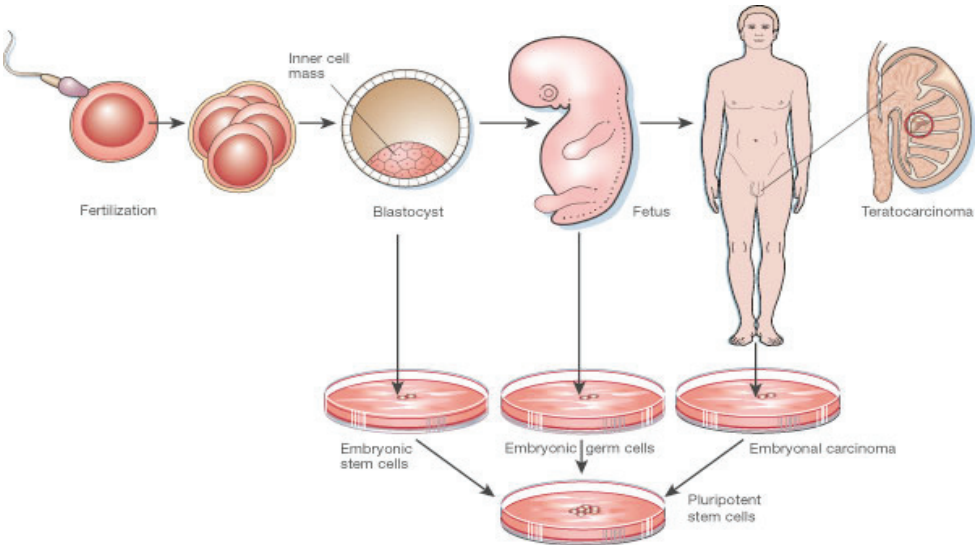


Fig. 2. Origin of embryonic stem cells, embryonic germ cells and embryonal carcinoma cells. ES cells are derived from the ICM of blastocysts; EG cells are derived from primordial germ (PG) cells isolated from the embryonic gonad; EC cells are derived from PG cells in the embryonic gonad but usually are detected as components of testicular tumors in the adult. Taken from (Donovan and Gearhart, 2001).

1.2.1. Embryonic stem cells

ES cells have been derived by culturing ICMs of mouse, human, rat and monkey blastocysts under specific conditions (Buehr et al., 2008; Evans and Kaufman, 1981; Thomson et al., 1995; Thomson et al., 1998) ES cells have the potential to proliferate indefinitely in culture and give rise to cells of all three germ layers upon differention *in vitro* or in teratomas after injection in nude mice (Reubinoff et al., 2000; Thomson et al., 1998). At the morphology level, ES cells are characterized by growth in colonies with well-defined edges, although signs of differentiation on the edges of the colonies are frequent. Interestingly, colonies of human ES cells are larger and not as compact as mouse ES cell colonies **(Fig. 3)**. Unlike differentiated cells, ES cells have a high nucleus/cytoplasm ratio and the nucleus contains a large nucleolus.

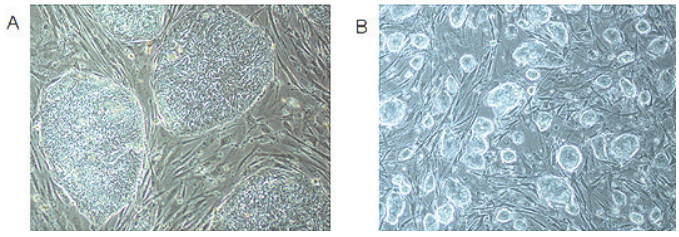


Fig 3. Brightfield images of (A) hES cell colonies and (B) mES cell colonies on inactivated mouse embryonic fibroblast (MEF) feeder layers. Both pictures are taken with a 10x objective. Taken from www.invitrogen.com

ES cells express surface markers including stage-specific embryonal antigens SSEA-3 and SSEA-4 (human) or SSEA-1 (mouse) (Boiani and Scholer, 2005). In addition, although molecular identities of tumor rejection antigens TRA-1-60 and TRA-1-81 are unknown, they are commonly used as markers of human ES cells (Schopperle and DeWolf, 2007). Expression of TRA-2-54 and Thy1 antigen also characterize human pluripotent cells and are downregulated during differentiation; however, Thy1 is also expressed on the surface of multipotent mesenchymal stromal cells (MSCs). ES cells also express the tissue-nonspecific isoform of alkaline phosphatase isozyme TRA-2-49 and Nanog (Takahashi et al., 2007).

Additionally, ES cells express transcription factors responsible for maintaining the undifferentiated state, among which Nanog, Oct4 and Sox2 have been proposed to constitute the core of an expanding transcriptional network controlling pluripotency (Boyer et al., 2005; Do and Scholer, 2009; Jaenisch and Young, 2008). Beside autoregulatory and feed-forward loops, Oct4, Nanog and Sox2 also target and regulate many downstream genes (Jaenisch and Young, 2008) **(Fig. 4)**. Oct4 (also called Octamer-4, Oct3, Oct3/4 or Pou5F1) belongs to the Octamer class of the Pit-Oct-Unc (POU) protein family (Scholer et al., 1990). Oct4 binds DNA through two DNA binding POU-domains that recognize an 8-bp DNA sequence with the consensus ATGCAAAT (Chambers and Tomlinson, 2009). Oct4 controls pluripotency in a dose-dependent manner (Niwa et al., 2000a). A 150% increase in *OCT4* gene expression turns pluripotent cells into primitive endoderm or mesoderm, while repression of *OCT4* induces formation of TE (Niwa et al., 2000a). Thus, pluripotent cells possess a network of regulators to keep *OCT4* expression at optimal level to ensure pluripotency.

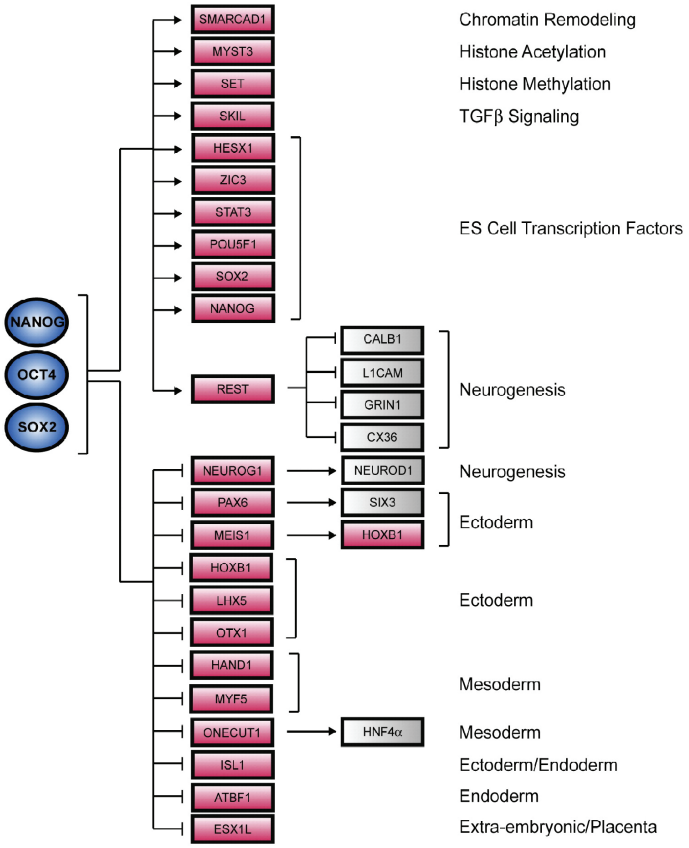


Fig. 4. A core transcriptional regulatory network maintaining pluripotency in human ES cells. Oct4, Nanog and Sox2 target genes that encode chromatin regulators or transcription factors. Some of the key genes co-occupied by the three altogether (blue) are shown. Bound promoters are in red and putative downstream targets are shown in grey. Taken from {Boyer, 2005 BOYER2005 /id].

Oct4 can form a heterodimer with Sox2, so that both proteins can bind DNA together {Rodda, 2005 RODDA2005 /id}. Sex determining region Y (SRY)-box 2, or Sox2, is a high mobility group (HMG) family member with a single HMG DNA-binding domain. This domain binds to the consensus sequence A/T A/T CAAAG in the minor groove of the DNA helix. Sox2 is required for epiblast and extraembryonic ectoderm formation, suggesting cooperativity with Oct4 to control the fate of pluripotency at implantation (Avilion et al., 2003). Sox2 is, in contrast to Oct4, also expressed in multipotent and unipotent stem cells and can be replaced by other members of the Sox-family (reviewed in (Welstead et al., 2008)) in the induction of pluripotent stem cells, although with reduced efficiency.

Oct4 frequently partners with Nanog in repressor complexes that control ES cell fate. Nanog was discovered based on its ability to sustain mouse ES cell renewal in the absence of leukemia inhibitory factor (Chambers et al., 2003; Niwa et al., 2000b). Nanog acts as a strong activator of the *OCT4* promoter, thus participating in the regulation of *OCT4* expression in pluripotent cells (Chambers et al., 2003; Niwa et al., 2000b).

The pluripotency of ES cells makes them a valuable tool for investigating differentiation. ES cells also constitute potentially material for testing novel therapies, drug screening and functional genomics applications. Because destruction of embryos is required for their derivation, there are however ethical concerns with derivation and use of human ES cells. Explantation of human blastocysts causes ethical difficulties in many countries and was prohibited in Norway until January, 2008. Moreover, there is to date no evidence to indicate that differentiated hES cells cannot revert to an undifferentiated, potentially tumorigenic state. Similarly, if cells are not fully differentiated, they may cause tumors. In this context, approaches to create pluripotent cells from sources other than embryos have been actively sought.

1.2.2 Primordial germ cells and embryonal carcinoma cells

Primordial germ (PG) cells are progenitors of the germ cell lineage. PG cells are not pluripotent but they are a source of pluripotent stem cells (Kerr et al., 2006). Under specific conditions, PG cells isolated from the gonadal ridge can convert into pluripotent EG cells (Shamblott et al., 1998) which contribute to all lineages in chimeric fetuses, including the germline.

Testicular cancer can arise from PG cells that have escaped normal differentiation. These tumors contain multiple differentiated tissues from all three germ layers, in addition to undifferentiated cells called embryonal carcinoma (EC) cells. EC cells are derived from teratocarcinomas and are considered to be the malignant counterparts of ES cells (Przyborski et al., 2004). Similarly to ES cells, EC cells are pluripotent and can form all three germ layers

(Przyborski et al., 2004). The gene expression profile of EC cells is similar to, but different from, that of ES cells. Both cell types express embryonic genes such as *OCT4*, *SOX2*, *FOXD3*, *NANOG*, *FZD7*, *DNMT3B* and *TERF1* (Sperger et al., 2003). These similarities may reflect a necessity to maintain a pluripotent phenotype, whereas differences may reflect adaption of EC cells to tumor growth. Given the issues associated with use of human embryonic material, EC cells constitute an alternative (and simpler) system to investigate differentiation.

Studies presented in this thesis rely on the use of the human EC cell line, NCCIT, as the basis for turning differentiated cells into ES-like cells. NCCIT cells have been characterized biochemically, immunochemically, functionally and at the gene expression level (Damjanov, 1993; Sperger et al., 2003). The core stemness gene *NANOG* is abnormally overexpressed in NCCIT cells and down-regulation of *NANOG* causes down-regulation of *OCT4*, *SOX2*, *REX1* and *FOXD3* expression, G2-M phase arrest, inhibition of colony formation and induction of differentiation into all three germ layers (You et al., 2009). NCCIT cells also display epigenetic differences with ES cells, notably at the level of DNA methylation and histone H3 and H4 modifications, at least in the *NANOG* gene locus (You et al., 2009). Epigenetic states of pluripotent cells are described in Section 2.

1.3. Cellular dedifferentiation

The terminally differentiated state of a cell has long thought to be stable. Several lines of evidence indicate however that fate of terminally differentiated cells is not necessarily locked, and that de-differentiation events can occur. Dedifferentiation refers to the progression of a differentiated cell to a less differentiated state. It is charachterized by a loss of developmental or differentiation markers and a gain of proliferative capacity (Sakai and Takeuchi, 1971).

Some lower vertebrates have the ability to regenerate organs through a dedifferentiation process. Most commonly, wound healing after injury involves migration of epithelial cells to the

wounded site and formation of scar tissue (Heber-Katz, 1999). For more serious injury, such as damaged or lost tissue, regeneration maintains or restores the original architecture by recapitulating part of its original embryonic development. Urodele amphibians can replace lost anatomical parts by a process of migration, dedifferentiation, proliferation and redifferentiation of epithelial cells in the wounded area (Morgan, 1901). They also have the ability to regenerate complex structures such as limbs, tails and jaws (Brockes and Kumar, 2002; Stocum, 2004).

The ability to regenerate is thought to be a lost property in mammals, although sporadic examples of mammalian regeneration exist. These include the regrowth of fingertips, antlers and examples of complete ear hole closure in specific rabbit and mouse strains (Clark et al., 1998; Heber-Katz et al., 2004a). For example, MRL mice not only repair ear holes, but can also spontaneously repair heart tissue after a normally irreversible infarction (Heber-Katz et al., 2004b; Leferovich and Heber-Katz, 2002). It is therefore reasonable to hypothesize that there is potential for restoring pluripotency in at least certain mammalian somatic cell types. Together with the motivation of producing ES-like cells without using embryos, the dedifferentiation ability of some cell types has triggered studies aiming at reprogramming cells to a pluripotent state. Approaches to reach this goal are addressed in Section 3.

2. Epigenetic states in embryonic stem cells

The extent of differentiation ability of stem cells is associated with the expression potential of developmentally- and differentiation-regulated genes. Such potential is regulated by epigenetic processes on DNA and chromatin. Epigenetic mechanisms refer to heritable modifications of DNA and chromatin that do not affect DNA sequence. Genome-wide mapping of DNA methylation and post-translational histone modifications, two primary epigenetic determinants, in stem- and differentiated cells have provided chromatin “maps” unveiling regulatory mechanisms by which genes are poised for transcription in undifferentiated cells. Potential for gene

expression is thus believed to be controlled by epigenetic processes that confer a specific chromatin configuration on regulatory, coding and intergenic regions. In addition to epigenetic modifications, positioning of transcriptional activators or repressors, ATP-dependent chromatin remodeling enzymes and small interfering RNAs on target genes also regulate expression.

2.1. DNA methylation and gene expression

DNA methylation consists of the addition of a methyl group to the 5 position of a cytosine in a cytosine-phosphate-guanine (CpG) dinucleotide. CpG methylation is symmetrical and targets isolated CpGs, clustered CpGs, or even CpGs within a CpG island (see (Gardiner-Garden and Frommer, 1987) and (Takai and Jones, 2002) for definitions of a CpG island) **(Fig. 5)**.

Fig. 5. CpG methylation. (A) Mechanism of DNA methylation. (B) CpG methylation is symmetrical and occurs on both DNA strands. (C) Simplified textbook view of the relationship between DNA methylation and gene expression. This relationship is clearly more complex (see main text). Taken from (Collas et al., 2007)

CpG methylation is catalyzed by DNA methyltransferase (DNMTs). Maintenance DNA methyltransferase DNMT1 recognizes hemi-methylated DNA and methylates the daughter strand, ensuring fidelity of methylation profile after replication (Jaenisch and Bird, 2003). In contrast to DNMT1, DNMT3a and DNMT3b are implicated in *de novo* DNA methylation that

takes place during development (Turek-Plewa and Jagodzinski, 2005), as a means of shutting down genes whose activity is no longer required. DNMT2 has no ascribed function in DNA methylation (Liu et al., 2003) but has transfer RNA methyltransferase activity (Goll et al., 2006).

DNA methylation is as a hallmark of long-term gene silencing. Methyl groups create target sites for methyl-binding proteins which induce transcriptional repression by recruiting transcriptional co-repressor complexes including histone deacetylases (HDACs) (Klose and Bird, 2006). DNA methylation is essential for development (Morgan et al., 2005; Razin and Shemer, 1995), X chromosome inactivation (Hellman and Chess, 2007), genomic imprinting (Reik et al., 1987; Sapienza et al., 1987), and repression of transposable elements (Yoder et al., 1997).

Genome-wide DNA methylation profiling has shown that the relationship between promoter DNA methylation and promoter activity depends on CpG content (Weber et al., 2007) **(Table 1)**. Notably, low CpG content promoters (LCPs) show no correlation between promoter activity and methylation, and most LCPs are methylated regardless of their activity. On the contrary, activity of intermediate CpG promoters (ICPs) is inversely correlated with methylation, arguing that ICP methylation is incompatible with transcription. High CpG promoters (HCPs) in contrast display no or weak methylation even when inactive **(Table 1)**.

Table 1. Relationship between promoter DNA methylation and activity depends on CpG content.

Promoter class[a]	Promoter activity	Methylation status
HCP	Active	Unmethylated
	Inactive	No or weakly methylated
ICP	Active	Unmethylated
	Inactive	Methylated
LCP	Active	Unmethylated or methylated
	Inactive	Unmethylated or methylated

[a]Promoter class is defined by promoter CpG content (Weber et al., 2007). HCP, high CpG content promoter; ICP; intermediate CpG content promoter; LCP; low CpG content promoter.

2.2. Post-translational histone modifications

The amino-terminal tails of core histones are post-translationally modified to confer physical properties that affect interaction with DNA on regulatory sequences **(Fig. 6)**. Histone modifications influence chromatin packaging and are read by chromatin modifying enzymes and transcription factors, and thus contribute to the regulation of transcription (Jenuwein and Allis, 2001).

Epigenetic histone modifications have been best characterized for histones H3 and H4 and include combinatorial phosphorylation, ubiquitination, sumoylation, acetylation, methylation, proline isomerization, ADP-ribosylation and deamination (Kouzarides, 2007). Notably, di- and trimethylation of H3 lysine 9 (H3K9me2/me3) and trimethylation of H3K27 (H3K27me3) elicit the formation of repressive heterochromatin through the recruitment of heterochromatin protein 1 (Lachner et al., 2001) and polycomb group (PcG) proteins, respectively (Cao et al., 2002). However, whereas H3K9me3 marks constitutive heterochromatin (Lachner and Jenuwein, 2002), H3K27me3 characterizes facultative heterochromatin, or chromatin domains harboring transcriptionally repressed genes that can potentially be activated (Azuara et al., 2006; Bernstein et al., 2006).

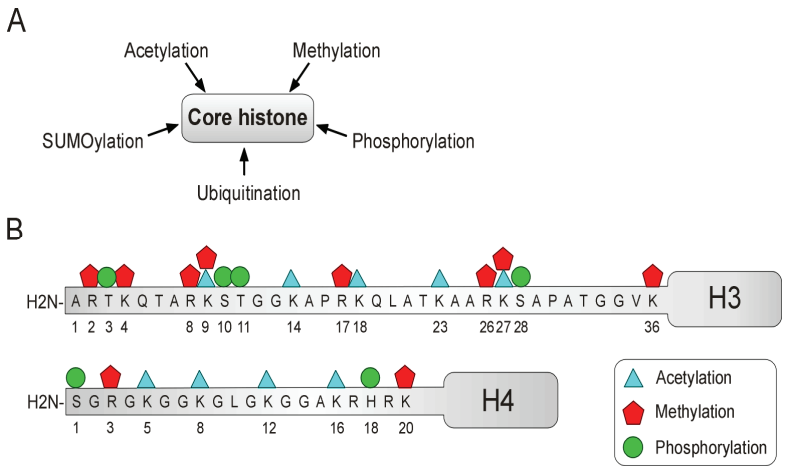


Fig. 6. Post-translational histone modifications. (A) Core histones can be methylated, acetylated, phosphorylated, ubiquitinated or SUMOylated, to modulate gene expression. (B) Known modifications on the amino-terminal tails of core histones H3 and H4. Taken from (Collas et al., 2007)

In contrast, histone acetylation loosens interaction with DNA and creates a conformation suitable for targeting transcriptional activators. Thus, acetylation on H3K9 (H3K9ac) and H4K16 (H4K16ac) (together with H3K4me2/me3) is often found in association with active genes (Bernstein et al., 2006; Schubeler et al., 2004; Struhl, 1998; Zhao et al., 2007). H3K4me3 and H3K9ac mediate recruitment of transcriptional activators (Kingston and Narlikar, 1999; Pray-Grant et al., 2005). Taken together, methylation and acetylation of specific lysine residues on amino-terminal histone tails are fundamental for the formation of euchromatin and heterochromatin. The large number of combinatorial histone modifications mediated by acetylation and methylation (even only considering these two modifications) illustrate the enormous regulatory potential of post-translational histone modifications.

2.3. DNA methylation patterns in ES cells

The DNA methylation signature of ES cells is distinct from that of differentiated cells; however whether this reflects differences in gene expression or the pluripotent nature of ES cells is unclear. Mouse ES cells are less methylated than differentiated cells (Jackson et al., 2004; Zvetkova et al., 2005), and this hypomethylation affects repetitive and unique sequences including differentially methylated regions which regulate expression of paternally imprinted loci (Zvetkova et al., 2005). Methylation profiles of ES cells are also distinct from those of cancer cell lines and somatic stem cells, reflecting an epigenetic distance between ES cells and other cell types (Bibikova et al., 2006). Of note, genes differentially methylated in human ES cells relative to somatic cells include *OCT4* and *NANOG*, which are unmethylated in hES cells. Thus the methylation pattern of a small number of developmentally-controlled genes may constitute an epigenetic mark of ES cells.

A recent genome-wide DNA methylation profiling across promoters in mouse ES cells annotated >5,000 unmethylated genes and >6,100 genes methylated in the promoter regions

examined (Fouse et al., 2008). Methylation was found to occur primarily in ICPs and LCPs or in non-CpG island regions of HCPs (**Fig. 7A)**. Methylated genes included late-differentiation and signal transduction genes (not expressed in ES cells) **(Fig. 7B)**. In contrast, unmethylated genes were associated with transcription, RNA and protein metabolic processes, cell survival and proliferation. Thus unmethylated promoters show good correlation with genes active in ES cells. In addition though, 10-15% of unmethylated genes are involved in developmentally-regulated and differentiation processes and not expressed in ES cells (Fouse et al., 2008).

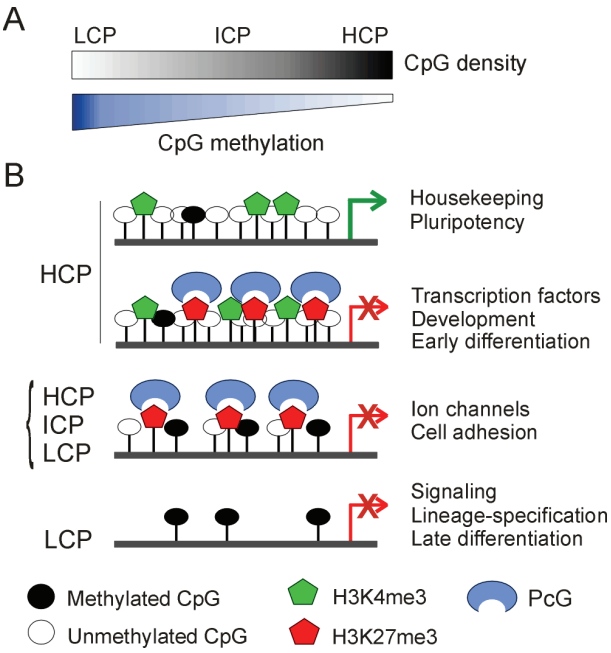


Fig. 7. Epigenetic states and transcriptional regulation in mouse embryonic stem cells. (A) Methylation state (blue gradient) relative to CpG density (black bar). (B) DNA methylation and state of H3K4me3/H3K27me3 "bivalency" in undifferentiated mouse ES cells. PcG refers to the polycomb group repressor complex (PRC) 2, which methylates H3K27 through activity of the histone methyltransferase Ezh2. Taken from (Collas, 2009)

2.4. Chromatin states in ES cells

2.4.1. Post-translational histone modifications

Recent mapping of histone modifications has shown that lineage-specific genes, which are either silent or active in differentiated somatic cells, are in a potentially active state in pluripotent ES cells. Genome-wide and locus-specific chromatin immunoprecipitation (ChIP) analyses reveal that repressed but potentially active promoters in mouse ES cells are associated with "bivalent"

histone modifications characterized by H3K4me3, a mark of active genes, and H3K27me3, a mark of inactive genes (Azuara et al., 2006; Bernstein et al., 2006) **(Fig. 7B)**. These bivalent domains consist of large regions of H3K27me3 embedding smaller areas of H3K4me3 (Bernstein et al., 2006; Mikkelsen et al., 2007; Zhao et al., 2007). These domains include transcription factor-encoding genes and early differentiation genes that are not expressed or expressed at low levels **(Fig. 7B)**. The role of these genes in lineage determination suggests that they are in a poised state and await transcriptionally inductive cues.

DNA methylation and histone modification profiles in mouse ES cells reveal four classes of genes whose promoters are enriched in either H3K4me3 and H3K27me3, H3K4me3 alone, H3K27me3 alone, or neither of these modifications (Fouse et al., 2008) **(Fig. 7B)**. Most promoters lacking both H3K4m3 and H3K27m3 are often methylated, suggesting exclusive H3K27 methylation and DNA methylation mechanisms of transcriptional repression. Indeed, about half of promoters with the H3K4me3/H3K27me3 marks are hypomethylated, arguing that transcriptional repression on these promoters is imposed by PcG proteins (see below). The remainder of these promoters appears to be hypermethylated, suggesting that DNA methylation in these instances may constitute an additional program of long-term transcriptional repression in undifferentiated ES cells.

2.4.2. Polycomb-group proteins

Polycomb group proteins (PcGs) are transcriptional repressors (Kennison, 1995; Ringrose and Paro, 2007) found in two distinct and conserved polycomb repressor complexes (PRC1 and PRC2) working cooperatively (Otte and Kwaks, 2003). Involvement of PRCs in pluripotency has been suggested by the requirement of PcG proteins for patterning of gene expression during development, and for establishing pluripotent ES cell cultures (Boyer et al., 2006a).

In ES cells, PcGs preferentially occupy genes that are activated upon differentiation, consistent with the view that these genes are poised for transcription (Boyer et al., 2006b; Bracken et al., 2006; Lee et al., 2006). Histone methyltransferase activity of enhancer of zeste homologue 2 (Ezh2; a PRC2 component) is responsible for trimethylation of H3K27 on these target genes (Cao et al., 2002; Cao and Zhang, 2004) **(Fig. 7B)**. Trimethylation of H3K4 is mediated by Trithorax group (Trx) proteins (Ringrose and Paro, 2007). Thus, the interplay between PcG and Trx proteins is likely to establish bivalent histone modifications in pluripotent cells. For genes activated upon differentiation, PcGs are displaced from promoters (Bracken et al., 2006). Furthermore, genes that are repressed during differentiation have also been found to be occupied by PcGs in undifferentiated cells. These findings suggest that PRCs constitute a programmed memory system established during embryogenesis (Bracken et al., 2006). This program would mark certain genes for transcriptional repression upon differentiation, while other genes would be primed for activation. These observations suggest that combinations of CpG methylation, histone modifications, PcG occupancy, as well as nucleosome positioning (Ozsolak et al., 2007; Pusarla et al., 2007) on developmentally-regulated promoters, in the context of hyperdynamic chromatin (Meshorer et al., 2006), define a pluripotent genomic organization in ES cells.

3. Reprogramming cells to pluripotency

The reprogramming of a differentiated cell into a pluripotent cell that in turn could repopulate or repair sick or damaged tissue would present beneficial applications in regenerative medicine. Over the years, several strategies have been developed to reprogram cells to a pluripotent state **(Fig. 8)**. Somatic cell nuclear transfer (SCNT) may offer this possibility **(Fig. 8A)** and is in principle possible in humans. However, technical hurdles and ethics regulations currently prevent application of this technology in a number of countries. As a result, alternative strategies to

reprogramming cell fate have been developed. In this section, we briefly address SCNT as a means of nuclear reprogramming and focus on recent non-SCNT approaches to reprogram cells and enhance their differentiation potential **(Fig. 8B-D)**. These include fusion with ES cells, and forced expression of pluripotency factors in somatic cells to elicit overexpression of pluripotency-associated genes. Another approach consists in the treatment of somatic cells with extracts of pluripotent cells; this approach has been developed in the course of this work and is addressed at large in this thesis.

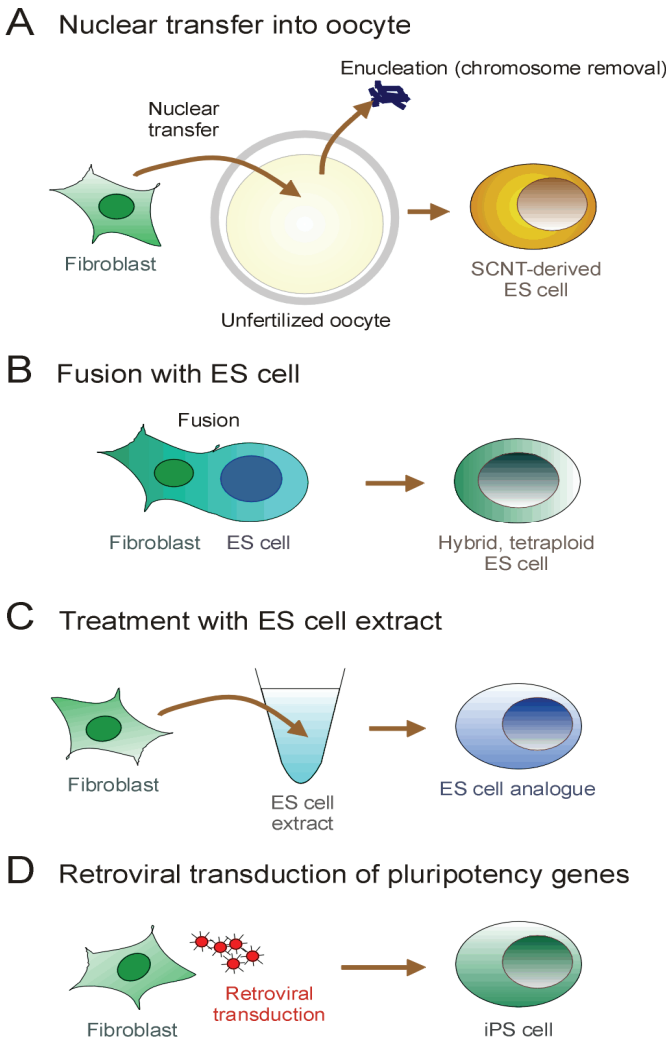


Fig. 8. Approaches to reprogramming somatic cells. (A) Transplantation of a somatic cell nucleus into an unfertilized oocyte (therapeutic cloning). Growth of cloned embryos to the blastocyst stage and derivation of SCNT ES cells may be one option to create genetically matched replacement cells. (B) Fusion of somatic cells with ES cells results in tetraploid hybrids with ES cell properties. (C) Transient incubation of somatic cells with extracts of ES cells elicits some nuclear reprogramming events and enhances pluripotency *in vitro* (work presented in this thesis). (D) Retroviral transduction of ES cell transcription factors (Oct4, Sox2, Klf4 and c-Myc) is sufficient to generate pluripotent induced pluripotent stem (iPS) cells. Taken from (Collas, 2007)

3.1. Reprogramming by somatic cell nuclear transfer (SCNT)

A classical experimental example of dedifferentiation is the reprogramming of a differentiated cell nucleus by transplantation into an unfertilized oocyte, a procedure referred to as SCNT (**Fig. 8A**). SCNT can lead to derivation of pluripotent ES cells from cloned embryos (Cibelli et al., 1998; Munsie et al., 2000; Wakayama et al., 2001) and in the birth of cloned offspring (Gurdon and Byrne, 2003; Rideout, III et al., 2002; Wilmut et al., 2002). The mechanisms of nuclear reprogramming by SCNT point to a process requiring DNA demethylation for reactivation of embryonic genes (Simonsson and Gurdon, 2004). However, SCNT is an inefficient process affected by differentiation and epigenetic state of the donor nucleus (Blelloch et al., 2006). Other approaches have been shown to elicit nuclear reprogramming and have been supported by modifications of the somatic cell's epigenome.

3.2. Reprogramming by fusion of a somatic cell with an ES cell

Fusion of mouse thymocytes with EG or ES cells have shown that epigenetic reprogramming could be triggered in the thymocyte nuclei (Tada et al., 1997; Tada et al., 2001) **(Fig. 8B)**. Notably, EG cell-thymocyte hybrids are characterized by heritable demethylation of imprinted and non-imprinted genes, and transcriptional activation of these genes. Epigenetic changes in the thymocyte nucleus are consistent with induction of pluripotency markers in the hybrids such as ability to differentiate into all three germ layers (Tada et al., 1997).

Somatic-ES cell hybrids also elicit markers of nuclear reprogramming such as thymocyte-derived X chromosome reactivation (Tada et al., 2001). Like EG cells, ES cells can induce pluripotency in somatic cells including thymocytes (Tada et al., 2001), neuronal progenitor cells (Pells et al., 2002; Ying et al., 2002) or bone marrow-derived cells (Terada et al., 2002). Similarly, fusion of EC cells with T-lymphoma cells promotes the formation of colonies expressing pluripotent cell transcripts from the lymphoma cell genome (Flasza et al., 2003).

Thus, factors originating from the undifferentiated cell can elicit some epigenetic reprogramming in a more differentiated cell type.

Further development in the cell fusion approach was reported by Cowan et al. (Cowan et al., 2005) to reprogram normal diploid human fibroblasts into human ES (or ES-like) cells. ES cells fused with the fibroblasts result in hybrids that maintain a tetraploid chromosome complement and display a morphology, growth rate, and surface molecules expression patterns characteristic of ES cells. Examination of genome-wide transcriptional changes, allele-specific gene expression and DNA methylation shows that the fibroblast genome is reprogrammed to near completion towards an embryonic state (Cowan et al., 2005). Further, differentiation of ES-fibroblasts hybrids *in vivo* produces cell types from each germ layer. These results support the mouse studies and show that human ES cells can reprogram differentiated nuclei.

Cell fusion systems provide tools for investigating mechanisms of reprogramming. Epigenetic analyses of the somatic cell genome following fusion with ES cells have confirmed the ability of ES cells to reprogram DNA methylation and histone modification patterns (see e.g. (Do et al., 2006; Do et al., 2007; Do et al., 2008; Do et al., 2009a; Do et al., 2009b; Han et al., 2008; Kimura et al., 2004)). These changes are compatible with acquisition of a pluripotent state. Notable changes were the CpG demethylation of *Oct4* and *Nanog* regulatory regions and acquisition of a histone marks compatible with transcriptional activation of these genes. As illustrated in this thesis, such changes were also noted in our own nuclear reprogramming studies. An additional remarkable feature of nuclear reprogramming is reactivation of the inactive X chromosome in the somatic cell, a hallmark of the pluripotent state (Do et al., 2009a).

Despite these successes, a limitation of cell fusion-mediated reprogramming is that the tetraploid state of the reprogrammed cell makes epigenetic analyses of the reprogrammed somatic genome challenging. This can be dealt with at the sequence level (e.g., under analysis of DNA methylation by bisulfite sequencing) by taking advantage of polymorphism between the ES

and the somatic cell (Cowan et al., 2005) but limits the analysis to restricted genomic sites. Persistence of the ES cell genome is also undesirable for therapeutic applications. Targeted elimination of chromosomes from mouse somatic-ES cell hybrids once reprogramming of the somatic genome is completed has been reported (Matsumura et al., 2006), however efficient elimination of all ES cell chromosomes remains challenging.

3.3. Reprogramming somatic cells with extracts

SCNT and somatic-ES cell hybridization have provided a rationale for the development of egg or cell extracts for reprogramming cells **(Fig. 8C**), because extracts should contain all necessary "reprogramming factors". Two advantages of extract-mediated reprogramming are the absence of introduction of ES cell chromosomes into the target cell, and the possibility of identifying reprogramming factors by manipulation of extract components.

Nuclear and cytoplasmic extracts from several cell types have been developed, which elicit changes in cell fate to various extents (Collas and Håkelien, 2003). Prior to the start of this thesis work, our laboratory developed a procedure to turn one differentiated cell type into another differentiated cell type (Håkelien et al., 2002). The approach involves the reversible permeabilization of a somatic cell with the bacterial toxin Streptolysin O (SLO), which cluster cholesterol in the plasma membrane, exposure of the permeabilized cells to the "reprogramming extract" for a defined period of time, and calcium-mediated resealing of the extract-treated cells (**Fig. 8C**). Using this approach, the group has shown that epithelial cells treated with extract of Jurkat T cells can take on T cell properties, including expression of T cell-specific genes, chromatin remodeling at a T cell-specific locus and induction of T cell signaling pathways including secretion of interleukin 2 (Håkelien et al., 2002; Håkelien et al., 2005) (**Fig. 9**).

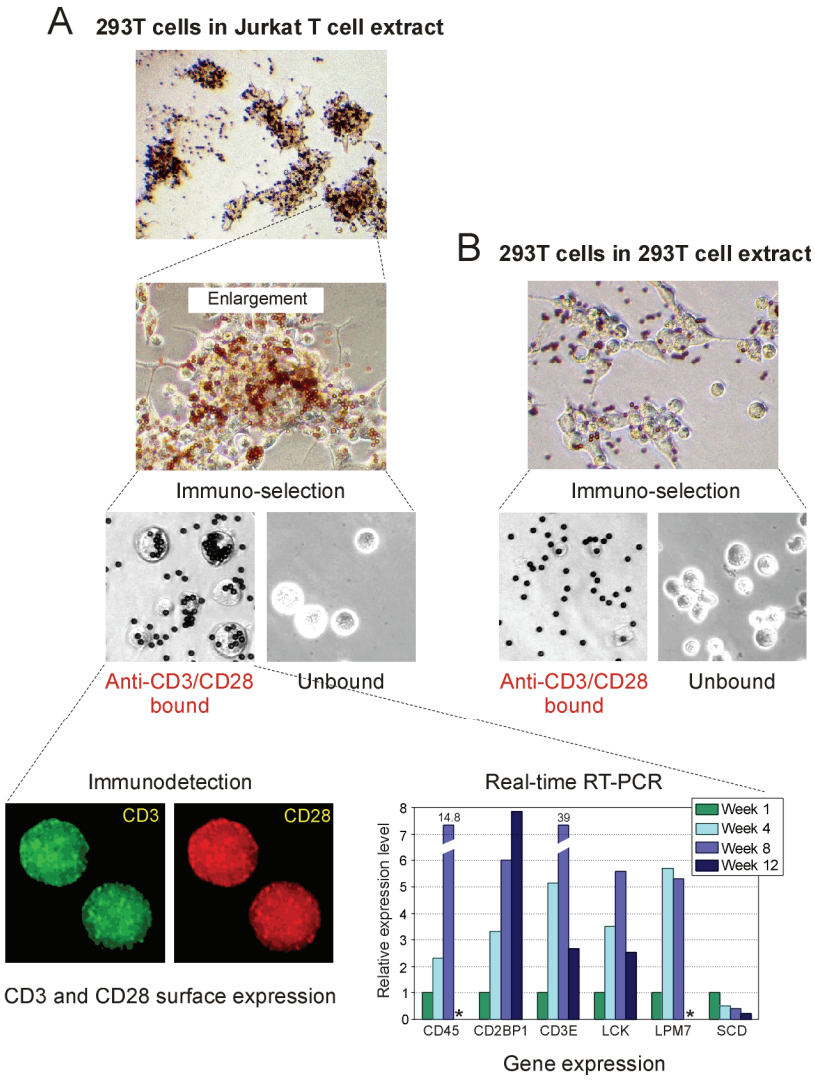


Fig. 9. Indications of reprogramming of epithelial 293T cells in extract of Jurkat T cells. (A) 293T cells treated with an extract of activated Jurkat T cells, as opposed to (B) an extract of 293T cells, causes cells to bind beads (brown spots) bearing anti-CD3 and anti-CD28 antibodies (shown in the enlargement; compare pictures in (A) and (B)). Antibody-bound cells can be separated from unbound cells (Immuno-selection). Immunolabeling of antibody-bound and released cells confirms the expression of CD3 and CD28 antigens on the cell surface (Immunodetection). The sorted cells also display T cell-specific genes, as shown by quantitative RT-PCR. Data compiled from (Håkelien et al., 2005).

A similar strategy has shown induction of cardiomyocyte functions in human adipose stem cells using extracts of rat fetal cardiomyocytes (Gaustad et al., 2004), and in another laboratory, the differentiation of mouse ES cells into pneumocytes using mouse pneumocyte extracts (Qin et al., 2005). Nonetheless, the long-term stability of the new program remains an issue (see Discussion).

Cell extracts may also be useful for reprogramming cells to pluripotency. A first example is the induction of dedifferentiation with extracts of regenerating newt limbs (McGann et al., 2001). When continuously exposed to cultured differentiated C2C12 myotubes, these extracts promote cell cycle reentry and approximately half of these continue proliferating as mononucleated cells. This is accompanied by a downregulation of muscle-specific markers in some of the myotubes. These findings imply that the dedifferentiated phenotype is maintained even after removal of the extract, suggesting that reprogramming events have taken place.

As anticipated from SCNT work in *Xenopus* (Byrne et al., 2003; Simonsson and Gurdon, 2004), extracts of *Xenopus* eggs can also induce expression of pluripotency markers in 293T cells and primary leukocytes while downregulating differentiation markers (Hansis et al., 2004). However, reprogrammed leukocytes have a limited life span and do not express surface markers characteristic of ES cells, so as observed with extract of other cell types, reprogramming under these conditions seems to be partial. *Xenopus* egg extract-treated porcine fibroblasts showed morphological changes, expression of *Oct4* and *Sox2*, and deacetylation of H3K9 (Miyamoto et al., 2007). The ability of the *Xenopus* egg extract to reactivate *Oct4* was also shown by EGFP expression in bovine fetal fibroblasts transformed by the *Oct4*-EGFP construct (Miyamoto et al., 2007). Interestingly, deacetylation of H3K9 also occured in non-permeabilized porcine fibroblasts (Miyamoto et al., 2007), suggesting that reprogramming also can take place in non-permeabilized cells. This was also the case in a very recent study where non-permeabilized mouse fibroblasts exposed to fish-egg extract (Zhu et al., 2009) reactivated *Oct4* and *Nanog*,

were able to differentiate into a variety of cells, and to induce teratoma formation while the genomic imprinting status of insulin-like growth factor II (Igf2) and H19 was stable (Zhu et al., 2009). When using SCNT, oocytes in metaphase II (mII) have shown to be most efficient whereas germinal vesicle (GV) oocytes are considered to be inadequate for use as recipients (Gao et al., 2002). Surprisingly, the opposite seems to be the case when it comes to extract-treatment because somatic cells exposed to mammalian mII-oocyte extract do not show any clear signs of reprogramming after culture and do not express *Nanog* after day 7 (Miyamoto et al., 2009). In contrast, mammalian GV oocyte-extract activates pluripotency genes as well as induces expected histone modification events, suggesting that MII and GV oocytes have different roles in nuclear reprogramming (Miyamoto et al., 2009). Mammalian cross-species experiments have also been reported where human somatic cells have been reprogrammed by mouse ES cell extract to reexpress *Oct4*, *Sox2*, *c-Myc* and *Klf4* (Bru et al., 2008). This was associated with increased recruitment of RNA polymerase II (RNA pol II) at the promoters, removal of lamin A/C and loss of repressive H3 modifications on *Nanog* and *Oct4* promoters. Alltogether, this shows that reprogramming of somatic cells using extracts is an efficient way of creating high potency cells without genetically altering them.

Today, many laboratories use the method for reprogramming cells with extracts based on the work done in our lab (Bru et al., 2008; Miyamoto et al., 2007; Miyamoto et al., 2009; Zhu et al., 2009). We show in this thesis that extracts of ES or EC cells can provide regulatory components required to direct a nuclear program characteristic of the pluripotent cell (Papers I, III). Changes in cell fate are accompanied by changes in DNA methylation and histone modifications on developmentally-regulated genes, indicating that functional epigenetic of the somatic genome can also occur in this way (Paper II).

3.4. Reprogramming by transduction of defined pluripotency factors

The transcription factors Oct4, Sox2, Klf4 and c-Myc have been reported to reprogram primary mouse or human fibroblasts in culture (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). To induce pluripotency, combinations of first 24, then 10, then 4 factors normally expressed in ES cells were co-transduced in fibroblasts using retroviral vectors, each bearing one transgene (**Fig. 8D**). Oct4 and Sox2, have been known to be required for induction and maintenance of self-renewal and pluripotency in ES cells (Mitsui et al., 2003; Pesce and Scholer, 2000). Overexpression of Nanog, however, another central component of self-renewal and pluripotency was against all expectations not necessary. It turns out that endogenous Nanog is activated in the transduced cells because Klf4 represses p53, which in turn represses Nanog upon differentiation of ES cells (Lin et al., 2005). A drawback of Klf4 overexpression, however, is that it also activates the tumor suppressor $p21^{CIP1}$ and abolishes cell proliferation. Overexpression of c-Myc, however, is there to suppress expression of $p21^{CIP1}$. So a balance between Klf4 and c-Myc is in all likelihood necessary to generate stable reprogramming in induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006).

iPS cells display all indicators of pluripotency. Cells are morphologically similar to ES cells, display a transcription profile nearly identical to that of ES cells, express ES cell surface markers and contribute to all germ layers in teratomas and in chimeras (Takahashi and Yamanaka, 2006). Importantly, adult fibroblasts have been shown to be able to generate a mouse with its genetics entirely derived from the fibroblast (Boland et al., 2009; Zhao et al., 2009)}. Of note, followup studies based on induction of pluripotency factors have shown that the number of transduced factors can be reduced to two or even one, when using target cell types already expressing some of these pluripotency factors (see e.g., (Feng et al., 2009a; Kim et al., 2008; Kim and Buratowski, 2009; Li et al., 2009; Utikal et al., 2009a)).

Nevertheless, limitations have until recently prohibited the use of first generation iPS cells in a clinical setting. 1) viral-induction of reprogramming factors creates risks of stable transgene integration into the genome; 2) c-Myc and Klf4 are oncogenic; and 3) production of iPS cells is inefficient with reprogramming often incomplete. For iPS cells to fulfill their potential in cell therapy, disease modeling or drug screening, non-genetic strategies have been devised. These include the use of inhibitors or epigenetic modifiers and signaling pathways that can replace the reprogramming factors or efficiently enhance genetic reprogramming (Feng et al., 2009b) **(Fig. 10)**. A promising approach involves the use of small molecules for reprogramming **(Table 2)**. Notable examples include the DNMT inhibitors 5-azacytidine (5aza) or RG108, which rescue cells trapped in a partially reprogrammed state (Mikkelsen et al., 2008). The histone deacetylase inhibitors (HDACi's) trichostatin A (TSA), valproic acid (VPA) or suberoylanilide hydroxamic acid (SAHA), the histone methyltransferase (HMT) inhibitor BIX, or the L-calcium channel agonist BayK also enhance reprogramming efficiency **(Fig. 10)**.

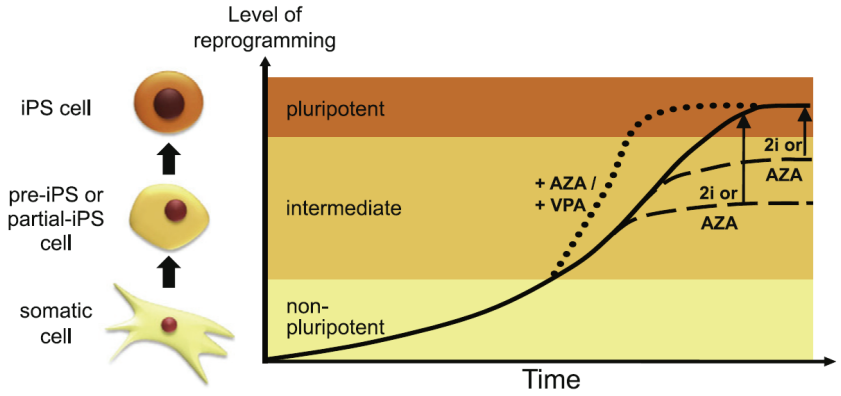


Fig. 10. Chemical-induced full reprogramming to pluripotency. Taken from (Feng et al., 2009b).

Inhibitors (together referred to as '2i') of the mitogen-activated protein kinase (MEK) and glycogen synthase kinase-3 (GSK3) pathways, involved in maintaining pluripotency in ES cells, have also been shown to elicit complete reprogramming of partially reprogrammed cells (Silva et al., 2008). Fibroblasts were also reprogrammed into iPS cells by combining 2i with A-83-01, an

inhibitor of TGF- signaling. These studies demonstrate the interplay between small molecules and epigenetic factors in inducing pluripotency.

Table 2. Chemicals used to replace core reprogramming factors or enhance reprogramming

Chemical	Function	Core factors used*	Cell type
5aza	DNMT inhibitor	OSKM	Mouse fibroblast
RG108	DNMT inhibitor	OSKM	Mouse fibroblast
TSA	HDAC inhibitor	OSKM	Mouse fibroblast
VPA	HDAC inhibitor	OSKM, OSK, OS	Mouse / human fibroblast
SAHA	HDAC inhibitor	OSKM	Mouse fibroblast
BIX-01294	G9a HMT inhibitor	OK	Mouse fibroblast / neuronal progenitors
BayK8644	L-Ca^{2+} channel agonist	OSK	Mouse fibroblast
Dexamethasone	Steroid glucocorticoid	OSK/ OSK	Mouse / human fibroblast
PD0325901 + CHIR99021 (2i)	MEK inhibitor GSK3 inhibitor	OK	Mouse neuronal progenitor cells
A-83-01	TGF- inhibitor	OSK	Rat liver progenitors / human fibroblasts

* O, Oct4; S, Sox2; K, Klf4; M, c-Myc

A prime application of the iPS cell technology in humans is the generation of disease- and patient-specific pluripotent cells suitable for the study of disease mechanisms *in vitro* and drug testing. Several such models have been recently published. Particularly relevant are generation of a large number of patient-specific iPS cell (Park et al., 2008), treatment of sickle cell anemia in a mouse model with autologous iPS cells (Hanna et al., 2007) and generation of motor neurons from a child with spinal muscular atrophy, with the demonstration of restoration of a defect phenotype in diseased iPS cell-derived motor neurons by treatment with VPA (Ebert et al., 2009). These and other studies demonstrate the usefulness of iPS cells in drug testing and disease study.

The area of nuclear reprogramming to pluripotency has literally ‘exploded’ while work reported in this thesis was ongoing, in particular with the advent of the ‘iPS technology’ and its recent derivatives. Work presented here relies on a cell-free, non-genetic approach initially developed in the laboratory to initially induce a ‘transdifferentiation’ of epithelial 293T cells into other types of differentiated cells (Håkelien et al., 2002). The approach has been tailored to produce cells with ES-like properties (Papers I-III).

AIMS OF THE STUDY

A differentiated cell can be reprogrammed to pluripotency by nuclear transplantation into oocytes, fusion with an ES cell or forced expression of pluripotency genes. Some of these genes can be substituted by proteins, suggesting that reprogramming cells by non-genetic means is possible. We rationalized that introduction into a target cell, of factors derived from pluripotent cells in the form of an extract could epigenetically and functionally reprogram the target cell. Aims of this study were therefore to:

1. Determine whether extracts from EC cells and ES cells are capable of reprogramming epithelial cells and fibroblasts to a pluripotent-like state.
2. Intitiate a characterization of components in ES cell extract that contribute to turning on Oct4 expression in extract-treated cells
3. Demonstrate that EC cell extract treatment elicits a reprogramming of DNA methylation and histone modifications on genes associated with pluripotency
4. Carry out a proteomic characterization of the nuclear reprogramming process in epithelial cells

SUMMARY OF PUBLICATIONS

Paper I:

Taranger, C.K., A.Noer, A.L.Sørensen, A.M.Håkelien, A.C.Boquest, and P.Collas. 2005. Induction of dedifferentiation, genome-wide transcriptional programming, and epigenetic reprogramming by extracts of carcinoma and embryonic stem cells. Mol. Biol. Cell. 16, 5719-5735

Reprogramming of a differentiated cell into a pluripotent cell may have long term applications in regenerative medicine. We report in this paper the induction of dedifferentiation, associated with genome-wide programming of gene expression and epigenetic reprogramming of an embryonic gene, in epithelial 293T cells treated with an extract of human NCCIT carcinoma cells. 293T cells exposed for 1 h to extract of NCCIT cells, but not of 293T or Jurkat T cells, form colonies that are maintained for at least 23 passages in culture. Microarray and quantitative analyses of gene expression reveal that transition from a 293T to a pluripotent cell phenotype involves the dynamic upregulation of hundreds of NCCIT genes, concomitant with the downregulation of 293T genes and of indicators of differentiation such as A-type nuclear lamins. Upregulated genes encompass stem cell markers including *OCT4* and Oct4-responsive genes. *OCT4* activation is associated with DNA demethylation in the *OCT4* promoter and nuclear targeting of Oct4 protein. In 3T3 fibroblasts exposed to an extract of embryonic stem cells, Oct4 activation is biphasic and RNA-PolII-dependent, with the first transient wave of *Oct4* upregulation being necessary for the long term transcriptional activation of *Oct4*. Genes characteristic of multilineage differentiation potential are also upregulated in NCCIT extract-treated cells, suggesting establishment of 'multilineage priming'. Retinoic acid triggers Oct4 gene and protein downregulation, activation of A-type lamins and nestin, and promotes differentiation towards neurogenic, adipogenic, endothelial and osteogenic lineages in vitro. These data indicate that an extract of undifferentiated carcinoma cells can elicit differentiation plasticity in a developmentally restricted cell type.

Paper II:

Freberg, C.T., Dahl, J.A., Timoskainen, S., and Collas, P. 2007. Epigenetic reprogramming of *OCT4* and *NANOG* by embryonal carcinoma cell extract. Mol. Biol. Cell. 18, 1543-1553.

We reported in our previous paper the reprogramming of epithelial cells by extract of undifferentiated embryonal carcinoma NCCIT cells. We show in this paper the reprogramming of DNA methylation and histone modifications on regulatory regions of the developmentally regulated *OCT4* and *NANOG* genes by exposure of 293T cells to NCCIT cell extract. *OCT4* and *NANOG* are transcriptionally upregulated and undergo mosaic CpG demethylation. *OCT4* demethylation occurs as early as week 1, is enhanced by week 2, and is most prominent in the proximal promoter and distal enhancer. Targeted *OCT4* and *NANOG* demethylation does not occur in 293T extract-treated cells. Retinoic acid-mediated differentiation of reprogrammed cells elicits *OCT4* promoter remethylation and transcriptional repression. Chromatin immunoprecipitation analyses of lysines K4, K9 and K27 of histone H3 on *OCT4* and *NANOG* indicate that primary chromatin remodeling determinants are acetylation of H3K9 and demethylation of dimethylated H3K9. H3K4 remains di- and trimethylated. Demethylation of trimethylated H3K9 and H3K27 also occurs; however, trimethylation appears more stable than dimethylation. We conclude that a central epigenetic reprogramming event is relaxation of chromatin at loci associated with pluripotency in order to create a conformation compatible with transcriptional activation.

Paper III:

Pewsey, E., Bruce, C., Georgiou, A.S., Jones, M., Baker, D., Ow, S.Y., Wright, P.C., Freberg, C.T., Collas, P. and Fazeli, A. Proteomic analysis of epithelial cells reprogramming in cell-free extract. Mol. Cell Proteomics. 8, 1401-1412.

We report in this publication the proteomic profile of epithelial cells reprogrammed to a more pluripotent state using undifferentiated embryonal carcinoma cellular extracts. 293T cells were reversibly permeabilised with Streptolysin O, transiently incubated in extract of NCCIT cells or, as a control extract of 293T cells, resealed and cultured. *OCT4* and *SOX2* gene expression was upregulated in NCCIT extract-teated cells relative to control cells, while there was no alteration in *DNMT3B* gene expression. Thirty percent of NCCIT extract-treated cells were positive for SSEA-4 and karyotyping confirmed their 293T origin, excluding the possibility of contamination from NCCIT cells. Two-dimensional PAGE revealed ~400 protein spots for each cell type studied. At least 10 protein spots in the proteome of NCCIT extrat-treated cells had an expression profile similar to NCCIT and remained unaltered in control cells. These proteins were identified using tandem mass spectrometry and include 78 kDa glucose-regulated protein precursor and Tropomyosin alpha-3 chain. This study constitutes to our knowledge the first report on the proteomic characterization of the nuclear reprogramming process.

DISCUSSION

This thesis reports the reprogramming of function of somatic cells by transient exposure to a cytoplasmic and nuclear (whole-cell) extract from pluripotent cells. In light of previous work in our laboratory on transdifferentiation attempts using extracts from differentiated cells to redirect somatic cell fate (Håkelien et al., 2002; Håkelien et al., 2005; Landsverk et al., 2002), we set out to investigate whether extracts from pluripotent cells would de-differentiate cells and induce pluripotency. Altogether, morphological observations, gene expression microarray and RT-qPCR, immunolabeling, *in vitro* differentiation assays, epigenetic alterations such as DNA methylation pattern and changes of histone modifications, as well as changes in surface markers and protein expression analysed by 2D gel electrophoresis and mass spectrometry are consistent with long-lasting alterations in somatic cell fate as a result of transient treatment with EC or ES cell extracts. Factors affecting efficiency of reprogramming, the nature of extract-derived reprogramming molecules, how epigenetic reprogramming of pluripotency-associated genes might take place in our system, and the extent of reprogramming to the pluripotent state, are discussed.

1. Factors affecting the efficiency of reprogramming cells with extracts

Reprogramming of a somatic nucleus in an amphibian egg has been shown to depend on the exchange of factors between the somatic nucleus and the egg cytoplasm (reviewed in (Kikyo and Wolffe, 2000). In heterokaryons and hybrid cells, factors from the “donor” cell, e.g. the ES cell), co-exist with the target cell nucleus, leading to transcriptional and epigenetic reprogramming (Tada et al., 1997). Moreover, the other known nuclear reprogramming approaches rely on the transfer of reprogramming factors and accompanying molecules (such as viruses, transposons, cytokines, small molecules) through the target somatic cell membrane (Huangfu et al., 2008; Shi et al., 2008; Woltjen et al., 2009). In contrast, removal of the ES cell genome from ES cell-

fibroblast heterokaryons within 24 h of fusion is not conducive of reprogramming (Pralong et al., 2006). Collectively, this shows that for reprogramming to occur, it is essential that the responsible factors gain access to the donor cell genome.

Permeabilization of the cell membrane using the bacterial (*Streptococcus pyogenes*) toxin Streptolysin O (SLO) allows for the delivery of extract components across the cell membrane. Our laboratory has earlier tried other plasma membrane-disrupting approaches such as gentle physical sharing with acid-washed beads or passing through a needle, or submicromolar concentrations of non-ionic detergents such as Nonidet P-40 or digitonin, without success: in short, cells were either not permeabilized, permeabilized in very low proportion, or irreversibly lysed (A.-M. Håkelien, K. Gaustad and P. Collas, unpublished data). Thus, SLO has been the reagent of choice for reversible taget cell permabilization for this project. SLO is a cholesterol-binding toxin that forms pores in the plasma membrane (Bhakdi et al., 1985; Bhakdi et al., 1993). Permeabilization is reversible as resealing can occur in a Ca^{2+}-dependent pathway (Walev et al., 2001). SLO-mediated permeabilization has proven valuable for delivery of macromolecules to cells (Fawcett et al., 1998; Walev et al., 2001) as pore size formed by SLO can be up to 30 nm in diameter (Bhakdi et al., 1993). This allows for uptake of proteins of over 100 kDa (Walev et al., 2001). Futhermore, endotoxins delivered to SLO-permeabilized cells remain biologically active (Walev et al., 2001); hence, import through SLO-induced pores does not appear to affect protein activity. Moreover, properties other than molecular size (e.g. molecule conformation) are important for uptake through SLO-formed pores as Fura-2 free acid (M_r 832) is not taken up by permeabilized cells (Fawcett et al., 1998). Consequently, permeabilization elicited by SLO appears to impose a restriction on the nature of molecules to be taken up.

Our protocol does not include any removal of excess SLO after binding of SLO to cholesterol and prior to the pore-forming step, and therefore some SLO can enter the cell. However, intracellular membranes, such as the endoplasmatic reticulum/nuclear envelope

network, contain much lower amounts of cholesterol than the plasma membrane and thus serve as poor substrates for SLO (Fawcett et al., 1998). Because diffusion through SLO-formed pores is expected to be bi-directional, soluble cytosolic components may leak out. We have observed that SLO-treated cells incubated in extract have higher survival rate than cells incubated in culture medium only. Cell survival could be greatly improved however when bovine serum albumin was added to the culture medium (A.M. Håkelien, unpublished observations), arguing that intracellular molecular crowding and/or maintenance of and isotonic osmonit pressure within the permeabilized cells was beneficial.

Recently, a study showing reprogramming of mouse fibroblasts to induced multipotent stem (iMS) cells using fish oocyte extracts, based on work performed in our lab, showed that the cells were induced to express pluripotency markers regardless of plasma membrane permeabilization (Zhu et al., 2009). Without SLO permeabilization, a passive entry of large molecules, like Oct4, Sox2 and Nanog is unlikely and theoretically only small molecules can enter the cells. However, it is possible that large molecules are actively transported into the cells, but this has not been examined. Moreover, the fish oocyte extracts used in these experiments lack the additional ATP generating system, which we add to our extracts to improve the active transport of factors across the membranes and to increase the chromatin remodelling. There is a possibility that membrane receptors and intracellular transport molecules cooperate in delivering information from the new surroundings of the cells. It is known that cell microenvironment is able to change cell genotype and epigenotype, and the best example of that is the reprogramming of intact cells when inserted into enucleated oocytes (Chang et al., 2003; Lin et al., 2008). In either case, a detailed analysis of the extracts used for reprogramming should be performed. It will be of interest to determine which factors or fractions of the extract are responsible for the various events during reprogramming, whether the extract contains inhibitors that can be

excluded, and whether the extract reprograms cells using different active factors than the known factors used for creating iPS cells.

The extracts used in the reprogramming experiments described in this thesis contain 25-35 mg/ml protein (Bradford assay). Experiments using NCCIT extract with 12 mg/ml protein or less did not show signs of reprogramming other than upregulation of the *REX1* (*ZFP42*) gene in 293T cells (our unpublished data). In addition, it was recently shown that fish egg extract at <25 μg/ml protein was able to reprogram mouse fibroblasts and that the "mild" reprogramming observed (see below) lead to lower cell death (Zhu et al., 2009). However, the treated cells showed stronger Oct4 labeling and a higher rate of colony formation after exposure to extract with higher protein concentration (10 mg/ml) (Zhu et al., 2009). However, a lower protein concentration also means a more diluted extract, hence a less viscous extract with an altered hydration status, which in our hands has showed to be highly important for the success of reprogramming (reduction of protein concentration was achieved by diluting concentrated extracts prepared as described in Paper I 1:1 with H_2O). The results from Zhu et al. (2009) are therefore consistent with our findings that dilution of the extract gives a higher survival rate than a non-diluted extract. Nonetheless, whether the beneficial effect of dilution on cell survical and reprogramming efficiency was due to a more appropriate concentration of (as yet undefined) factors or to reduced osmotic pressure upon exposure of the target cells to the extract, remains uncertain. Notably, NCCIT extracts prepared in this work were ~500-600 mOsM prior to dilution in MilliQ H_2O. This proved to be rather detrimental despite the permeabilized (and there "open" or leaky state of the target cells). Indeed, maintaining the extract in the range of 280-300 mOsM elevated survival rate and reprogramming efficiency (Paper I). Thus, the combination of keeping the highest possible protein concentration and the balanced hydration status is most efficient when reprogramming cells using extracts.

2. What component(s) in the extract might elicit nuclear reprogramming?

Induction of reprogramming by fusion with ES or EC cells or by exposure to pluripotent cell extracts indicates that the ES and EC cell contains the factors necessary to initiate this event. Experiments reported in Paper I together with recent published advancements point to possible candidates.

Protein components are likely candidates because treatment of ES cell extract with Proteinase K or trypsin abolishes the short- and long-term expression of Oct4 in target cells (Paper I). Moreover these proteins are heat-labile because heat treatment of the extract also abolishes Oct4 expression. The nature of proteins implicated in extract-based induction of pluripotency is currently unknown (see below). Nonetheless, a likely candidate is the BRG1 component of the SWI-SNF complex, whose depletion from *Xenopus* egg extract abolishes reprogramming (Hansis et al., 2004). Similar work in our laboratory also suggests that BRG1 is also implicated in reprogramming NIH3T3 cells in ES cell extract (our unpublished data) It is likely, therefore, that chromatin remodeling enzymes play an active role in the reprogramming process.

Could nucleic acids mediate extract-based reprogramming? This possibility has been examined to show by PCR that extracts contain no detectable genomic *OCT4* DNA, and that DNase treatment of extracts does not affect Oct4 detection in the target cells (Paper I). Thus, it is unlikely that DNA transfer is a component of the reprogramming mechanism. It is possible, however, that RNAs are implicated. RT-PCR amplification of various transcripts indicates that the NCCIT extract contains RNAs; this was also evidenced by RNase treatment of the extract followed by agarose gel electrophoresis (our unpublished data). Thus, there is a formal possibility for transfer of RNAs from the extract into the permeabilized cells.

Whether RNAs of extract origin play a role in reprogramming remains to be thoroughly investigated. Previous work has shown that RNase A-treated T cell extract was able to mediate

expression of T cell markers in 293T cells (Hakelien and Collas, 2002), and similarly, ES cell extract treatment with RNase A elicits the same pattern of Oct4 protein expression in target cells as untreated extract (Paper I). Further, pretreatment of fish oocyte extracts with RNase did not significantly alter efficiency of fibroblast reprogramming of to iMS cells (Zhu et al., 2009). This would suggest that mRNAs are not essential components of the mechanism of reprogramming. Alternatively, it is possible that RNAase treatment did not degrade RNA species that might be implicated in the reprogramming process.

Of note, our extract preparation procedure does not include RNase treatment because it might have affected transcription or translation in the target cell. Thus, RNAs may be involved in the initial process of dedifferentiation either as templates for translation or through their involvement in forming an appropriate chromatin structure (Stevenson and Jarvis, 2003). However, the possibility that detection of pluripotency-specific transcripts in reprogrammed 293T cells is due to mRNA contamination can reasonably be excluded. First, NCCIT extract-treated cells harbor only approximately half of the number distinct transcripts detected on microarrays in NCCIT cells. Second, many transcripts are not detectable before week 2 after extract treatment, wich would not be expected from a direct mRNA uptake. Third, the dynamics of up- and downregulation of gene expression and the persistence of transcripts for over at least 50 population doublings in extract-treated cells are inconsistent with uptake of mRNAs as the sole source of transcripts detected in these cells. Fourth, many 293T cell genes are downregulated after exposure to NCCIT extract, a feature difficult to explain merely by mRNA uptake. Lastly, as mentioned above, RNAse A treatment of ES cell extract is compatible with long term Oct4 expression and maintenance of an ES cell-like phenotype in NIH3T3 cells. Thus, we argue that transcription of pluripotency genes cannot occur as a result of direct mRNA uptake, but rather by altered transcription dynamics from the 293T cell genome.

It is tempting to speculate that non-coding RNAs, including microRNAs might be involved in eliciting nuclear reprogramming. Non-coding RNAs have been implicated in regulating expression of target genes by promoting the degradation of transcripts or by binding to regulatory sequences on target genes (Clark, 2007; Filipowicz et al., 2008; Jacquier, 2009; Mercer et al., 2009; Nesterova et al., 2008)thereby modulating transcription. Small non-coding RNAs also play a role in development (Stefani and Slack, 2008). Interestingly, non-coding RNAs have also recently been shown to regulate promoter methylation in ES cells by downregulating Dnmt3a (Nesterova et al., 2008) Thus, because a single non-coding RNA can have multiple (up to several hundreds) targets, it is conceivable that even a small number of such RNAs would affect the regulation of many genes, sufficiently to alter cell fate. An interesting approach would be to transfect various somatic cell types with a library of ES-cell derived microRNAs or long non-coding RNAs and assess any effect on induction of pluripotency.

Another class of molecules possibly involved in extract-based nuclear reprogramming includes small molecules. These would easily be diffusible through pores generated with SLO, or could be taken up by intact cells. Earlier work from our laboratory has shown that cyclic AMP was likely involved in inducing Oct4 protein expression in cells juxtaposed to embryonic blastomeres, within mouse preimplantation embryos (Burnside and Collas, 2002). The effect was blocked with a gap junction inhibitor, arguing for a mechanism involving cAMP signaling through gap junctions (Burnside and Collas, 2002). A role of small molecules in induction of plutipotency is supported by the replacement of some of the established reprogramming factors with inhibitors of signaling pathways or epigenetic modifiers (see Introduction).

3. A role of Oct4 in induction of pluripotency?

The nature of protein factor(s) required for extract-based reprogramming remains currently unknown. Our studies however suggest that extract-derived Oct4 is probably not required:

immunodepletion of Oct4 and Oct4-associated proteins from ES cell extract maintains the biphasic Oct4 expression pattern, including its long-term expression, in the target cells (Paper I). This contradicts more recent findings that among the factors shown to be sufficient to elicit iPS cell formation (Oct4, Sox2, Klf4, c-Myc), Oct4 seems to be the only one required. Indeed, Nanog, Sox2, Klf4, C-myc or Lin28, but not Oct4, have shown to be dispensable (Kim et al., 2009; Li et al., 2009). That makes Oct4 the only reprogramming factor that to date has not been replaced by other factors or drugs. It is however possible that low levels of Oct4 remain in the ES cell extract after immunodepletion (even though Western blotting shows undetectable Oct4; Paper I) that may be sufficient to elicit reprogramming. Alternatively, the early and short-term wave of Oct4 protein induction in the target cell may serve as a "primer" for subsequent long-term Oct4 expression. Interestingly, we found that transient inhibition of RNA Pol II with DRB long enough to inhibit the first wave of Oct4 expression (24 h), also prevented the long-term induction of Oct4 (Paper I). This suggests that this first transient induction is necessary for subsequent long-term Oct4 expression, and thereby reprogramming, at least in these experiments.

The time-lapse between extract treatment and detection of Oct4 mRNA and protein after 1-2 weeks is consistent with the establishment of epigenetic modifications necessary for transcriptional activation of *Oct4* shown in *Xenopus* egg extract (Simonsson and Gurdon, 2004). Timing of initiation and completion of these changes may vary with the systems investigated. In another study, SWI/SNF comlex dependent activation of *Oct4* transcription in permeabilized somatic cells incubated in *Xenopus* egg extract occurs after 30 min (Hansis et al., 2004), indicating that not only chromatin remodeling events, but also transcriptional activation of a silent gene can occur within minutes *in vitro*. In contrast, when injecting permeabilized cells into the germinal vesicle of Amphibian oocytes, *OCT4* transcription was not detected until after 2 days of incubation (Byrne et al., 2003). Also, in ES cell-thymocyte hybrids, an *Oct4*-GFP transgene was activated 2 days after fusion (Tada et al., 2001). Thus the time frame for onset of

reprogramming events appears to depend on the system used. Conceivably, timing of Oct4 expression in reprogrammed cells may be affected by the concentration of reprogramming factors required to turn on *OCT4*, and/or to the duration of exposure of the somatic nucleus to such factors. Collectively, these results suggest that induction of Oct4 expression in reprogrammed cells elicits a transcriptional activation pathway linked to Oct4 function.

An interesting observation among 293T cells treated with NCCIT extract in the present work is the correlation between down-regulation and disappearance of perinuclear lamin A/C staining (a marker of differentiated cells; (Guilly et al., 1990)) and appearance of intranuclear Oct4 protein (Paper I). Detection of Oct4 and loss of lamin A/C staining in 60% of extract-treated cells indicates that the extract is capable of inducing the start of translation of one protein and end translation of another. Nonetheless this does not imply that loss of lamin A/C stimulates Oct4 induction and vice versa. TSA-induced histone hyperacetylation decreases the amount of peripheral heterochromatin. The same pattern is shown in lamin A/C-deficient fibroblasts which display reorganization of centromeric heterochromatin (Galiova et al., 2008). Additionally, HDAC inhibition compensates for lamin A/C-dependent chromatin reorganization (Galiova et al., 2008), suggesting that interactions between lamins and specifically modified histones may play an important role in higher-order chromatin organization. These are in turn likely to influence transcriptional activity. Whether lamin A/C downregulation occurs before or after induction of *OCT4* in our system is not known. However, it is tempting to speculate that pluripotent cell extract-elicited loss of lamin A/C in target cells leads to rearrangement of chromatin domains promoting expression of plrupotency-associated genes. To support this view, ES cells, EC cells, and other types of immature cells such as lymphoblasts (Guilly et al., 1990), do not express the *LMNA* gene (encoding lamins A and C). Noteably, differentiation of human ES cells starts with the downregulation of Tra-1-60, Tra-1-81 and SSEA-4. Next, and prior to downregulation of *OCT4*, lamin A/C is upregulated (Constantinescu et al., 2006). This does not

nessecarily mean that this is accurately reversed during reprogramming. But if this is the situation, the early activation of Oct4 may lead to lamin A/C downregulation and further Tra-1-60, Tra-1-81 and SSEA-4 upregulation. The proteomic analyses of extract-treated cells (paper III) show that 30% are positive for SSEA-4, but not for Tra-1-60 or Tra-1-81. Perhaps the extract-treated cells have managed to go through the first steps, upregulating OCT4, downregulating lamin A/C and upregulating SSEA4 and are on their way to, but was investigated prior to, their upregulation of Tra-1-60 and Tra-1-81.

4. Epigenetic mechanisms of reprogramming pluripotency-associated genes

Epigenetic states of somatic cells and pluripotent cells are different. Histone modifications act in concert with DNA methylation to produce epigenetic modifications that regulate the balance of expression of pluripotency-associated genes and lineage-restricted genes. Thus, remodeling of the epigenome is essential in reprogramming.

Genes associated with pluripotency and not expressed in differentiated cells are, prior to reprogramming, methylated in their promoters (except for CpG island promoters such as *SOX2*; (Barrand and Collas, 2010), show strong enrichment in repressive H3K27me3 and association with PcG proteins. During reprogramming, the promoters of these genes are DNA-demethylated and lose H3K27me3 so exhibit H3K4me3 (presumably in addition to acetylated epitopes) (Amabile and Meissner, 2009). Changes in H3K27me3 are highly significant for the reprogramming to iPS cells and suggest an important role for PcG proteins in reprogramming (Maherali et al., 2007). Genome-wide mapping studies of human and mouse ES cells divide genes into four classes based on their association with either one, two or none of the H3K4me3 and H3K27me3 marks (Guenther et al., 2007; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). The class of genes associated with H3K4me3 only is most actively transcribed in pluripotent cells (Mikkelsen et al., 2007) and in mES cells, 60% of these genes have a high

proportion of unmethylated CpGs in the promoter region (Fouse et al., 2008). Notably, DNA methylation in mES cells primarily takes place on ICP and LCP promoters or on non-CpG island regions of HCP promoters (Fouse et al., 2008).

Reprogramming of somatic cells also requires silencing of certain lineage-specific genes. Genome-wide mapping of Oct4, Sox2 and Nanog target genes revealed a large group of genes that are co-regulated by these factors in different combinations (Boyer et al., 2005; Loh et al., 2006). Surprisingly, many of these target genes are not expressed in ES cells. In ES cells, many differention-associated genes as well as Oct4, Sox2 and Nanog target genes are silenced by PcG complexes and hold the bivalent H3K4me3/H3K27me3. PcG-mediated H3K27 trimethylation silences these genes in ES cells; nonetheless, they are primed for expression later during differentiation. This is consistent with the fact that pluripotent cells have a much higher amount of bivalent domains than have more lineage-restricted cells (Meissner et al., 2008). Interestingly, the class of genes associated with neither H3K4me3 nor H3K27me3 contains as much as one third of the genes, and of these the majority contain low- ro intermediate CpG content promoters. Further, 87% show a methylated proximal promoter and of these 80% are not expressed in mES cells (Fouse et al., 2008). In addition, a ChIP-chip study on H3K27me3- and H3K4me3 patterns in promoters of 16,500 genes in one iPS cell line found that reprogramming is largely associated with changes in H3K27me3 rather than H3K4me3 (Maherali et al., 2007), and failure in establishing repressive marks results in incompletely reprogrammed cells (Mikkelsen et al., 2008; Sridharan et al., 2009). Thus, both activation of pluripotency-associated genes and repression of lineage-specific genes are two important events required for, or being a process of, complete nuclear reprogramming.

5. Epigenetic reprogramming of *OCT4* in somatic cells by EC- and ES cell extracts

In ES cells, the *OCT4* TSS region contains unmethylated CpGs and is packaged with H3K9ac and H3K4me3, consistent with high expression of the gene. Conversely, *OCT4* in 293T cells is highly CpG methylated, associated with low H3K9ac, high H3K9me2 and H3K27me3, and is not expressed. Extracts of pluripotent cells retain the ability to epigenetically reprogram *OCT4* in 293T cells (Papers I-II, (Bru et al., 2008)).

In the NCCIT-extract system, nine amplicons of the *OCT4* region examined by bisulfite sequencing (Paper II) included 47 CpGs that were largely methylated in 293T cells and largely unmethylated in NCCIT cells. 293T cells treated with NCCIT extract showed mosaic CpG demethylation in the *OCT4* promoter. It is however not possible at present to determine whether some cells exhibited complete demethylation on all *OCT4*-containing alleles in the entire promoter region (as a side note, 293T cells are aneuploid so there might be more than two alleles bearing *OCT4*). However, whole regions within one amplicon are completely demethylated compared to 293T cells, indicating that when demethylation occurs, large promoter areas are affected. Moreover, differentiation of the reprogrammed 293T cells towards the neurogenic pathway showed "whole-amplicon" methylation on *OCT4*, suggesting that the cells that respond to the extract-treatment de-methylate the majority of the *OCT4* promoter.

Interestingly, as reported in another EC cell line (NT2) (Deb-Rinker et al., 2005), region of the *OCT4* proximal enhancer (PE) show mosaic methylation in NCCIT cells. Further, NCCIT extract-treated cells do not show complete demethylation in this region, in contrast to all others examined. In mES cells, the *Oct4* PE is hypomethylated and the gene highly expressed (Hattori et al., 2004). Distal regulatory elements such as enhancers may activate transcription over long distances. Their action must be restricted to prevent illegitimate activation of non-target genes. An *Oct4*-GFP transgene not containing the *Oct4* PE was reactivated 40-48 h after cell fusion-

induced reprogramming, wheras the effect of including the PE region was shown by activation already within 22 h (Han et al., 2008). Only the PE-containing transgene corresponded to endogenous *Oct4* activation and DNA demethylation occurring 24 h after fusion. This suggests that the PE region may positively regulate *Oct4* re-expression (Han et al., 2008) and that methylation of this region may affect the potential of the extracts used to reprogram cells to pluripotency.

Oct4 DNA demethylation is required for *Oct4* transcription after nuclear transplantation into *Xenopus* oocytes (Simonsson and Gurdon, 2004). Additionally, culture of NIH3T3 cells with TSA and/or 5aza showed that both chemicals were necessary for reactivation of *Oct4* (Hattori et al., 2004). This treatment altered the enhancer/promoter region of *Oct4* to become hyperacetylated and CpG demethylated (Hattori et al., 2004). This indicates that DNA demethylation is required but not sufficient to reprogram expression of *Oct4*. Activating H3K4me3 is crucial for complete reprogramming, especially at the *Oct4* and *Nanog* promoters (Mikkelsen et al., 2008). There are only minor differences in the H3K4me3 level between 293T cells and NCCIT cells (Paper II). However, an exception is found in the proximal promoter (PP) of *OCT4* (covered by ChIP primer-pair OCT4E in Paper II) where NCCIT cells and extract-treated cells show a significantly higher level of H3K4me3 compared to 293T cells and the control. Further, bioinformatic analysis of the *OCT4* promoter region using Genomatix MatInspector (www.genomatix.de) showed a putative overlapping binding site for both CTCF and Sp1 in the PP of *OCT4* (our unpublished data). The transcription factor Sp1 is activated in mouse embryogenesis and activates *Oct4* gene expression (Hattori et al., 2004). CTCF physically links cohesin to chromatin (Rubio et al., 2008) and is often bound (together with subunit of cohesin RAD21 (Hallson et al., 2008)) near the boundaries of regions rich in RNA Pol II and H3K4me3 (Wada et al., 2009). Thus, an interaction between Sp1, CTCF and H3K4me3 in the regulation of the PP/TSS region of *OCT4* is possible. Also, the fact that 293T cells incubated in

mES extract induce *OCT4* expression starting already after 4 h with the level of *OCT4* increasing after return to cell culture (Bru et al., 2008) could indicate that ES cell extract triggers *OCT4* activation eaerlier than NCCIT extract (see also Paper I). The OCT4D ChIP amplicon covers this locus in our experiments (Paper II) and this region shows a similar H3K9ac level in extract-treated cells as in NCCIT cells. Our ChIP results also show that the level of H3K9ac in extract-treated cells in the more upstream region, but yet within the PE (covered by the OCT4C primer pair), shows only half the level of H3K9ac as NCCIT cells this region. DNA methylation, as well as histone acetylation and methylation, occur concominant with or prior to DNA replication and subsequent to cell division (Han et al., 2008). The extract-treated cells divide every 24 h. It is thus possible that reprogramming of the epigenome occurs immediately after extract treatment.

Using an extract of mES cells showed that the re-expression of *Oct4* is biphasic, RNA Pol II-dependent and occurs as early as 1 h after recovery from the extract with the first peak at 24 h (Paper I). This biphasic Oct4 expression could be explained by nuclear uptake of transcription factors and chromatin remodelers that promptly target the *Oct4* promoter (see Discussion of Paper I). However, re-expression of genes can also start with incomplete elongation at first, then complete elongation after some time (Wada et al., 2009). Focusing on the first cycle of transcription after re-expression of five long human genes, an accumulation of RNA Pol II was seen at the TSS. These sites also contain boundaries marked with RAD21/CTCF binding. Knockdown of RAD21 abolished this accumulation suggesting that elongation by RNA Pol II can be regulated by an epigenetic mechanism (Wada et al., 2009). Alternatively, as a result of RNA Pol II stalling at sites where RAD21/CTCF are bound, the second wave of Oct4 can be expressed some time after extract-treatment. This suggests a link between RNA Pol II, H3K4me3 and other factors bound close to the *Oct4* TSS. A map of the histone methylations in the human genome was aligned with a map of CTCF binding sites (Bao et al., 2008). These authors found that the CTCF binding sites reside at the boundary between H3K4me3 and H3K27me3. It is

tempting to speculate that when PcG complexes and H3K27me3 are no longer present on the promoter, H3K4me3 will have a function in eliminating CTCF bound silencing of the gene, allowing RNA Pol II to elongate the gene. Stalling at the TSS may be different in a possible heterogenous population of donor cells and make the biphasic pattern of gene expression in the reprogrammed cells.

6. How complete and stable is extract-based reprogramming?

An issue under current debate is the extent to which induction (or maintenance) of pluripotency can be relied upon by mere morphology of cell colonies. Mouse and human ES cell colonies display distinct morphologies though growth in well-delimited "islands" is a common characteristic. Differentiation is often manifested by changes in cell shape at the ediges of the colonies. Colonies of distinct morphologies have also been reported during the generation of mouse and human iPS cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Nonetheless, identification of reprogrammed colonies based on morphology among heterogenous types of colonies has been reported (Meissner et al., 2007; Sridharan et al., 2009; Wernig et al., 2007). Delayed onset of selection for reprogrammed cells has been shown to be a critical factor to obtain fully reprogrammed cells, and pluripotent colonies have been successfully selected solely based on morphology (Blelloch et al., 2007; Meissner et al., 2007; Wernig et al., 2007) Interestingly, hES colonies form a flat monolayer, while mouse pluripotent colonies form thicker, multilayered colonies (Maherali and Hochedlinger, 2008). The morphology of the pluripotent reprogrammed cells should be round colonies with smooth edges. A recent live imaging study comparing ES cells, iPS cells and partial iPS cells characterized three types of colonies (Chan et al., 2009). Analysis of gene expression and epigenetic state showed that colonies of type I and II resembled intermediate stages of reprogramming while colonies of type III contained true iPS cells **(Fig. 11)**.

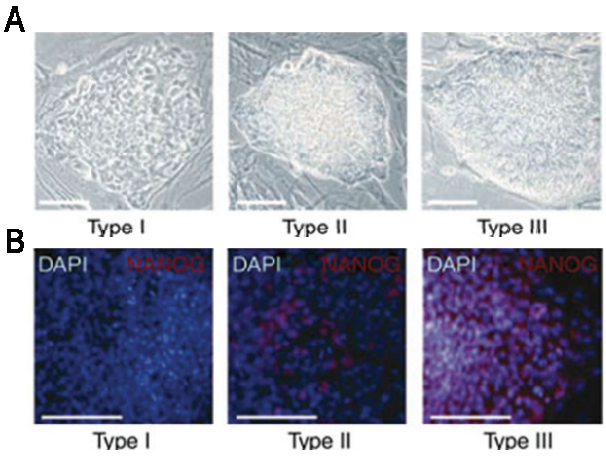


Fig. 11. Three types of colonies in cultures of human iPS cells. (A) Phase contrast examination. (B) DAPI (blue) and Nanog (red) staining of colonies shown in (A). Taken from (Chan et al., 2009). Truly reprogrammed iPS cells are those of Type III.

We observed a clear difference in morphology of extract-treated cells over time relative to 293T cells (Paper I; **Fig. 1**). 293T cells undergo a morphological change when treated with NCCIT extract compared to control 293T extract already at week 1, and this change is not reversed even after 12 weeks (Paper 1) and beyond (our unpublished data). However, in the first 4 weeks, our colonies resemble the Type I colonies in this paper (Chan et al., 2009) lacking the smooth, round edges of a completely reprogrammed colony (**Fig. 11A**; Type III). At week 8, our colonies round up to become more like Type II/III colonies, and are maintained at least through week 12. Notably, NCCIT cell cultures also contain colonies of all three types in addition to adherent cells growing like fibroblasts. Thus, an extract of this heterogenous source of cells can make the monolayer 293T cells round up to grow in Type II/III colonies. Establishment and maintenance of morphology requires the regulated expression of several genes involved in cytoskeletal organization, cell adhesion and cell locomotion. Thus, maintenance of round islands of reprogrammed cells for >12 weeks of culture shows that extract treatment is sufficient to maintain complex features of cell function, including cytoskeletal functions regulating cell shape.

The increased expression of 78-kDa glucose-regulated protein (GRP78) precursor in NCCIT extract-treated cells compared to 293T cells for as long as 12 weeks suggests that GRP78 is an important component of the reprogrammed cells (Paper III). GRP78 is localized to the endoplasmic reticulum in normal tissues, and functions in Ca^{2+} homeostasis and the correct

folding of proteins (Shani et al., 2008). However, GRP78 is localized to the plasma membrane of tumor cells and plays a role in cellular proliferation, motility and survival by blocking the antiproliferative effects of TGF-β signalling(Gray et al., 2006; Shani et al., 2008). Moreover, GRP78 is upregulated at the 2-cell stage and silenced at the blastula stage in early mouse development, and knock-out studies have shown that GRP78 is required for cell proliferation and protecting the ICM from apoptosis (Luo et al., 2006). GRP78 co-localizes with the developmental oncoprotein Cripto (Shani et al., 2008) where GRP78 is required for correct Cripto signalling in human tumor, mammary epithelial and embryonic stem cells via both the MAPK/PI3K and Smad2/3 pathways (Kelber et al., 2009). The Cripto/GRP78 complex is expressed at the surface of hES cells and NCCIT cells and knockdown of GRP78 disrupts Cripto effects on Smad2/3 signaling (Kelber et al., 2009). 293T cells also express GRP78, but at very low levels and not to the same extent as NCCIT (Paper III, (Shani et al., 2008). However, 293T cells do not express endogenous Cripto (Kelber et al., 2009; Shani et al., 2008). It would be interesting to know whether the increased expression of GRP78 in extract-treated cells is due to initiation of translation of Cripto in these cells. If this is the case, these two proteins could be potential candidates for promoting cellular proliferation and maintaining pluripotency in the extract-treated cells.

CONCLUSIONS AND PERSPECTIVES

An important remaining question is whether extract-based reprogramming is effective in primary cells? Attempts reported in Papers I and II use epithelial 293T cells and NIH3T3 fibrobasts. One possibility is that the immortalized or transformed state of NIH3T3 cells creates an environment favorable to reprogramming. Support for this view is lent by recent reports that the immortalization state of cells to be reprogrammed by viral transduction enhances the efficiency of reprogramming by promoting the proliferative state (Marion et al., 2009; Utikal et al., 2009b). Furthermore, 293T cells are modified to constitutively express the large tumor-antigen (T-antigen) of simian virus 40 (SV40). The SV40 large T-antigen is a multifunctional viral protein involved in viral and cellular transcriptional regulation, viron assembly, viral DNA replication and alteration of the cell cycle (Sullivan and Pipas, 2002). Incidently, it was reported that, when used in conjunction with transcription factors such as Oct4 and Sox2, large T could replace Klf4 and c-Myc in the generation of human iPS cells (Mali et al., 2008). 293T cells already express some *OCT4* and *SOX2* transcripts and might therefore be easier to reprogram than, e.g., primary fibroblasts. Preliminary work from our laboratory supports this view.

Extract-based reprogramming constitutes an attractive method elucidating molecular mechanisms of reprogramming. Experimental approaches and results from Paper I indicate that the levels of some extract factors can be manipulated, for example by immunodepletion. It should also be possible to prepare extracts from mES cells with a gene knock-out for potentially critical components, such as Dnmts or histone methyltransferases, and thereby deermine the effect of these factors on nuclear reprogramming. Conversely, it is also straightforward to add derfine factors to the extract, which migh be expected to enhance reprogramming efficiency. It would also be relatively easy to treat target cells prior to, or after (or both), extract treatment with e.g., epigenetic modifiers, as discussed above in the iPS system. One migh hope that such combinations will assist in reprogramming primary cells. This option would be attractive in a

therapeutic context because, unlike retroviral-mediated transduction of reprogramming factors (the “Yamanaka approach”), extract-mediated reprogramming is non-genetic – and thus may be more easily ethically acceptable.

REFERENCES

Amabile, G. and Meissner, A. (2009). Induced pluripotent stem cells: current progress and potential for regenerative medicine. *Trends Mol. Med.* 15, 59-68.

Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N. and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* 17, 126-140.

Azuara, V., Perry, P., Sauer, S., Spivakov, M., Jorgensen, H. F., John, R. M., Gouti, M., Casanova, M., Warnes, G., Merkenschlager, M. et al. (2006). Chromatin signatures of pluripotent cell lines. *Nat. Cell Biol.* 8, 532-538.

Bao, L., Zhou, M. and Cui, Y. (2008). CTCFBSDB: a CTCF-binding site database for characterization of vertebrate genomic insulators. *Nucleic Acids Res.* 36, D83-D87.

Barrand, S. and Collas, P. (2010). Chromatin states of core pluripotency-associated genes in pluripotent, multipotent and differentiated cells. *Biochem. Biophys. Res. Commun.* 391, 762-767.

Bernstein, B. E., Mikkelsen, T. S., Xie, X., Kamal, M., Huebert, D. J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K. et al. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 125, 315-326.

Bhakdi, S., Tranum-Jensen, J. and Sziegoleit, A. (1985). Mechanism of membrane damage by streptolysin-O. *Infect. Immun.* 47, 52-60.

Bhakdi, S., Weller, U., Walev, I., Martin, E., Jonas, D. and Palmer, M. (1993). A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes. *Med. Microbiol. Immunol.* 182, 167-175.

Bibikova, M., Chudin, E., Wu, B., Zhou, L., Garcia, E. W., Liu, Y., Shin, S., Plaia, T. W., Auerbach, J. M., Arking, D. E. et al. (2006). Human embryonic stem cells have a unique epigenetic signature. *Genome Res.* 16, 1075-1083.

Blelloch, R., Venere, M., Yen, J. and Ramalho-Santos, M. (2007). Generation of induced pluripotent stem cells in the absence of drug selection. *Cell Stem Cell* 1, 245-247.

Blelloch, R., Wang, Z., Meissner, A., Pollard, S., Smith, A. and Jaenisch, R. (2006). Reprogramming efficiency following somatic cell nuclear transfer is influenced by the differentiation and methylation state of the donor nucleus. *Stem Cells* 24, 2007-2013.

Boiani, M. and Scholer, H. R. (2005). Regulatory networks in embryo-derived pluripotent stem cells. *Nat. Rev. Mol. Cell Biol.* 6, 872-884.

Boland, M. J., Hazen, J. L., Nazor, K. L., Rodriguez, A. R., Gifford, W., Martin, G., Kupriyanov, S. and Baldwin, K. K. (2009). Adult mice generated from induced pluripotent stem cells. *Nature* 461, 91-94.

Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G. et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122, 947-956.

Boyer, L. A., Mathur, D. and Jaenisch, R. (2006a). Molecular control of pluripotency. *Curr. Opin. Genet. Dev.* 16, 455-462.

Boyer, L. A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L. A., Lee, T. I., Levine, S. S., Wernig, M., Tajonar, A., Ray, M. K. et al. (2006b). Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 441, 349-353.

Bracken, A. P., Dietrich, N., Pasini, D., Hansen, K. H. and Helin, K. (2006). Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. *Genes Dev.* 20, 1123-1136.

Brockes, J. P. and Kumar, A. (2002). Plasticity and reprogramming of differentiated cells in amphibian regeneration. *Nat. Rev. Mol. Cell Biol.* 3, 566-574.

Bru, T., Clarke, C., McGrew, M. J., Sang, H. M., Wilmut, I. and Blow, J. J. (2008). Rapid induction of pluripotency genes after exposure of human somatic cells to mouse ES cell extracts. *Exp. Cell Res.* 314, 2634-2642.

Buehr, M., Meek, S., Blair, K., Yang, J., Ure, J., Silva, J., McLay, R., Hall, J., Ying, Q. L. and Smith, A. (2008). Capture of authentic embryonic stem cells from rat blastocysts. *Cell* 135, 1287-1298.

Burnside, A. S. and Collas, P. (2002). Induction of Oct-3/4 expression in somatic cells by gap junction-mediated cAMP signaling from blastomeres. *Eur. J. Cell Biol.* 81, 585-591.

Byrne, J. A., Simonsson, S., Western, P. S. and Gurdon, J. B. (2003). Nuclei of adult mammalian somatic cells are directly reprogrammed to oct-4 stem cell gene expression by amphibian oocytes. *Curr. Biol.* 13, 1206-1213.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S. and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 298, 1039-1043.

Cao, R. and Zhang, Y. (2004). The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. *Curr. Opin. Genet. Dev.* 14, 155-164.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113, 643-655.

Chambers, I. and Tomlinson, S. R. (2009). The transcriptional foundation of pluripotency. *Development.* 136, 2311-2322.

Chan, E. M., Ratanasirintrawoot, S., Park, I. H., Manos, P. D., Loh, Y. H., Huo, H., Miller, J. D., Hartung, O., Rho, J., Ince, T. A. et al. (2009). Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. *Nat. Biotechnol.* 27, 1033-1037.

Chang, K. H., Lim, J. M., Kang, S. K., Lee, B. C., Moon, S. Y. and Hwang, W. S. (2003). Blastocyst formation, karyotype, and mitochondrial DNA of interspecies embryos derived from nuclear transfer of human cord fibroblasts into enucleated bovine oocytes. *Fertil. Steril.* 80, 1380-1387.

Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce, d. L. F. and Robl, J. M. (1998). Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. *Nat. Biotechnol.* 16, 642-646.

Clark, L. D., Clark, R. K. and Heber-Katz, E. (1998). A new murine model for mammalian wound repair and regeneration. *Clin. Immunol. Immunopathol.* 88, 35-45.

Clark, S. J. (2007). Action at a distance: epigenetic silencing of large chromosomal regions in carcinogenesis. *Hum. Mol. Genet.* 16 Spec No 1, R88-R95.

Collas, P. (2007). Dedifferentiation of cells: new approaches. *Cytotherapy* 9, 236-244.

Collas, P. (2009). Epigenetic states in stem cells. *Biochim. Biophys. Acta* 1790, 900-905.

Collas, P. and Håkelien, A. M. (2003). Teaching cells new tricks. *Trends Biotechnol.* 21, 354-361.

Collas, P., Noer, A. and Timoskainen, S. (2007). Programming the genome in embryonic and somatic stem cells. *J. Cell Mol. Med.* 11, 602-620.

Constantinescu, D., Gray, H. L., Sammak, P. J., Schatten, G. P. and Csoka, A. B. (2006). Lamin A/C expression is a marker of mouse and human embryonic stem cell differentiation. *Stem Cells* 24, 177-185.

Cowan, C. A., Atienza, J., Melton, D. A. and Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science* 309, 1369-1373.

Damjanov, I. (1993). Teratocarcinoma: neoplastic lessons about normal embryogenesis. *Int. J. Dev. Biol.* 37, 39-46.

Deb-Rinker, P., Ly, D., Jezierski, A., Sikorska, M. and Walker, P. R. (2005). Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. *J. Biol. Chem.* 280, 6257-6260.

Do, J. T., Han, D. W., Gentile, L., Sobek-Klocke, I., Stehling, M., Lee, H. T. and Scholer, H. R. (2007). Erasure of cellular memory by fusion with pluripotent cells. *Stem Cells.* 25, 1013-1020.

Do, J. T., Han, D. W., Gentile, L., Sobek-Klocke, I., Stehling, M. and Scholer, H. R. (2008). Enhanced reprogramming of Xist by induced upregulation of Tsix and Dnmt3a. *Stem Cells.* 26, 2821-2831.

Do, J. T., Han, D. W., Gentile, L., Sobek-Klocke, I., Wutz, A. and Scholer, H. R. (2009a). Reprogramming of Xist against the pluripotent state in fusion hybrids. *J. Cell Sci.* 122, 4122-4129.

Do, J. T., Han, D. W. and Scholer, H. R. (2006). Reprogramming somatic gene activity by fusion with pluripotent cells. *Stem Cell Rev.* 2, 257-264.

Do, J. T., Joo, J. Y., Han, D. W., rauzo-Bravo, M. J., Kim, M. J., Greber, B., Zaehres, H., Sobek-Klocke, I., Chung, H. M. and Scholer, H. R. (2009b). Generation of Parthenogenetic iPS Cells from Parthenogenetic Neural Stem Cells. *Stem Cells.*

Do, J. T. and Scholer, H. R. (2009). Regulatory circuits underlying pluripotency and reprogramming. *Trends Pharmacol. Sci.* 30, 296-302.

Donovan, P. J. and Gearhart, J. (2001). The end of the beginning for pluripotent stem cells. *Nature* 414, 92-97.

Ebert, A. D., Yu, J., Rose, F. F., Jr., Mattis, V. B., Lorson, C. L., Thomson, J. A. and Svendsen, C. N. (2009). Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature* 457, 277-280.

Evans, M. J. and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154-156.

Fawcett, J. M., Harrison, S. M. and Orchard, C. H. (1998). A method for reversible permeabilization of isolated rat ventricular myocytes. *Exp. Physiol* 83, 293-303.

Feng, B., Jiang, J., Kraus, P., Ng, J. H., Heng, J. C., Chan, Y. S., Yaw, L. P., Zhang, W., Loh, Y. H., Han, J. et al. (2009a). Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. *Nat. Cell Biol.* 11, 197-203.

Feng, B., Ng, J. H., Heng, J. C. and Ng, H. H. (2009b). Molecules that promote or enhance reprogramming of somatic cells to induced pluripotent stem cells. *Cell Stem Cell* 4, 301-312.

Filipowicz, W., Bhattacharyya, S. N. and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* 9, 102-114.

Flasza, M., Shering, A. F., Smith, K., Andrews, P. W., Talley, P. and Johnson, P. A. (2003). Reprogramming in inter-species embryonal carcinoma-somatic cell hybrids induces expression of pluripotency and differentiation markers. *Cloning Stem Cells* 5, 339-354.

Fouse, S. D., Shen, Y., Pellegrini, M., Cole, S., Meissner, A., Van, N. L., Jaenisch, R. and Fan, G. (2008). Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. *Cell Stem Cell* 2, 160-169.

Galiova, G., Bartova, E., Raska, I., Krejci, J. and Kozubek, S. (2008). Chromatin changes induced by lamin A/C deficiency and the histone deacetylase inhibitor trichostatin A. *Eur. J. Cell Biol.* 87, 291-303.

Gao, S., Gasparrini, B., McGarry, M., Ferrier, T., Fletcher, J., Harkness, L., De, S. P. and Wilmut, I. (2002). Germinal vesicle material is essential for nucleus remodeling after nuclear transfer. *Biol. Reprod.* 67, 928-934.

Gardiner-Garden, M. and Frommer, M. (1987). CpG islands in vertebrate genomes. *J. Mol. Biol.* 196, 261-282.

Gaustad, K. G., Boquest, A. C., Anderson, B. E., Gerdes, A. M. and Collas, P. (2004). Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes. *Biochem. Biophys. Res. Commun.* 314, 420-427.

Goll, M. G., Kirpekar, F., Maggert, K. A., Yoder, J. A., Hsieh, C. L., Zhang, X., Golic, K. G., Jacobsen, S. E. and Bestor, T. H. (2006). Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science.* %20;311, 395-398.

Gray, P. C., Shani, G., Aung, K., Kelber, J. and Vale, W. (2006). Cripto binds transforming growth factor beta (TGF-beta) and inhibits TGF-beta signaling. *Mol. Cell Biol.* 26, 9268-9278.

Guenther, M. G., Levine, S. S., Boyer, L. A., Jaenisch, R. and Young, R. A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. *Cell* 130, 77-88.

Guilly, M.-N., Kobl, J.-P., Gosti, F., Godeau, F. and Courvalin, J.-C. (1990). Lamins A and C are not expressed at early stages of human lymphocyte differentiation. *Exp. Cell Res.* 189, 145-147.

Gurdon, J. B. and Byrne, J. A. (2003). The first half-century of nuclear transplantation. *Proc. Natl. Acad. Sci. U. S. A* 100, 8048-8052.

Hakelien, A. M. and Collas, P. (2002). Novel approaches to transdifferentiation. *Cloning Stem Cells* 4, 379-387.

Håkelien, A. M., Gaustad, K. G., Taranger, C. K., Skålhegg, B. S., Kuntziger, T. and Collas, P. (2005). Long-term in vitro, cell-type-specific genome-wide reprogramming of gene expression. *Exp. Cell Res.* 309, 32-47.

Håkelien, A. M., Landsverk, H. B., Robl, J. M., Skålhegg, B. S. and Collas, P. (2002). Reprogramming fibroblasts to express T-cell functions using cell extracts. *Nat. Biotechnol.* 20, 460-466.

Hallson, G., Syrzycka, M., Beck, S. A., Kennison, J. A., Dorsett, D., Page, S. L., Hunter, S. M., Keall, R., Warren, W. D., Brock, H. W. et al. (2008). The Drosophila cohesin subunit Rad21 is a trithorax group (trxG) protein. *Proc. Natl. Acad. Sci. U. S. A* 105, 12405-12410.

Han, D. W., Do, J. T., Gentile, L., Stehling, M., Lee, H. T. and Scholer, H. R. (2008). Pluripotential reprogramming of the somatic genome in hybrid cells occurs with the first cell cycle. *Stem Cells.* 26, 445-454.

Hanna, J., Wernig, M., Markoulaki, S., Sun, C. W., Meissner, A., Cassady, J. P., Beard, C., Brambrink, T., Wu, L. C., Townes, T. M. et al. (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* 318, 1920-1923.

Hansis, C., Barreto, G., Maltry, N. and Niehrs, C. (2004). Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. *Curr. Biol.* 14, 1475-1480.

Hattori, N., Nishino, K., Ko, Y. G., Hattori, N., Ohgane, J., Tanaka, S. and Shiota, K. (2004). Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. *J. Biol. Chem.* 279, 17063-17069.

Heber-Katz, E. (1999). The regenerating mouse ear. *Semin. Cell Dev. Biol.* 10, 415-419.

Heber-Katz, E., Chen, P., Clark, L., Zhang, X. M., Troutman, S. and Blankenhorn, E. P. (2004a). Regeneration in MRL mice: further genetic loci controlling the ear hole closure trait using MRL and M.m. Castaneus mice. *Wound. Repair Regen.* 12, 384-392.

Heber-Katz, E., Leferovich, J., Bedelbaeva, K., Gourevitch, D. and Clark, L. (2004b). The scarless heart and the MRL mouse. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 359, 785-793.

Hellman, A. and Chess, A. (2007). Gene body-specific methylation on the active X chromosome. *Science* 315, 1141-1143.

Huangfu, D., Maehr, R., Guo, W., Eijkelenboom, A., Snitow, M., Chen, A. E. and Melton, D. A. (2008). Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat. Biotechnol.* 26, 795-797.

Jackson, M., Krassowska, A., Gilbert, N., Chevassut, T., Forrester, L., Ansell, J. and Ramsahoye, B. (2004). Severe global DNA hypomethylation blocks differentiation and induces histone hyperacetylation in embryonic stem cells. *Mol. Cell Biol.* 24, 8862-8871.

Jacquier, A. (2009). The complex eukaryotic transcriptome: unexpected pervasive transcription and novel small RNAs. *Nat. Rev. Genet.* 10, 833-844.

Jaenisch, R. and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* 33 Suppl, 245-254.

Jaenisch, R. and Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell.* 132, 567-582.

Jenuwein, T. and Allis, C. D. (2001). Translating the histone code. *Science* 293, 1074-1080.

Kelber, J. A., Panopoulos, A. D., Shani, G., Booker, E. C., Belmonte, J. C., Vale, W. W. and Gray, P. C. (2009). Blockade of Cripto binding to cell surface GRP78 inhibits oncogenic Cripto signaling via MAPK/PI3K and Smad2/3 pathways. *Oncogene* 28, 2324-2336.

Kennison, J. A. (1995). The Polycomb and trithorax group proteins of Drosophila: trans-regulators of homeotic gene function. *Annu. Rev. Genet.* 29:289-303., 289-303.

Kerr, C. L., Shamblott, M. J. and Gearhart, J. D. (2006). Pluripotent stem cells from germ cells. *Methods Enzymol.* 419, 400-426.

Kikyo, N. and Wolffe, A. P. (2000). Reprogramming nuclei: insights from cloning, nuclear transfer and heterokaryons. *J. Cell Sci.* 113, 11-20.

Kim, J., Chu, J., Shen, X., Wang, J. and Orkin, S. H. (2008). An extended transcriptional network for pluripotency of embryonic stem cells. *Cell* 132, 1049-1061.

Kim, J. B., Sebastiano, V., Wu, G., rauzo-Bravo, M. J., Sasse, P., Gentile, L., Ko, K., Ruau, D., Ehrich, M., van den, B. D. et al. (2009). Oct4-induced pluripotency in adult neural stem cells. *Cell.* 136, 411-419.

Kim, T. and Buratowski, S. (2009). Dimethylation of H3K4 by Set1 recruits the Set3 histone deacetylase complex to 5' transcribed regions. *Cell.* 137, 259-272.

Kimura, H., Tada, M., Nakatsuji, N. and Tada, T. (2004). Histone code modifications on pluripotential nuclei of reprogrammed somatic cells. *Mol. Cell Biol.* 24, 5710-5720.

Kingston, R. E. and Narlikar, G. J. (1999). ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev.* 13, 2339-2352.

Klose, R. J. and Bird, A. P. (2006). Genomic DNA methylation: the mark and its mediators. *Trends Biochem. Sci.* 31, 89-97.

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell* 128, 693-705.

Lachner, M. and Jenuwein, T. (2002). The many faces of histone lysine methylation. *Curr. Opin. Cell Biol.* 14, 286-298.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 410, 116-120.

Landsverk, H. B., Håkelien, A. M., Küntziger, T., Robl, J. M., Skålhegg, B. S. and Collas, P. (2002). Reprogrammed gene expression in a somatic cell-free extract. *EMBO Rep.* 3, 384-389.

Lee, T. I., Jenner, R. G., Boyer, L. A., Guenther, M. G., Levine, S. S., Kumar, R. M., Chevalier, B., Johnstone, S. E., Cole, M. F., Isono, K. et al. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* 125, 301-313.

Leferovich, J. M. and Heber-Katz, E. (2002). The scarless heart. *Semin. Cell Dev. Biol.* 13, 327-333.

Li, W., Zhou, H., Abujarour, R., Zhu, S., Joo, J. Y., Lin, T., Hao, E., Scholer, H. R., Hayek, A. and Ding, S. (2009). Generation of Human Induced Pluripotent Stem Cells in the Absence of Exogenous Sox2. *Stem Cells.*

Lin, T., Chao, C., Saito, S., Mazur, S. J., Murphy, M. E., Appella, E. and Xu, Y. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat. Cell Biol.* 7, 165-171.

Lin, T. A., Tsay, C., Chen, C. H., Tang, P. C. and Ju, J. C. (2008). Nuclear and cytoskeletal dynamics during oocyte maturation and development of somatic cell cloned pig embryos injected with membrane disintegrated donor cells. *Anim Reprod. Sci.* 103, 107-119.

Liu, K., Wang, Y. F., Cantemir, C. and Muller, M. T. (2003). Endogenous assays of DNA methyltransferases: Evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells in vivo. *Mol. Cell Biol.* 23, 2709-2719.

Loh, Y. H., Wu, Q., Chew, J. L., Vega, V. B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J. et al. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.* 38, 431-440.

Luo, S., Mao, C., Lee, B. and Lee, A. S. (2006). GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early mouse embryonic development. *Mol. Cell Biol.* 26, 5688-5697.

Maherali, N. and Hochedlinger, K. (2008). Guidelines and techniques for the generation of induced pluripotent stem cells. *Cell Stem Cell* 3, 595-605.

Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R. et al. (2007). Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell.* 1, 55-70.

Mali, P., Ye, Z., Hommond, H. H., Yu, X., Lin, J., Chen, G., Zou, J. and Cheng, L. (2008). Improved efficiency and pace of generating induced pluripotent stem cells from human adult and fetal fibroblasts. *Stem Cells* 26, 1998-2005.

Marion, R. M., Strati, K., Li, H., Murga, M., Blanco, R., Ortega, S., Fernandez-Capetillo, O., Serrano, M. and Blasco, M. A. (2009). A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. *Nature* 460, 1149-1153.

Matsumura, H., Tada, M., Otsuji, T., Yasuchika, K., Nakatsuji, N., Surani, A. and Tada, T. (2006). Targeted chromosome elimination from ES-somatic hybrid cells. *Nat. Methods* 4, 23-25.

McGann, C. J., Odelberg, S. J. and Keating, M. T. (2001). Mammalian myotube dedifferentiation induced by newt regeneration extract. *Proc. Natl. Acad. Sci. U. S. A* %20;98, 13699-13704.

Meissner, A., Mikkelsen, T. S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X., Bernstein, B. E., Nusbaum, C., Jaffe, D. B. et al. (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 454, 766-770.

Meissner, A., Wernig, M. and Jaenisch, R. (2007). Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nat. Biotechnol.* 25, 1177-1181.

Mercer, T. R., Dinger, M. E. and Mattick, J. S. (2009). Long non-coding RNAs: insights into functions. *Nat. Rev. Genet.* 10, 155-159.

Meshorer, E., Yellajoshula, D., George, E., Scambler, P. J., Brown, D. T. and Misteli, T. (2006). Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. *Dev. Cell* 10, 105-116.

Mikkelsen, T. S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B. E., Jaenisch, R., Lander, E. S. and Meissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. *Nature* 454, 49-55.

Mikkelsen, T. S., Ku, M., Jaffe, D. B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim, T. K., Koche, R. P. et al. (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* 448, 553-560.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M. and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell.* 113, 631-642.

Miyamoto, K., Furusawa, T., Ohnuki, M., Goel, S., Tokunaga, T., Minami, N., Yamada, M., Ohsumi, K. and Imai, H. (2007). Reprogramming events of mammalian somatic cells induced by Xenopus laevis egg extracts. *Mol. Reprod. Dev.* 74, 1268-1277.

Miyamoto, K., Tsukiyama, T., Yang, Y., Li, N., Minami, N., Yamada, M. and Imai, H. (2009). Cell-free extracts from mammalian oocytes partially induce nuclear reprogramming in somatic cells. *Biol. Reprod.* 80, 935-943.

Morgan, H. D., Santos, F., Green, K., Dean, W. and Reik, W. (2005). Epigenetic reprogramming in mammals. *Hum. Mol. Genet.* 14, R47-R58.

Morgan, T. H. (1901). REGENERATION AND LIABILITY TO INJURY. *Science* 14, 235-248.

Munsie, M. J., Michalska, A. E., O'Brien, C. M., Trounson, A. O., Pera, M. F. and Mountford, P. S. (2000). Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. *Curr. Biol.* 10, 989-992.

Nesterova, T. B., Popova, B. C., Cobb, B. S., Norton, S., Senner, C. E., Tang, Y. A., Spruce, T., Rodriguez, T. A., Sado, T., Merkenschlager, M. et al. (2008). Dicer regulates Xist promoter methylation in ES cells indirectly through transcriptional control of Dnmt3a. *Epigenetics. Chromatin.* 1, 2.

Niwa, H., Miyazaki, J. and Smith, A. G. (2000a). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* 24, 372-376.

Niwa, H., Miyazaki, J. and Smith, A. G. (2000b). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* 24, 372-376.

Otte, A. P. and Kwaks, T. H. (2003). Gene repression by Polycomb group protein complexes: a distinct complex for every occasion? *Curr. Opin. Genet. Dev.* 13, 448-454.

Ozsolak, F., Song, J. S., Liu, X. S. and Fisher, D. E. (2007). High-throughput mapping of the chromatin structure of human promoters. *Nat. Biotechnol.* 25, 244-248.

Pan, G., Tian, S., Nie, J., Yang, C., Ruotti, V., Wei, H., Jonsdottir, G. A., Stewart, R. and Thomson, J. A. (2007). Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. *Cell Stem Cell* 1, 299-312.

Park, I. H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., Lensch, M. W., Cowan, C., Hochedlinger, K. and Daley, G. Q. (2008). Disease-specific induced pluripotent stem cells. *Cell* 134, 877-886.

Pells, S., Di Domenico, A. I., Callagher, E. J. and McWhir, J. (2002). Multipotentiality of neuronal cells after spontaneous fusion with embryonic stem cells and nuclear reprogramming in vitro. *Cloning Stem Cells* 4, 331-338.

Pesce, M. and Scholer, H. R. (2000). Oct-4: control of totipotency and germline determination. *Mol. Reprod. Dev.* 55, 452-457.

Pralong, D., Trounson, A. O. and Verma, P. J. (2006). Cell fusion for reprogramming pluripotency: toward elimination of the pluripotent genome. *Stem Cell Rev.* 2, 331-340.

Pray-Grant, M. G., Daniel, J. A., Schieltz, D., Yates, J. R., III and Grant, P. A. (2005). Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. *Nature* 433, 434-438.

Przyborski, S. A., Christie, V. B., Hayman, M. W., Stewart, R. and Horrocks, G. M. (2004). Human embryonal carcinoma stem cells: models of embryonic development in humans. *Stem Cells Dev.* 13, 400-408.

Pusarla, R. H., Vinayachandran, V. and Bhargava, P. (2007). Nucleosome positioning in relation to nucleosome spacing and DNA sequence-specific binding of a protein. *FEBS J.* 274, 2396-2410.

Qin, M., Tai, G., Collas, P., Polak, J. M. and Bishop, A. E. (2005). Cell extract-derived differentiation of embryonic stem cells. *Stem Cells* 23, 712-718.

Razin, A. and Shemer, R. (1995). DNA methylation in early development. *Hum. Mol. Genet.* 4, 1751-1755.

Reik, W., Collick, A., Norris, M. L., Barton, S. C. and Surani, M. A. (1987). Genomic imprinting determines methylation of parental alleles in transgenic mice. *Nature* 328, 248-251.

Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A. and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotechnol.* 18, 399-404.

Rideout, W. M., III, Hochedlinger, K., Kyba, M., Daley, G. Q. and Jaenisch, R. (2002). Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* 109, 17-27.

Ringrose, L. and Paro, R. (2007). Polycomb/Trithorax response elements and epigenetic memory of cell identity. *Development* 134, 223-232.

Rubio, E. D., Reiss, D. J., Welcsh, P. L., Disteche, C. M., Filippova, G. N., Baliga, N. S., Aebersold, R., Ranish, J. A. and Krumm, A. (2008). CTCF physically links cohesin to chromatin. *Proc. Natl. Acad. Sci. U. S. A* 105, 8309-8314.

Sakai, Y. and Takeuchi, I. (1971). Changes of the prespore specific structure during dedifferentiation and cell type conversion of a slime mold cell. *Dev. Growth Differ.* 13, 231-240.

Sapienza, C., Peterson, A. C., Rossant, J. and Balling, R. (1987). Degree of methylation of transgenes is dependent on gamete of origin. *Nature* 328, 251-254.

Scholer, H. R., Ruppert, S., Suzuki, N., Chowdhury, K. and Gruss, P. (1990). New type of POU domain in germ line-specific protein Oct-4. *Nature.* 344, 435-439.

Schopperle, W. M. and DeWolf, W. C. (2007). The TRA-1-60 and TRA-1-81 human pluripotent stem cell markers are expressed on podocalyxin in embryonal carcinoma. *Stem Cells* 25, 723-730.

Schubeler, D., MacAlpine, D. M., Scalzo, D., Wirbelauer, C., Kooperberg, C., van, L. F., Gottschling, D. E., O'Neill, L. P., Turner, B. M., Delrow, J. et al. (2004). The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. *Genes Dev.* 18, 1263-1271.

Shamblott, M. J., Axelman, J., Wang, S., Bugg, E. M., Littlefield, J. W., Donovan, P. J., Blumenthal, P. D., Huggins, G. R. and Gearhart, J. D. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl. Acad. Sci. U. S. A* 95, 13726-13731.

Shani, G., Fischer, W. H., Justice, N. J., Kelber, J. A., Vale, W. and Gray, P. C. (2008). GRP78 and Cripto form a complex at the cell surface and collaborate to inhibit transforming growth factor beta signaling and enhance cell growth. *Mol. Cell Biol.* 28, 666-677.

Shi, Y., Do, J. T., Desponts, C., Hahm, H. S., Scholer, H. R. and Ding, S. (2008). A combined chemical and genetic approach for the generation of induced pluripotent stem cells. *Cell Stem Cell.* 2, 525-528.

Silva, J., Barrandon, O., Nichols, J., Kawaguchi, J., Theunissen, T. W. and Smith, A. (2008). Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS. Biol.* 6, e253.

Simonsson, S. and Gurdon, J. (2004). DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. *Nat. Cell Biol.* 6, 984-990.

Sperger, J. M., Chen, X., Draper, J. S., Antosiewicz, J. E., Chon, C. H., Jones, S. B., Brooks, J. D., Andrews, P. W., Brown, P. O. and Thomson, J. A. (2003). Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. *Proc. Natl. Acad. Sci. U. S. A.* 100, 13350-13355.

Sridharan, R., Tchieu, J., Mason, M. J., Yachechko, R., Kuoy, E., Horvath, S., Zhou, Q. and Plath, K. (2009). Role of the murine reprogramming factors in the induction of pluripotency. *Cell* 136, 364-377.

Stefani, G. and Slack, F. J. (2008). Small non-coding RNAs in animal development. *Nat. Rev. Mol. Cell Biol.* 9, 219-230.

Stevenson, D. S. and Jarvis, P. (2003). Chromatin silencing: RNA in the driving seat. *Curr. Biol.* 13, R13-R15.

Stocum, D. L. (2004). Amphibian regeneration and stem cells. *Curr. Top. Microbiol. Immunol.* 280, 1-70.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev.* 12, 599-606.

Sullivan, C. S. and Pipas, J. M. (2002). T antigens of simian virus 40: molecular chaperones for viral replication and tumorigenesis. *Microbiol. Mol. Biol. Rev.* 66, 179-202.

Tada, M., Tada, T., Lefebvre, L., Barton, S. C. and Surani, M. A. (1997). Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *EMBO J.* 16, 6510-6520.

Tada, M., Takahama, Y., Abe, K., Nakastuji, N. and Tada, T. (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Curr. Biol.* 11, 1553-1558.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861-872.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663-676.

Takai, D. and Jones, P. A. (2002). Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc. Natl. Acad. Sci. U. S. A.* 99, 3740-3745.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E. and Scott, E. W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* 416, 542-545.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145-1147.

Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., Becker, R. A. and Hearn, J. P. (1995). Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. U. S. A* 92, 7844-7848.

Turek-Plewa, J. and Jagodzinski, P. P. (2005). The role of mammalian DNA methyltransferases in the regulation of gene expression. *Cell Mol. Biol. Lett.* 10, 631-647.

Utikal, J., Maherali, N., Kulalert, W. and Hochedlinger, K. (2009a). Sox2 is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. *J. Cell Sci.* 122, 3502-3510.

Utikal, J., Polo, J. M., Stadtfeld, M., Maherali, N., Kulalert, W., Walsh, R. M., Khalil, A., Rheinwald, J. G. and Hochedlinger, K. (2009b). Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. *Nature* 460, 1145-1148.

Wada, Y., Ohta, Y., Xu, M., Tsutsumi, S., Minami, T., Inoue, K., Komura, D., Kitakami, J., Oshida, N., Papantonis, A. et al. (2009). A wave of nascent transcription on activated human genes. *Proc. Natl. Acad. Sci. U. S. A* 106, 18357-18361.

Wakayama, T., Tabar, V., Rodriguez, I., Perry, A. C., Studer, L. and Mombaerts, P. (2001). Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. *Science* 292, 740-743.

Walev, I., Bhakdi, S. C., Hofmann, F., Djonder, N., Valeva, A., Aktories, K. and Bhakdi, S. (2001). Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. *Proc. Natl. Acad. Sci. U. S. A* 98, 3185-3190.

Weber, M., Hellmann, I., Stadler, M. B., Ramos, L., Paabo, S., Rebhan, M. and Schubeler, D. (2007). Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat. Genet.* 39, 457-466.

Welstead, G. G., Schorderet, P. and Boyer, L. A. (2008). The reprogramming language of pluripotency. *Curr. Opin. Genet. Dev.* 18, 123-129.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B. E. and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448, 318-324.

Wilmut, I., Beaujean, N., De Sousa, P. A., Dinnyes, A., King, T. J., Paterson, L. A., Wells, D. N. and Young, L. E. (2002). Somatic cell nuclear transfer. *Nature* 419, 583-586.

Woltjen, K., Michael, I. P., Mohseni, P., Desai, R., Mileikovsky, M., Hamalainen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M. et al. (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature.* 458, 766-770.

Ying, Q. L., Nichols, J., Evans, E. P. and Smith, A. G. (2002). Changing potency by spontaneous fusion. *Nature* 416, 545-548.

Yoder, J. A., Walsh, C. P. and Bestor, T. H. (1997). Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* 13, 335-340.

You, J. S., Kang, J. K., Seo, D. W., Park, J. H., Park, J. W., Lee, J. C., Jeon, Y. J., Cho, E. J. and Han, J. W. (2009). Depletion of embryonic stem cell signature by histone deacetylase inhibitor in NCCIT cells: involvement of Nanog suppression. *Cancer Res.* 69, 5716-5725.

Zhao, X. D., Han, X., Chew, J. L., Liu, J., Chiu, K. P., Choo, A., Orlov, Y. L., Sung, W. K., Shahab, A., Kuznetsov, V. A. et al. (2007). Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. *Cell Stem Cell* 1, 286-298.

Zhao, X. Y., Li, W., Lv, Z., Liu, L., Tong, M., Hai, T., Hao, J., Guo, C. L., Ma, Q. W., Wang, L. et al. (2009). iPS cells produce viable mice through tetraploid complementation. *Nature.* 461, 86-90.

Zhu, X. Q., Pan, X. H., Wang, W., Chen, Q., Pang, R. Q., Cai, X. M., Hoffman, A. R. and Hu, J. F. (2009). Transient in vitro epigenetic reprogramming of skin fibroblasts into multipotent cells. *Biomaterials*.

Zvetkova, I., Apedaile, A., Ramsahoye, B., Mermoud, J. E., Crompton, L. A., John, R., Feil, R. and Brockdorff, N. (2005). Global hypomethylation of the genome in XX embryonic stem cells. *Nat. Genet.* 37, 1274-1279.

I

Molecular Biology of the Cell
Vol. 16, 5719–5735, December 2005

Induction of Dedifferentiation, Genomewide Transcriptional Programming, and Epigenetic Reprogramming by Extracts of Carcinoma and Embryonic Stem Cells[D]

Christel K. Taranger, Agate Noer, Anita L. Sørensen, Anne-Mari Håkelien, Andrew C. Boquest, and Philippe Collas

Department of Biochemistry, Institute of Basic Medical Sciences, University of Oslo, 0317 Oslo, Norway

Submitted June 28, 2005; Revised September 15, 2005; Accepted September 16, 2005
Monitoring Editor: Carl-Henrik Heldin

Functional reprogramming of a differentiated cell toward pluripotency may have long-term applications in regenerative medicine. We report the induction of dedifferentiation, associated with genomewide programming of gene expression and epigenetic reprogramming of an embryonic gene, in epithelial 293T cells treated with an extract of undifferentiated human NCCIT carcinoma cells. 293T cells exposed for 1 h to extract of NCCIT cells, but not of 293T or Jurkat T-cells, form defined colonies that are maintained for at least 23 passages in culture. Microarray and quantitative analyses of gene expression reveal that the transition from a 293T to a pluripotent cell phenotype involves a dynamic up-regulation of hundreds of NCCIT genes, concomitant with down-regulation of 293T genes and of indicators of differentiation such as A-type lamins. Up-regulated genes encompass embryonic and stem cell markers, including *OCT4*, *SOX2*, *NANOG*, and Oct4-responsive genes. *OCT4* activation is associated with DNA demethylation in the *OCT4* promoter and nuclear targeting of Oct4 protein. In fibroblasts exposed to extract of mouse embryonic stem cells, Oct4 activation is biphasic and RNA-PolII dependent, with the first transient rise of *Oct4* up-regulation being necessary for the second, long-term activation of *Oct4*. Genes characteristic of multilineage differentiation potential are also up-regulated in NCCIT extract-treated cells, suggesting the establishment of "multilineage priming." Retinoic acid triggers Oct4 down-regulation, de novo activation of A-type lamins, and nestin. Furthermore, the cells can be induced to differentiate toward neurogenic, adipogenic, osteogenic, and endothelial lineages. The data provide a proof-of-concept that an extract of undifferentiated carcinoma cells can elicit differentiation plasticity in an otherwise more developmentally restricted cell type.

INTRODUCTION

Differentiated cells are thought to be stably committed to their fate; however, there is evidence to indicate that dedifferentiation events can take place. Urodele amphibians and teleost fish can replace lost anatomical parts by a process of migration, dedifferentiation, proliferation, and redifferentiation of epithelial cells in the wounded area. Functional reprogramming of differentiated cell nuclei has also been illustrated by the derivation of pluripotent embryonic stem (ES) cells (ESCs) (Cibelli *et al*., 1998; Munsie *et al*., 2000; Wakayama *et al*., 2001; Hwang *et al*., 2004) and by the live birth of cloned animals (Wilmut *et al*., 2002; Gurdon and Byrne, 2003) after nuclear transplantation into unfertilized eggs. Notably, *Xenopus* eggs can reprogram mammalian somatic nuclei to express the POU family member homeodomain transcription factor gene *Oct4* (Byrne *et al*., 2003) by a process requiring DNA demethylation (Simonsson and Gurdon, 2004). DNA demethylation also occurs after fusion of mouse thymocytes with embryonic germ (EG) cells (EGCs) or ESCs (Tada *et al*., 1997, 2001; but interestingly, only EG cells are capable of demethylating imprinted genes (Tada *et al*., 1997). Similarly, fusion of neuronal progenitor cells (Pells *et al*., 2002; Ying *et al*., 2002) or bone marrow-derived cells (Terada *et al*., 2002) with ESCs results in hybrids that express markers of pluripotency (Pells *et al*., 2002), contribute to chimeras (Ying *et al*., 2002), and form teratomas (Terada *et al*., 2002). Similar observations resulted from fusing human fibroblasts with ESCs (Cowan *et al*., 2005). Fusion of embryonal carcinoma (EC) cells (ECCs) with T-lymphoma cells also promotes the formation of colonies expressing pluripotent cell transcripts from the lymphoma genome (Flasza *et al*., 2003). Thus, components of pluripotent EG, ES, or EC cells have the potential of eliciting reprogramming events in a somatic genome.

As an alternative to fusion, somatic nuclear function may also be altered using nuclear and cytoplasmic extracts, with the rationale that extracts provide the necessary regulatory components. Notably, extracts of regenerating newt limbs

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05–06–0572) on September 29, 2005.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

Address correspondence to: Philippe Collas (philippe.collas@medisin.uio.no).

Abbreviations used: CHX, cycloheximide; DRB, 5,6-dichloro-1-D-ribofuranosyl benzimidazole; ECC, embryonal carcinoma cell; EGC, embryonal germ cell; EGFP, enhanced green fluorescent protein; ESC, embryonic stem cell; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; LIF, leukemia inhibitory factor; NTP, nucleotide triphosphate; PBS, phosphate-buffered saline; SLO, streptolysin O.

promote cell cycle reentry and down-regulation of myogenic markers in differentiated myotubes (McGann *et al*., 2001). Furthermore, we have shown that kidney epithelial 293T cells permeabilized with streptolysin O (SLO) and briefly exposed to an extract of Jurkat T-cells take on T-cell properties, including growth in aggregates, chromatin remodeling, expression of T-cell-specific genes and surface receptors, secretion of interleukin-2, and stimulation-dependent assembly of the interleukin-2 receptor (Håkelien *et al*., 2002, 2005; Landsverk *et al*., 2002). Similarly, lysates of cardiomyocytes or insulinoma cells elicit expression of cardiomyocyte or β-cell markers in adipose stem cells (Gaustad *et al*., 2004) and fibroblasts (Håkelien *et al*., 2004), and a pneumocyte extract was recently shown to induce differentiation of ESCs into a pneumocyte phenotype (Qin *et al*., 2005). 293T cells were also shown to express pluripotency markers such *OCT4* and *GCAP* and down-regulate a kidney marker after coculture with extract of *Xenopus* eggs (Hansis *et al*., 2004). Despite these observations, evidence for induction of epigenetic reprogramming events in large numbers of cells by extracts is lacking.

Teratocarcinomas are a particular type of germ cell tumors that contain undifferentiated stem cells and differentiated derivatives that can include endoderm, mesoderm, and ectoderm germ layers (Chambers and Smith, 2004). Undifferentiated carcinoma cells can be cultured to give rise to lines of ECCs. ECCs form malignant teratocarcinomas when transplanted into ectopic sites; however, some ECC lines can also contribute to tissues of the developing fetus when introduced into a blastocyst (Blelloch *et al*., 2004). Undifferentiated human teratocarcinoma NCCIT cells were established from a mediastinal mixed germ cell tumor (Teshima *et al*., 1988). NCCIT is at a stage intermediate between a seminoma (a precursor of germ cell tumors) and an embryonal carcinoma (Damjanov *et al*., 1993). NCCIT is a developmentally pluripotent cell line that can differentiate into derivatives of all three embryonic germ layers and extraembryonic cell lineages (Damjanov *et al*., 1993).

This study tests the hypothesis that an extract of undifferentiated somatic cells can elicit dedifferentiation in a somatic cell line. Based on morphological and immunolabeling observations, gene expression profiling, DNA methylation assays, and functional assessments, we show that 293T and NIH3T3 cells can be programmed by extracts of undifferentiated NCCIT cells or mouse ES cells to acquire characteristics of pluripotency.

MATERIALS AND METHODS

Cells

NCCIT, Jurkat (clone E6-1), and 293T cells (American Type Culture Collection, Vanassas, MD) were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, and nonessential amino acids (complete RPMI 1640 medium). NIH3T3 Swiss-Albino fibroblasts (American Type Culture Collection) were cultured in DMEM (Sigma-Aldrich) with 10% FCS, L-glutamine, and 0.1 mM β-mercaptoethanol. Mouse ESCs were isolated from inner cell masses of strain sv129 blastocysts and plated on mouse fibroblast γ-irradiated feeder layers in ESC medium (DMEM, 15% FCS, 0.1 mM β-mercaptoethanol, nonessential amino acids, and 1% penicillin/streptomycin) supplemented with 1000 U/ml (10 ng/ml) recombinant leukemia inhibitory factor (LIF; Sigma-Aldrich) on gelatin-coated plates. Before harvesting for preparing extracts, ESCs were passaged and cultured under feeder-free conditions in RPMI 1640 medium containing 10 ng/ml LIF. Cells treated with NCCIT or 293T extract were seeded at 100,000 cells per well in a 48-well plate and cultured in 250 μl of complete RMPI 1640 medium with antibiotics. Cells exposed to ESC extract were cultured as ESCs with 10 ng/ml LIF under feeder-free conditions.

Cell Extracts

To prepare NCCIT extracts, cells were washed in phosphate-buffered saline (PBS) and in cell lysis buffer (100 mM HEPES, pH 8.2, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors), sedimented at 400 × *g*, resuspended in 1 volume of cold cell lysis buffer, and incubated for 30–45 min on ice. Cells were sonicated on ice in 200-μl aliquots using a Labsonic-M pulse sonicator fitted with a 3-mm-diameter probe (B. Braun Biotech, Melsungen, Germany) until all cells and nuclei were lysed, as judged by microscopy (our unpublished data). The lysate was sedimented at 15,000 × *g* for 15 min at 4°C to pellet the coarse material. The supernatant was aliquoted, frozen in liquid nitrogen, and stored for up to 9 mo at −80°C. Lysate of 95,583 ± 10,966 NCCIT cells was used to generate 1 μl of extract. Protein concentration of the NCCIT extract was 29.5 ± 4.6 mg/ml (Bradford) and pH was 7.0 ± 0.0 (4 batches). ESC extracts (25–30 mg/ml protein) were similarly prepared from LIF-adapted ESC cultures. 293T, Jurkat, and NIH3T3 extracts were also prepared as described above. If necessary, extracts were diluted with H_2O before use to adjust osmolarity to ~300 mOsM.

SLO-mediated Permeabilization and Cell Extract Treatment

293T and 3T3 cells were washed in cold PBS and in cold Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA). Cells were resuspended in aliquots of 100,000 cells/100 μl of HBSS, or multiples thereof; placed in 1.5-ml tubes; and centrifuged at 120 × *g* for 5 min at 4°C in a swing-out rotor. Sedimented cells were suspended in 97.7 μl of cold HBSS, tubes were placed in a H_2O bath at 37°C for 2 min, and 2.3 μl of SLO (Sigma-Aldrich) (100 μg/ml stock diluted 1:10 in cold HBSS) was added to a final SLO concentration of 230 ng/ml. Samples were incubated horizontally in a H_2O bath for 50 min at 37°C with occasional agitation and set on ice. Samples were diluted with 200 μl of cold HBSS, and cells were sedimented at 120 × *g* for 5 min at 4°C. Permeabilization was assessed by monitoring uptake of a 70,000-M_r Texas Red-conjugated dextran (50 μg/ml; Invitrogen) in a separate sample 24 h after resealing and replating the cells (our unpublished data). Permeabilization efficiency under these conditions was ~80%.

After permeabilization, cells were suspended at 1000 cells/μl in 100 μl of NCCIT, ESC or indicated control extract (or multiples thereof) containing an ATP-regenerating system (1 mM ATP, 10 mM creatine phosphate, and 25 μg/ml creatine kinase; Sigma-Aldrich), 100 μM GTP (Sigma-Aldrich), and 1 mM each nucleotide triphosphate (NTP; Roche Diagnostics, Mannheim, Germany). The tube containing cells was incubated horizontally for 1 h at 37°C in a H_2O bath with occasional agitation. To reseal plasma membranes, the extract was diluted with complete RPMI 1640 medium containing 2 mM $CaCl_2$ and antibiotics, and cells were seeded at 100,000 cells per well on a 48-well plate. After 2 h, floating cells were removed, and plated cells were cultured in complete RPMI 1640 medium.

Microarray Analysis of Gene Expression

Microarrays. Gene expression analysis was performed using Human Genome Affymetrix U133A GeneChips as described previously (Ji *et al*., 2004). Total RNA was isolated using a Stratagene RNA Nanoprep isolation kit, treated with DNAse I, and purified with RNeasy Mini columns (QIAGEN, Valencia, CA).

PCRcDNA. First-strand cDNA was prepared as described previously (Ji *et al*., 2004) using a SMART PCRcDNA synthesis kit (BE Biosciences Clontech, Palo Alto, CA). Briefly, total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). cDNA was amplified by PCR as follows: 95°C for 1 min, 20–30 cycles of 95°C for 15 s, 65°C for 30 s, and 68°C for 3 min. PCRcDNA was purified with QIAquick columns (QIAGEN) and ethanol precipitation and dissolved in H_2O.

Biotin-labeling of cRNA. Biotin-labeled cRNA was prepared from PCRcDNA using a T7 RNA polymerase MEGAscript T7 kit (Ambion, Austin, TX), purified with an RNeasy mini kit and fragmented as described (Ji *et al*., 2004). Fragmented cRNA was used for hybridization or stored at −80°C.

Hybridization to GeneChips, Labeling, and Scanning. Hybridization was performed using 50 μg of fragmented cRNA at 45°C for 16 h as described previously (Ji *et al*., 2004). Chips were washed, stained at 35°C for 15 min with a phycoerythrin-strepavidin conjugate (Invitrogen), washed, and scanned on an HP GeneArray scanner Hewlett Packard (Palo Alto, CA).

GeneChip Image Quantification and Data Processing. GeneChip images were quantified and gene expression values calculated using the Affymetrix Microarray suite version 5.0 (MAS 5.0; Affymetrix, Santa Clara, CA). Expression ratios were calculated relative to mean hybridization level of three glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide spots on the arrays, and plots were drawn using Microsoft Excel 2002 (Microsoft, Redmond, WA).

Polymerase Chain Reaction

PCR amplification of the simian virus SV40 large T antigen was performed using primers 5′-GTGGCTATGGGAACTGGAG-3′ and 5′-CTCTACAGAT-

GTGATATGGCTG-3′, which cover nucleotides 39–265 of GenBank locus AF168998. PCR conditions were 95°C for 3 min and 30 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s followed by 10 min at 72°C. PCR products were visualized by ethidium bromide staining in a 2% agarose gel.

Reverse transcription (RT)-PCR reactions were carried from 200 to 1000 ng of total RNA using the Iscript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR reactions were performed in triplicates on a MyiQ real-time PCR detection system using either IQ SYBR Green (Bio-Rad) or ProbeLibrary probes (Exiqon, Vedbæk, Denmark) as indicated in Table S1. SYBR Green PCR conditions were 95°C for 4.5 min and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, using *GAPDH* as normalization control. ProbeLibrary PCR conditions were 95°C for 7 min and 40 cycles of 94°C for 20 s and 60°C for 1 min using *ACTB* as standard.

Bisulfite Sequencing

DNA was purified by phenol-chloroform-isoamylalcohol extraction or by using the GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich). Bisulfite conversion was performed using the MethylEasy DNA bisulphite modification kit (Human Genetic Signatures, Sydney, Australia) as described by the manufacturer. Converted DNA was amplified by seminested PCR using primers (Human Genetic Signatures) specific for the human *OCT4*, *LMNA*, and *LMNB1* genes (see *Results*), and PCR products were sequenced. PCR conditions were, for each of the nested PCRs, 95°C for 3 min and 30 cycles of 95°C for 1 min, 50°C for 2 min, and 72°C for 2 min, followed by 10 min at 72°C.

Induction of Neuronal Differentiation

To generate neuronal derivatives (Stewart *et al.*, 2003), cells were seeded in complete RPMI 1640 medium at 5×10^5 cells per 90-mm sterile bacterial culture dish. Suspension cultures were maintained for 24 h before adding 10 μM all-*trans*-retinoic acid (Sigma-Aldrich). Cells were cultured for 3–5 wk in retinoic acid, replacing the medium every 2–3 d. Subsequently, cell aggregates were washed in complete RPMI 1640 medium and plated onto poly-L-lysine (10 μg/ml; Sigma-Aldrich)-coated plates in complete RPMI 1640 medium containing the mitotic inhibitors fluorodeoxyuridine (10 μM; Sigma-Aldrich), cytosine arabinosine (1 μM; Sigma-Aldrich), and uridine (10 μM; Sigma-Aldrich).

Mesodermal Lineage Differentiation

Cells were cultured for 21 d in complete RPMI 1640 medium containing 10 μM all-*trans*-retinoic acid and washed in complete RPMI 1640 medium. For adipogenic differentiation, cells were cultured for a further 21 d in DMEM/Ham's F-12 supplemented with 10% FCS, dexamethasone, insulin, and indomethacin (Boquest *et al.*, 2005). Cells were fixed with 4% formalin, washed in 5% isopropanol, and stained for 15 min with Oil-Red-O (Sigma-Aldrich). For osteogenic differentiation, cells were cultured for 21 d in DMEM containing 10% FCS, dexamethasone, β-glycerophosphate, and L-ascorbate-2-phosphate (Boquest *et al.*, 2005). Extracellular matrix mineralization nodules were visualized by Alzarin red staining. Endothelial differentiation was performed as described previously (Planat-Benard *et al.*, 2004). Briefly, NCCIT extract-treated cells and controls were harvested by flask shaking and plated at 2×10^5 cells per milliliter in 3 ml of methylcellulose (Methocult GF H4434; Stem Cell Technologies, Vancouver, British Columbia, Canada) and cultured for 7 d.

Immunological Procedures

For immunofluorescence, cells were seeded onto coverslips, fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with bovine serum albumin, and hybridized with relevant antibodies. Antibodies used were a rabbit polyclonal anti-Oct4 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), an anti-lamin A/C monoclonal antibody (mAb) (1:50 dilution; mAb XB10; BAbCO, Covance Research Products, Grand Rapids, MI), and a rabbit polyclonal antibody against a peptide of human B-type lamins (1:1000) (Chaudhary and Courvalin, 1993). Rabbit polyclonal antibodies (1:200) against neurofilament NF200 were from Sigma-Aldrich, and anti-NeuN (mAb377) and anti-nestin antibodies (mAb5362; 1:200) were from Chemicon International (Temecula, CA). Secondary antibodies were Cy2- and Cy3-conjugated anti-mouse and anti-rabbit antibodies and Cy3-conjugated anti-rabbit antibodies (1:1000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). For direct immunolabeling, cells (~300,000) were incubated with fluorescein isothiocyanate-conjugated mouse anti-human CD31 or rabbit anti-human CD144 antibodies (1:10 dilution; Serotec, Oxford, United Kingdom) in 100 μl of PBS. After extensive washing in PBS, cells were fixed with 3% paraformaldehyde before viewing. For Western blotting, antibodies used were anti-Oct4 (1:250), anti-lamin A/C (1:500), anti-lamin B (1:5000), and anti-tubulin (1:250; Santa Cruz Biotechnology). Immunodepletion of Oct4 from mouse ESC extract was performed using anti-Oct4 antibodies (1:50 dilution) bound to protein A/G-Sepharose beads. After 1-h incubation in extract at 4°C, bead complexes were removed by sedimentation at $4000 \times g$ for 5 min, and a second round of immunoprecipitation was carried out for 30 min at 4°C. After sedimentation of the beads, an aliquot of the supernatant was removed for Western blotting, whereas the extract was used for cell treatment.

Alkaline Phosphatase Assay

Relative intracellular ALP levels were determined using a dot-blot assay. Two microliters of soluble lysate ($15{,}000 \times g$ supernatant at 20 μg/μl protein) from indicated cell types were spotted on a dry 45-μm nitrocellulose membrane (Bio-Rad). The membrane was wetted in 50 mM NaCl, 10 mM Tris-HCl, pH 7.0, and ALP was revealed by applying an Alk-Phos Direct detection solution (GE Healthcare, Piscataway, NJ). Light emission on film was quantified by densitometry within a linear signal range.

RESULTS

Treatment of 293T Cells with NCCIT Extract Promotes Colony Formation

293T cells were permeabilized with SLO and exposed for 1 h to an extract of undifferentiated NCCIT cells. In vitro culture of extract-treated cells was accompanied by morphological, immunological, gene expression, and functional analyses over 8–12 wk to evaluate induction of dedifferentiation in two to six independent experiments. As controls, permeabilized 293T cells were treated with extract of 293T or Jurkat T-cells.

A first result of NCCIT extract exposure was a change in morphology of 293T cells. Within 2 wk, colonies with defined edges developed and resembled NCCIT colonies (Figure 1A and B, a–e). This phenotype was maintained for at least 12 wk in culture, corresponding to >50 population doublings and 23 passages (Figure 1B, a–e). The phenotype was not a mere consequence of treatment with any extract as 293T cells incubated in their own extract did not form colonies (Figure 1B, f–j), and 293T cells treated with an extract of Jurkat cells formed clearly morphologically distinct aggregates (Figure 1C). The latter were reminiscent of Jurkat T-cell clusters (Håkelien *et al.*, 2005).

The NCCIT Extract Elicits Expression of Oct4 and Oct4-responsive Genes, Expression of ESC Markers, and Repression of A-Type Lamins

Expression of the homeodomain protein Oct4, a POU family transcription factor, is restricted to germ cells, preimplantation embryos, the epiblast of early postimplantation embryos and ESCs (Chambers and Smith, 2004). As expected, Oct4 was detected in the nucleus of NCCIT but not 293T cells (Figure 2A), and identity of the protein was confirmed by immunoblotting (our unpublished data). One week after treatment with NCCIT extract, >60% of 293T cells displayed intranuclear Oct4, whereas Oct4 remained undetectable in 293T extract-treated cells (Figure 2, B and C).

We next monitored the loss A-type nuclear lamins, a marker of differentiated cells (Hutchison and Worman, 2004), from 293T cells. mAbs against lamins A and C (lamin A/C) decorated the nuclear periphery of 293T but not NCCIT cells (Figure 2A). In contrast, 1 wk after NCCIT extract treatment, lamin A/C was undetectable in >60% of the cells, whereas controls displayed lamin A/C labeling (Figure 2, B and C). Notably, Oct4 expression paralleled the loss of lamin A/C expression in the same cells (Figure 2C), suggesting that these cells were undergoing dedifferentiation. Expression of the ubiquitously expressed B-type lamins (Hutchison and Worman, 2004) remained unaltered (Figure 2, A–C).

Induction of *OCT4* (*POU5F1*) transcription and loss of *LMNA* (lamin A) gene expression over time were demonstrated by quantitative RT-PCR analysis 4 wk after extract treatment (Table 1). Several target genes of Oct4 were also up-regulated, including *UTF1*, *OXT2*, *REX1*, and *NANOG*. Interestingly, Oct4 is known to act in cooperation with Sox2

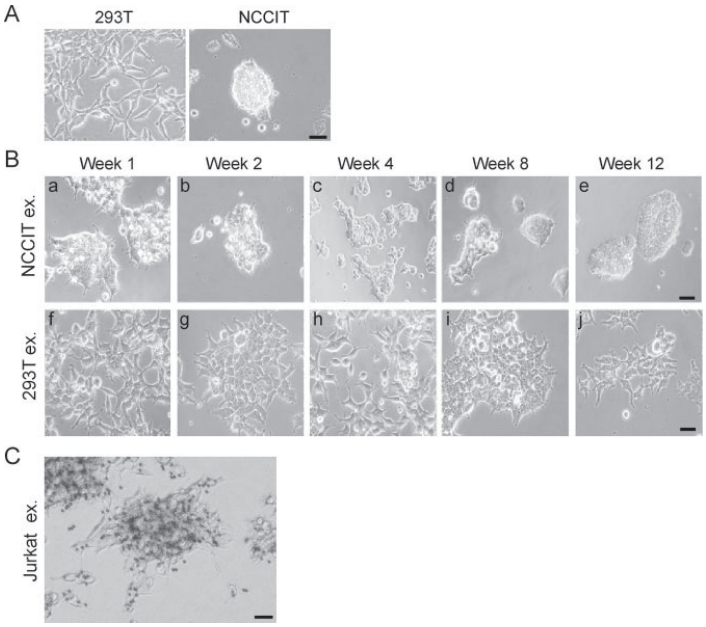


Figure 1. Morphology of 293T cells treated with NCCIT extract. (A) Untreated 293T and NCCIT cells. (B) 293T cells at indicated time points after exposure to NCCIT (a–e) or 293T (f–j) extract. (C) 293T cells 10 d after exposure to Jurkat extract and cultured under T-cell growth conditions. Dark spots are Dynal Biotech (Montebello, Norway) magnetic beads bearing antibodies against CD3 and CD28 surface antigens and used to promote T-cell expansion. Bars, 30 μm.

(Avilion *et al*., 2003), which was also found to be induced in extract-treated cells. Additional markers of pluripotentiality (Hoffman and Carpenter, 2005) up-regulated in NCCIT extract-treated cells and verified by quantitative RT-PCR included ALP 1 (*APL*), *STELLA*, *AC133*, *CD9*, *DMNT3B*, and *DNMT3L* (Table 1). Expression of these genes was examined and confirmed at 2, 4, and 6 wk after extract treatment but not examined thereafter (our unpublished data). However, genes such as *PDGFαR*, *FGF2*, *LEFTY1*, *LEFTY2*, *CD135*, or *CD117* were not expressed in any cell type or were not altered by extract treatment (our unpublished data). As expected, transcripts for the ubiquitously expressed lamin B1 (*LMNB1*) were not altered by exposure to either extract (Table 1), supporting our immunofluorescence observations. None of the pluripotency marker transcripts examined by real time RT-PCR were elicited in 293T cells treated with their own extract (our unpublished data).

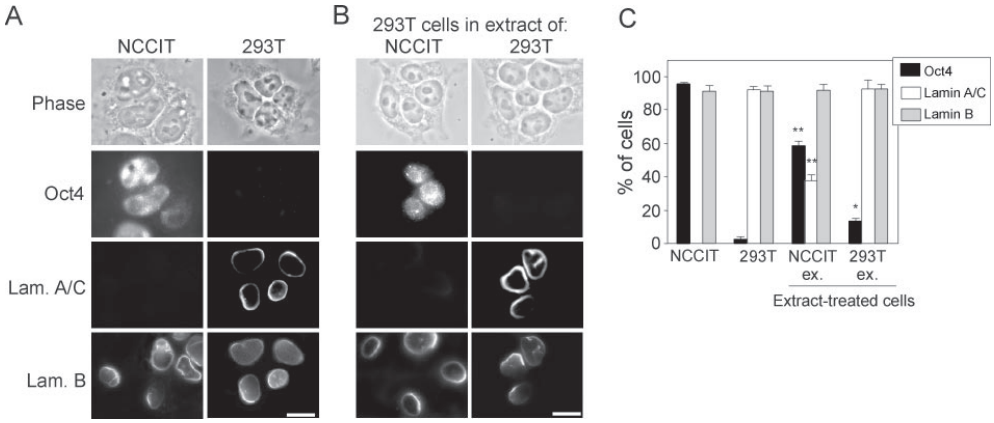


Figure 2. Immunofluorescence analysis of Oct4, lamin A/C and B-type lamin expression in 293T cells exposed to NCCIT extract. Untreated NCCIT and 293T cells (A) and 293T cells (B) treated with NCCIT or 293T extract were immunolabeled with antibodies against Oct4, lamin A/C, and B-type lamins (B, 1 wk after extract treatment). Bars, 20 μm. (C) Proportions (mean ± SD) of untreated NCCIT and 293T cells and of extract-treated cells expressing Oct4, lamin A/C, and B-type lamins. Three sets of 200 cells were examined for each marker. *$p < 0.05$ compared with 293T cells (*t* test); **$p < 0.001$ compared with 293T cells and 293T cells treated with 293T extract (*t* test).

Table 1. Quantitative RT-PCR analysis of expression of indicated stem cell genes in 293T cells treated with NCCIT extract

	Fold up- or down-regulation		
Gene	293T extract	NCCIT extract	NCCIT cells
AC133	2▲	260 ▲	271▲
APL	2▲	35 ▲	28▲
CD9	1	3 ▲	4▲
DNMT3B	1	16 ▲	11▲
DNMT3L	3▲	36 ▲	23▲
NANOG	1	2513 ▲	1541▲
OXT2	0	20 ▲	23▲
POU5F1	1	600 ▲	410▲
REX1	1	1994 ▲	1985▲
SOX2	1	18 ▲	27▲
STELLA	1	88 ▲	48▲
UTF1	2▲	121 ▲	133▲
LMNA	2▼	370 ▼	15▼
LMNB1	1	1.5▲	1

Table indicates fold up-regulation (▲) or down-regulation (▼) of indicated genes in 293T cells exposed to 293T or NCCIT extract and cultured for 4 wk. Reference level (1) was that of untreated 293T cells. Transcript levels in NCCIT cells relative to 293T cells are also shown.

To verify that expression of Oct4 and reduction of A-type lamin expression did take place in 293T cells, 293T cells stably expressing an enhanced green fluorescent protein (EGFP) and a geneticin-resistance gene (293T-EGFP-Gen^R cells) were treated with NCCIT extract. After 2 wk of culture with 700 μg/ml geneticin, which kills NCCIT cells (our unpublished data), the majority of 293T-EGFP-Gen^R cells stained positive for Oct4 (Figure 3A). Strong up-regulation of *OCT4*, SOX2, and *APL* and moderate up-regulation of *STELLA* (~2-fold) gene expression were also detected in these cells, whereas *LMNA* was repressed (Figure 3B). We also took advantage of the large T antigen marker carried by 293T cells. PCR analysis indicated that 293T-EGFP-Gen^R cells, but not NCCIT cells, contained the SV40 large T antigen transgene (Figure 3C), confirming that cells expressing Oct4 were of 293T origin. Of note, karyotyping analysis of 293T cells before and after extract treatment was inconclusive due to the severe aneuploidy and genomic instability of untreated 293T cells (our unpublished data). 293T cell aneuploidy was expected to occur as a result of large T antigen transformation, which is known to cause endoreplication (Wu *et al.*, 2004).

Last, treatment of NCCIT extract with 500 μg/ml DNAse I, which eliminates most detectable DNA in the extract (our unpublished data), before incubating 293T-EGFP-Gen^R cells did not affect induction of *OCT4*, *SOX2*, *APL*, and *STELLA* expression or *LMNA* repression (Figure 3B; +DNAse). This argues that Oct4 expression in 293T cells did not emanate from intact DNA derived from the NCCIT extract.

The NCCIT Extract Promotes OCT4 DNA Demethylation

Transfer of mammalian cells into the germinal vesicle of *Xenopus* oocytes elicits DNA demethylation in the *OCT4* promoter, a prerequisite for *OCT4* expression in this system (Simonsson and Gurdon, 2004). The *OCT4* region analyzed in our study was from nucleotide 1433–1671 (GenBank sequence AJ297527), encompassing eight potentially methylated cytosines in CpG dinucleotides between conserved regions CR2 and CR3 in the *OCT4* promoter (Nordhoff *et al.*, 2001) (Figure 4A). Bisulfite sequencing showed that this region was unmethylated in NCCIT but methylated in 293T cells (Figure 4A). In 293T cells exposed to their own extract, *OCT4* remained methylated. However, in NCCIT extract-treated cells, *OCT4* demethylation was evident after 4 and 9 wk of culture (Figure 4A) and provided molecular support for long-term transcriptional activation of *OCT4*. Two CpGs showed apparent partial demethylation (Figure 4A), which was interpreted as the expected presence of a mixed cell population in which cytosines −1686 and −1676 did not undergo demethylation. We concluded therefore that the NCCIT extract was capable of eliciting *OCT4* demethylation in 293T cells. *OCT4* demethylation was specific for the NCCIT extract and it did not occur in 293T extract (Figure 4A).

We also examined the DNA methylation status of *LMNA*, whose expression is virtually repressed in NCCIT extract-treated cells. The *LMNA* region analyzed spanned nucleo-

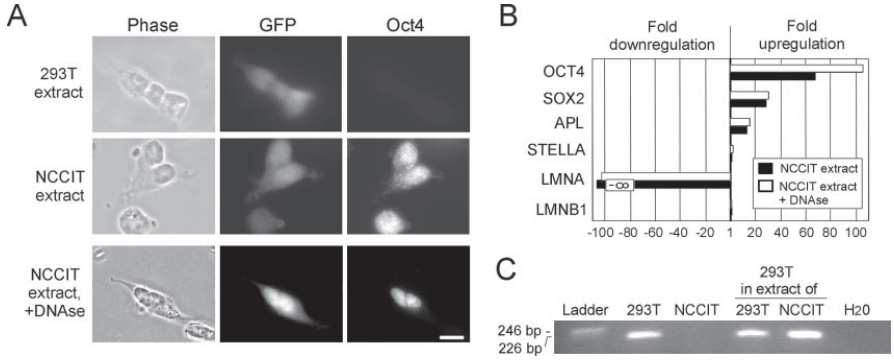


Figure 3. Oct4 expression in EGFP-labeled 293T cells. (A) 293T cells stably expressing EGFP and a geneticin resistance (Gen^R) gene were treated with 293T or NCCIT extract and cultured for 2 wk with 700 ng/ml geneticin before immunolabeling with anti-Oct4 antibodies. NCCIT extract was also treated with 500 μg/ml DNAse I before incubating cells (bottom row). Bar, 20 μm. (B) Quantitative RT-PCR analysis of expression of indicated genes in 293T-EGFP-Gen^R cells 2 wk after incubation in intact or DNAse I-treated NCCIT extract (relative to 293T extract-treated controls). (C) PCR analysis of the presence of SV40 large T antigen in 293T, NCCIT, and extract-treated cells. Ladder is a 123-base pair DNA ladder.

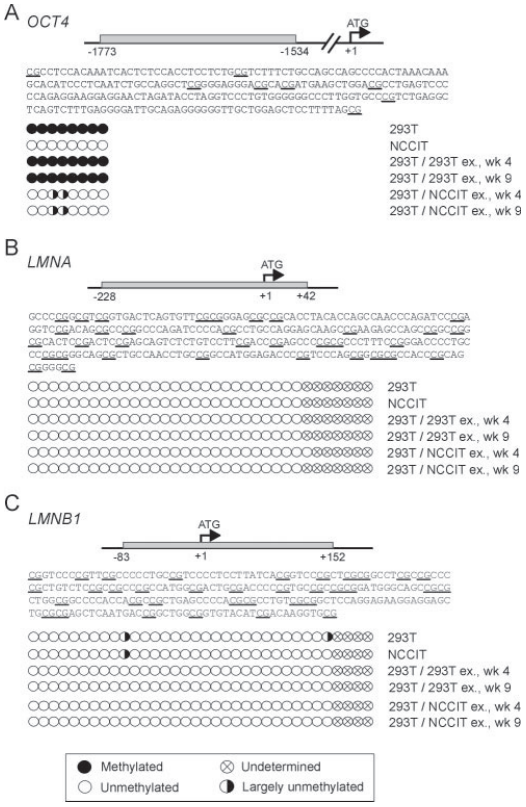


Figure 4. Bisulfite sequencing analysis of DNA methylation changes in extract-treated cells. 293T cells, NCCIT cells, and cells treated with 293T or NCCIT extract were examined for cytosine methylation in underlined CpG dinucleotides within shown genomic regions of the human *OCT4* (A), *LMNA* (B), and *LMNB1* (C) genes. Diagrams show localization (nucleotide numbers) of regions examined relative to the ATG translation start (+1).

tides 2379–2648 (GenBank L12399), encompassing 34 potentially methylated cytosines found in CpG dinucleotides flanking the ATG translation start (Figure 4B). This region is unmethylated in 293T cells and clearly remained unmethylated in NCCIT extract-treated cells (Figure 4B). However, we also found an absence of *LMNA* DNA methylation in this region in NCCIT cells despite the repression of *LMNA* in these cells (Figure 4B). Thus, we concluded that NCCIT extract-treated cells did not undergo aberrant DNA methylation in this region. We cannot ascertain, however, that other regions in the LMNA locus were modified by extract treatment. Last, *LMNB1* remained unmethylated regardless of extract treatment (Figure 4C), in agreement with its ubiquitous expression. The *LMNB1* region examined spanned nucleotides 1594–1828 (GenBank L37737), encompassing 35 potentially methylated cytosine nucleotides in a CpG island around the ATG start. *GAPDH*, also ubiquitously expressed and used as reference in our quantitative RT-PCR analyses, also remained largely unmethylated in any cell type examined (our unpublished data).

Transcriptional Profiling of NCCIT Cells Relative to 293T Cells

To evaluate the extent of transcriptional alterations elicited by the NCCIT extract, an Affymetrix U133A GeneChip microarray analysis of 293T cell gene expression was carried out for 8 wk after extract treatment. We took advantage of a SMART PCRcDNA approach that combines PCR amplification and T7 RNA polymerase to amplify submicrogram RNA samples (Ji *et al*., 2004). Although some distortion of within-sample stoichiometry occurs with this method, one can assume the same distortion between samples, thus maintenance of between-sample stoichiometry allows comparative analyses.

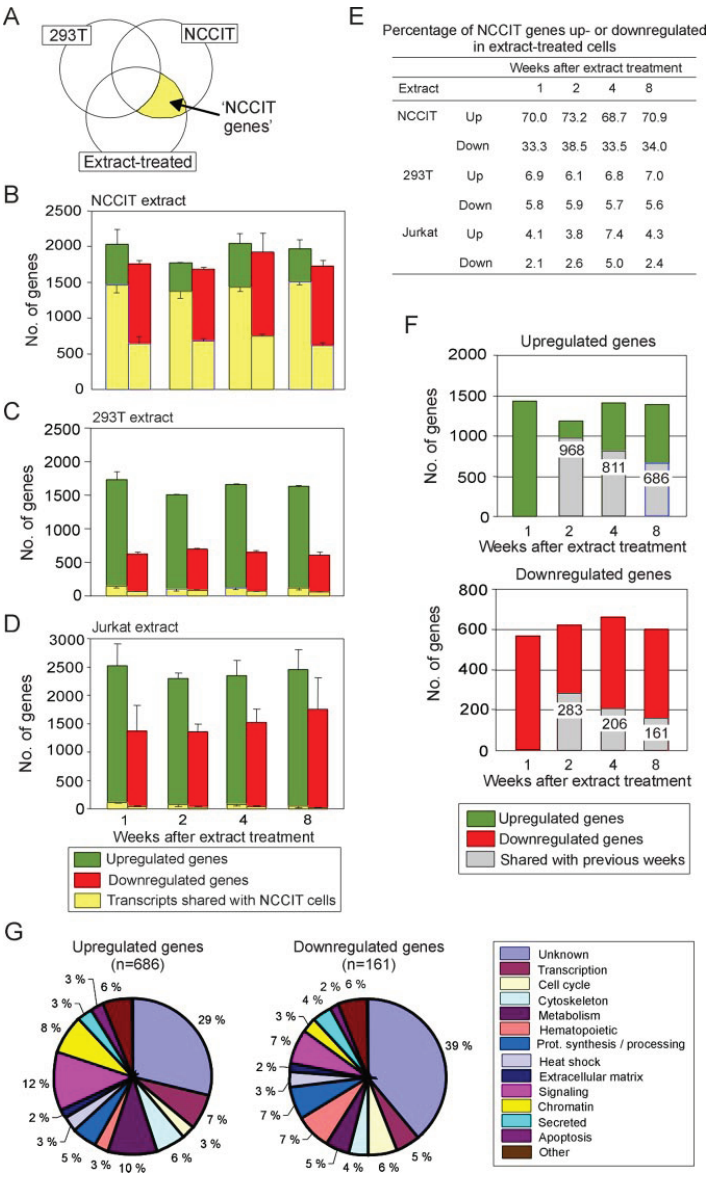
We first assessed genes significantly ($p < 0.001$) up- or down-regulated at a more than threefold difference level in NCCIT compared with 293T cells. A total of 2950 genes were up-regulated, whereas 2528 genes were down-regulated in NCCIT cells, in two independent analyses performed in duplicate. Distributions of up- and down-regulated genes into functional classes were similar, with most genes encoding elements involved in transcription regulation (22 and 19%, respectively), cytoskeletal organization (6 and 3%), metabolism (4 and 10%), protein synthesis and processing (6 and 4%), cell signaling (10 and 8%), and chromatin organization (6 and 4%) (Figure S1).

NCCIT Extract Induces Expression of NCCIT-specific Genes and Down-Regulation of 293T Genes

The NCCIT extract elicited up- and down-regulation of ~1700–2000 and ~1650–1800 genes, respectively, on any given week relative to 293T cells (Figure 5B, green and red bars). Of these, ~70 and ~34%, respectively, were shared with NCCIT cells and qualified as "NCCIT genes" (Figure 5, A and B, yellow bars; E). Furthermore, the likelihood that expression of these genes was altered by chance rather than as a result of extract treatment was extremely low ($p < 10^{-5}$ and $p < 10^{-4}$, respectively; *t* tests), indicating that changes were elicited by the NCCIT extract.

We then addressed the specificity of gene expression changes elicited by NCCIT extract. First, exposure of 293T cells to their own extract induced up- or down-regulation of ~1600 and ~600 genes, respectively (Figure 5C, green and red bars), of which only ~6% were identified as NCCIT genes (Figure 5C, yellow bars; E; listed in Table S2). Similarly, treatment of 293T cells with Jurkat T-cell extract altered expression of a negligible proportion of NCCIT genes (Figure 5, D and E). Furthermore, nearly all NCCIT genes affected by 293T or Jurkat extract were the same (Table S2; annotations[a,b]), and probabilities that these genes were altered by chance rather than by extract treatment were relatively high ($p > 0.07$ and $p > 0.08$, respectively; *t* tests). Resulting numbers of NCCIT genes specifically up- or down-regulated by NCCIT extract and reproducibly in both experiments are shown in Figure 5F (green and red bars, respectively). Thus, the NCCIT extract elicits specific alterations in the 293T cell expression profile. Genes not differentially expressed in NCCIT compared with 293T also seem to be affected.

The consistency of NCCIT gene expression changes in NCCIT extract-treated cells over time was subsequently assessed. Figure 5G (gray bars) shows that 686 genes were consistently up-regulated from weeks 1–8, whereas 161 genes were consistently down-regulated (these genes were shared between both experiments). These genes are listed in Table S3. The remaining affected genes included those with an onset of up- or down-regulation later than week 1, or



Extract		Weeks after extract treatment 1	2	4	8
NCCIT	Up	70.0	73.2	68.7	70.9
	Down	33.3	38.5	33.5	34.0
293T	Up	6.9	6.1	6.8	7.0
	Down	5.8	5.9	5.7	5.6
Jurkat	Up	4.1	3.8	7.4	4.3
	Down	2.1	2.6	5.0	2.4

Figure 5. Microarray analysis of gene expression in extract-treated 293T cells. (A) Venn diagram identifying "NCCIT-specific" genes (yellow area). Numbers of genes up- or down-regulated more than threefold (relative to input 293T cells) in cells incubated in extract of NCCIT (B), 293T (C), and Jurkat (D) cells (mean ± SD of two [B and C] and four [D] experiments). Yellow bars indicate genes up- or down-regulated in extract-treated cells and shared with NCCIT cells. In B, the likelihood that NCCIT genes are up- or down-regulated by chance rather than by extract treatment is extremely low ($p < 10^{-5}$ and $p < 10^{-4}$, respectively; *t* tests). By contrast, in C and D these probabilities are relatively high ($p > 0.07$ and $p > 0.08$, respectively; *t* tests). (E) Percentage of NCCIT genes up- or down-regulated in extract-treated cells (percentage of total up- or down-regulated genes). (F) Number of NCCIT genes specifically up- or down-regulated by treatment with NCCIT extract, over time. (G) Consistency of gene up- or down-regulation over time after treatment with NCCIT extract. Numbers of up- and down-regulated NCCIT genes in cells exposed to NCCIT extract are shown in green and red. Gray bars represent genes consistently up- or down-regulated at weeks 1 and 2 (gray bars at week 2), weeks 1, 2, and 4 (gray bars at week 4), etc., and shared between the two experiments. (H) Functional class distribution of genes consistently up- or down-regulated over 8 wk in two experiments (gray bars in G). These genes are listed in Table S3.

those with a more fluctuating expression level. For example, a large number of genes were up-regulated from week 2 onward, and thus they were not taken into account in the above-mentioned analysis. Functional class distribution of the consistently up- or down-regulated genes (Figure 5H) shows that most annotated up-regulated genes encoded elements involved in transcription, cytoskeletal organization, metabolism, signaling, and chromatin remodeling, whereas down-regulated genes were more evenly distributed across functional classes.

Treatment with NCCIT Extract Up-Regulates Markers of Pluripotency and Genes Indicative of Multilineage Priming

Table 2 lists markers of pluripotency represented in the array and that were up-regulated in extract-treated cells. In agreement with our immunolabeling and DNA methylation data, *OCT4* (*POU5F1*) was up-regulated from week 2 onward. Notably, the Oct4-responsive genes *UTF1* and *REX1/DRN3* (Hosler *et al.*, 1989; Okuda *et al.*, 1998) were also up-regulated together with *SOX2*, suggesting the induction

Table 2. Changes in expression level of selected markers of dedifferentiation and multi-lineage differentiation potential in NCCIT extract-treated cells

Name	GenBank accession no.	Description	NCCIT cells (fold up-regulation)[a]	NCCIT extract-treated cells (fold up-regulation)[a]			
				Wk 1	Wk 2	Wk 4	Wk 8
Somatic cell markers							
LMNA	M13452.1	Nuclear lamin A	0.0015	0.9	0.0011	0.0014	0.0011
LMNB1	NM_005573.1	Nuclear lamin B1	2.5	1.7	1.0	1.5	1.4
LMNB2	M94363	Nuclear lamin B2	0.8	1.1	1.1	0.9	1.1
NPR3	NM_000908.1	Atrionatriuretic peptide receptor C	0.001	0.001	0.001	0.001	0.001
Embryonic, germ cell and stem cell markers							
OCT4 (*POU5F1*) and Oct4-responsive genes							
POU5F1	NM_002701.1	POU domain, class 5, TF1 (Oct4)	36	2	17	35	36
UTF1	N_003577.1	Undifferentiated embryonic cell transcription factor 1	317	2	213	293	318
REX1	AJ243797	Deoxyribonuclease III (Drn3)	5	1	2.5	6	9
FOXD3	N_012183.1	Forkhead box D3 (FoxD3)	847	38	283	295	254
Telomerase and telomerase-associated factors							
TERT	N_003219.1	Telomerase reverse transcriptase	102	2.5	22	76	89
TERF1	N_017489.1	Telomerase-associated factor 1	3.5	3	3.2	4.5	3.5
TERF2	N_005652.1	Telomerase-associated factor 2	0.7	0.5	0.9	0.6	0.6
SOX2	AI356682	Sex determining region Y-box 2 (Sox2)	119	1	12	118	121
POU3F1	L26494.1	POU domain, class 3, TF1 (Oct-6)	164	1.5	3	106	84
ALP1	M13077	Placental alkaline phosphatase 1	1311	38	292	928	927
ALP1	J04948.1	Placental alkaline phosphatase 1	2124	56	344	651	1337
CD44	N_000610.1	CD44 antigen (homing function)	1028	102	205	622	663
LIF	N_002309.2	Leukemia inhibitory factor	221	7	27	127	271
FZD9	N_003508.1	Frizzled (*Drosophila*) homologue 9	331	10	101	102	107
TEF	U06935.1	Thyrotroph embryonic factor	423	69	126	222	273
SCGF	D86586.1	Stem cell growth factor β	934	234	917	939	906
GCNF	AF004291.1	Germ cell nuclear factor	1105	27	71	424	696
SPINK2	N_021114.1	Serine protease inhibitor, Kazal type, 2	662	2.5	65	257	555
DKK2	N_014421.1	Dickkopf (*Xenopus laevis*) homolog 2[b]	1122	345	457	473	476
INTA6	AV733308	Integrin α6	3.1	1.5	4.5	10	15
Markers of potential lineage- specific differentiation							
Osteogenic lineage							
BMP1	N_006129.2	Bone morphogenic protein 1	870	67	169	367	470
BMP2	NM_001200.1	Bone morphogenic protein 2	655	166	333	653	663
OGN	N_014057.1	Osteoglycin	17	2	4	14	17
CTSK	N_000396.1	Cathepsin K	8	4	5	5	6
TNFRSF11B	N_002546.1	Osteoprotegerin	82	1	14	94	65
Endothelial lineage							
VWF	N_000552.2	Von Willebrand Factor	629	604	598	734	647
NOS3	N_000603.1	Nitric oxide synthase 3 (endothelial cell)	421	127	167	129	306
Myogenic lineage							
MYF5	N_005593.1	Myogenic factor 5	13	10	13	30	23
TMP1	N_000366.1	Tropomyosin 1 alpha	46	14	12	11	16
MYH11	N_022844.1	Myosin, heavy polypeptide 11	98	4	22	28	69
Neurogenic lineage							
NTS	N_006183.2	Neurotensin	73	7	20	27	48
NRG1	N_004495.1	Neuregulin 1 isoform gamma	857	60	56	66	86
MBP	M13577.1	Myelin basic protein	21	2	7	11	14
MOBP	D28114.1	Myelin-associated oligodend. basic protein	43	22	24	24	22
NCAM1	BF348061	Neural cell adhesion molecule 1	18	1	7	17	20
CD56	U63041.1	Neural cell adhesion molecule CD56	16	1.5	2	4	11
Adipogenic lineage							
APOA2	N_001643.1	Apolipoprotein A-II	13	4	5	9	9
APOD	N_001647.1	Apolipoprotein D	11	3	11	10	12
APOE	N33009	Apolipoprotein E	21	2	11	22	2
APOC1	N_001645.2	Apolipoprotein C1	4	0.5	1.5	3	4
PPARG2	N_015869.1	Peroxisome prolif. activated receptor γ2	80	1	1.5	10	22
FAD1	BG165833	Fatty acid desaturase 1	10	3	4	7	5
Chondrogenic lineage							
COL4A3	U02520	Collagen type IV alpha 3	36	3	9	27	31
COL5A2	N_000393.1	Collagen, type V, alpha 2	44	2	4	12	16
COL8A1	BE877796	Collagen, type VIII, alpha 1	19	1	5	9	15
COL11A1	J04177	Collagen, type X1, alpha 1	251	1	10	42	230
CSPG2	N_004385.1	Chondroitin sulfate proteoglycan 2	85	1	10	92	87
AGC1	N_013227.1	Aggrecan 1	15	5.5	4.5	50	46
DSPG3	N_004950.2	Dermatan sulphate proteoglycan 3	310	1.5	3	52	157
CSPG1	X17406.1	Aggrecan 1	9	1	2	13	16
FNP	X02761.1	Fibronectin precursor	9	4	9	4	2
FN1	AK026737.1	Fibronectin 1	12	4	10	7	6

[a] Relative to 293Tcells.

[b] Also up-regulated in cells exposed to 293T or Jurkat extract.

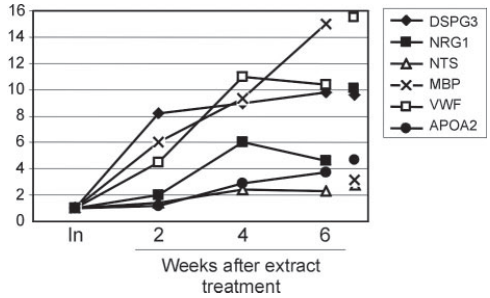


Figure 6. Quantitative RT-PCR analysis of expression of indicated multilineage priming genes in 293T cells treated with NCCIT extract relative to transcript levels in 293T cells exposed to 293T extract. Expression levels were adjusted to those of *GAPDH* in triplicate samples. Single data points show mean expression level in NCCIT cells.

of Oct4-dependent functions in reprogrammed cells. Telomerase (*TERT*) and telomerase-associated factor 1 (*TERF1*) were also increasingly up-regulated. Other pluripotency markers up-regulated were the CD44 stem cell antigen, placental ALP (*APL1*), *LIF*, stem cell growth factor β (*SCGF*), germ cell nuclear factor (*GCNF*), and integrin α6 (*INTA6*), a putative marker of "stemness" shared between three gene expression profile analyses of mouse ESCs (Fortunel *et al*., 2003). Remarkably, except for Dikkopf2 (*DKK2*), none of the stem cell marker genes listed in Table 2 was affected by treatment with 293T or Jurkat extract, illustrating the extract specificity of changes elicited (Table S4). In parallel, *LMNA* was essentially repressed, whereas expression of B-type lamins (*LMNB1* and *LMNB2*) persisted, consistent with our RT-PCR and immunolabeling data. The kidney-derived 293T cell marker natriuretic peptide receptor C (*NPR3*) was also strongly down-regulated (Hansis *et al*., 2004).

We also noted the up-regulation of markers of lineage-specific differentiation to levels comparable with NCCIT expression levels. These included markers of osteogenic, endothelial, myogenic, neurogenic, adipogenic, and chondrogenic lineages (Boquest *et al*., 2005). Expression of markers of chondrogenic (*DSPG3*), neuronal (*NRG1*, *NTS*, and *MBP*), endothelial (*VWF*), and adipocyte (*APOA2*) lineages was confirmed by real-time RT-PCR (Figure 6). Furthermore, expression of several housekeeping genes, including *18S*, *28S*, *GAPDH*, *HPRT1*, and *ACTB* and 35 genes for ribosomal proteins, was unaffected in extract-treated cells (Table S5). Collectively, these results indicate that the NCCIT extract promotes the up-regulation of markers of pluripotency typically expressed in ECCs or ESCs and suggest, in addition, the establishment of a "multilineage priming" in 293T cells treated with NCCIT extract.

Oncogenes and Tumor Suppressor Genes Are Not Affected by NCCIT Extract Treatment

NCCIT is a tumor cell line that bears genetic mutations required for its expansion and phenotypic characteristics. We determined whether mRNA levels for oncogenes and tumor suppressor genes were altered in 293T cells exposed to NCCIT extract, relative to untreated cells. We did not observe any up-regulation or induction of oncogene expression in NCCIT extract-treated cells. Genes such *c-MYC*, *c-MYC*-responsive genes, genes encoding Myc-interacting or Myc-regulated proteins, and genes encoding RAB and RAF isoforms were not significantly expressed in any of the cell types examined nor were they altered by NCCIT extract. Among tumor suppressor genes, *P53* was strongly up-regulated in NCCIT compared with 293T ($p < 10^{-4}$) but remained unaltered in NCCIT extract-treated cells ($p > 0.05$). Other tumor suppressor genes, however, were either not significantly expressed in 293T or NCCIT [*RB1*, *TSC1*, *TSC22*, *BRCA1*, *BRCA2*, *CDKN2A* (p16), *CDKN1A* (p21, Cip1), *CDKN1C* (P57, Kip2), *MSH2*, *STK11*, *MEN1*, and *MEN2*] or were expressed at similar levels in both cell types (*PTCH*, *PTEN*, and *WT1D*). These genes remained unaltered by treatment with NCCIT extract ($p > 0.05$). Similarly, genes encoding enzymes involved in DNA repair (*XPA*, *ERCC5*, *FANCA*, *-C*, *-E*, *-F*, and *-G*) were not significantly expressed in either cell types nor altered by extract treatment. *ATM* was highly expressed in 293T and NCCIT cells and remained unchanged in extract-treated cells. Because it is unlikely that NCCIT genetic lesions are passed onto the 293T cell genome through extract treatment, acquisition of an NCCIT phenotype by 293T cells implies that either the phenotype obtained is independent of NCCIT lesions or that genetic mutations that gave rise to the NCCIT tumor phenotype are dispensable for the maintenance of this state.

Retinoic Acid Stimulation of NCCIT Extract-treated Cells Induces Neuronal Differentiation

To determine whether NCCIT extract-treated 293T cells acquired a potential for pluripotency, we attempted to induce neuronal differentiation in vitro with retinoic acid (Stewart *et al*., 2003). 293T, NCCIT (our unpublished data), and extract-treated cells were exposed to 10 μM all-*trans*-retinoic acid and were maintained as suspended aggregation cultures (Figure 7A). Suspensions of all cell types formed disorganized aggregates in bacteriological dishes but after 2 wk in retinoic acid, the cells formed spheres that sometimes fused with one another (Figure 7A). This was particularly evident for NCCIT extract-treated 293T cells (Figure 7A, top). After washing and replating, cells adhered to poly-L-lysine-coated coverslips. However, only NCCIT and NCCIT extract-treated cells showed evidence of neurite outgrowth already after culture for 2 d in the absence of retinoic acid but in the presence of mitotic inhibitors (Figure 7A, bottom, and S2A). This suggested that neuronal progenitor cells emanated from NCCIT extract-treated 293T cells.

Immmunolabeling and real-time RT-PCR analyses confirmed the induction of a neuronal phenotype. Spheres of NCCIT cells and of NCCIT extract-treated cells showed a reduction of Oct4 protein to a level nearly undetectable by immunofluorescence in >90% of the cells (Figure 7B). This was confirmed by a threefold reduction of *OCT4* transcript levels in these cells compared with cells not stimulated with retinoic acid (Figure 7C). Moreover, expression of the intermediate filament nestin (*NES*), a marker of neuronal precursor cells (Cattaneo and McKay, 1990), was induced at the transcriptional and translational levels by retinoic acid in NCCIT extract-treated but not 293T extract-treated, cells (Figure 7, B and C). Furthermore, *LMNA*, repressed after exposure to NCCIT extract (Figure 2 and Table 1) was strongly up-regulated de novo by retinoic acid but only in NCCIT extract-treated cells (Figure 7C). This indicated that the cells redifferentiated upon retinoic acid exposure. Last, the majority of NCCIT extract-treated cells, as NCCIT cells (Figure S2B and S2C) were positive for the neuronal markers NeuN and NF200 (Figure 7D). We concluded that NCCIT extract-treated 293T cells can be induced to differentiate into a neuronal phenotype in vitro.

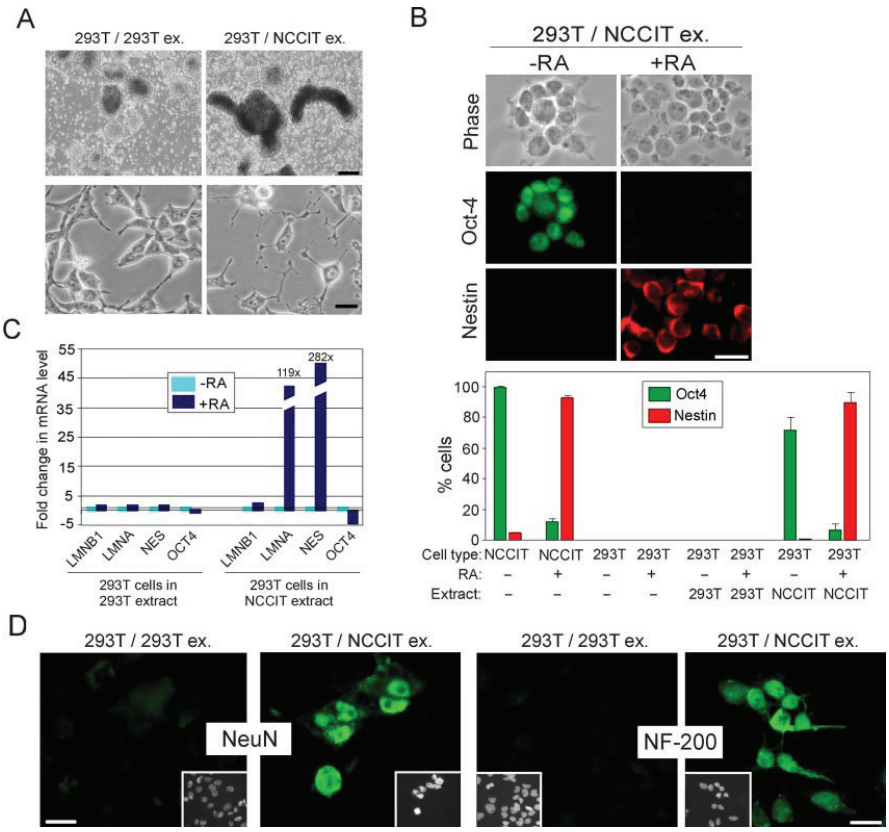


Figure 7. Neuronal differentiation of 293T cells treated with 293T or NCCIT extract. (A) Top, induction of differentiation. Suspended aggregates after 2 wk of culture with 10 μM all-*trans*-retinoic acid. Bottom, differentiation. Cells were plated onto poly-L-lysine-coated coverslips after 2 d of culture in the absence of retinoic acid but with mitotic inhibitors. Note neurite extensions in NCCIT extract-treated cells. (B) NCCIT extract-treated cells either treated with retinoic acid (+RA) or not (−RA) for 3 wk were immunolabeled using antibodies against Oct4 and nestin. Graph shows proportions (mean ± SD) of cells immunolabeled with anti-Oct4 and anti-nestin antibodies (n = 200 cells in each of a triplicate analysis for each treatment). (C) Quantitative RT-PCR analysis of expression of *OCT4*, *NES* (nestin), *LMNA* and *LMNB1* in 293T cells treated with 293T or NCCIT extract, in absence (−RA) or presence (+RA) or retinoic acid for 3 wk. (D) NeuN and NF200 immunofluorescence analysis of indicated cell types induced to differentiate as described in A. Insets, DNA labeled with Hoechst 33342. Bars, 400 μm (A, top); 40 μm (A, bottom); 40 μm (B and D).

NCCIT Extract Enhances Differentiation Potential toward Adipogenic, Osteogenic, and Endothelial Lineages

To provide additional evidence for induction of differentiation potential in 293T cells treated with NCCIT extract, we determined whether the cells would acquire phenotypic characteristics of adipocytes and osteoblasts. After 3 wk of retinoic acid treatment and 3 wk of stimulation in appropriate differentiation medium (see *Materials and Methods*), a proportion of 293T cells, NCCIT cells, and of cells treated with 293T or NCCIT extract were induced to differentiate toward adipogenic and osteogenic pathways (Figures 8, A and B, and S2D and S2E). Intracellular lipid staining with Oil-Red-O showed enhanced differentiation of NCCIT extract-treated cells relative to any other cell type toward the adipogenic pathway (Figure 8A). Moreover, significantly more Alzarin red-stained mineralized nodules were detected in NCCIT extract-treated 293T cells compared with any other cell type (Figure 8B, arrows; $p < 10^{-6}$; *t* tests). Additionally, induction of endothelial differentiation of NCCIT extract-treated cells in methylcellulose triggered the appearance of an endothelial cell phenotype after 7 d. With cells forming elongated "tracks" in methylcellulose (Figure 8C; see controls in Figure S2F). Morphological changes were supported by immunoreactivity to CD31 and CD144, two endothelial cell surface markers (Boquest *et al*., 2005) (Figure 8D), and by induction of expression of *CD31* (2 ± 0.15-fold up-regulation compared with undifferentiated, extract-treated cells) and *CD144* (347 ± 97.6-fold up-regulation). Collectively, these results indicate that treatment with NCCIT extract enhances the ability of 293T cells to differentiate into ectoderm and several mesoderm lineages.

A Mouse ES Cell Extract Promotes A-Type Lamin Down-Regulation and Oct4 Transcription in NIH3T3 Fibroblasts

A nuclear and cytoplasmic extract of mouse ESCs was prepared to determine whether it was capable of eliciting mark-

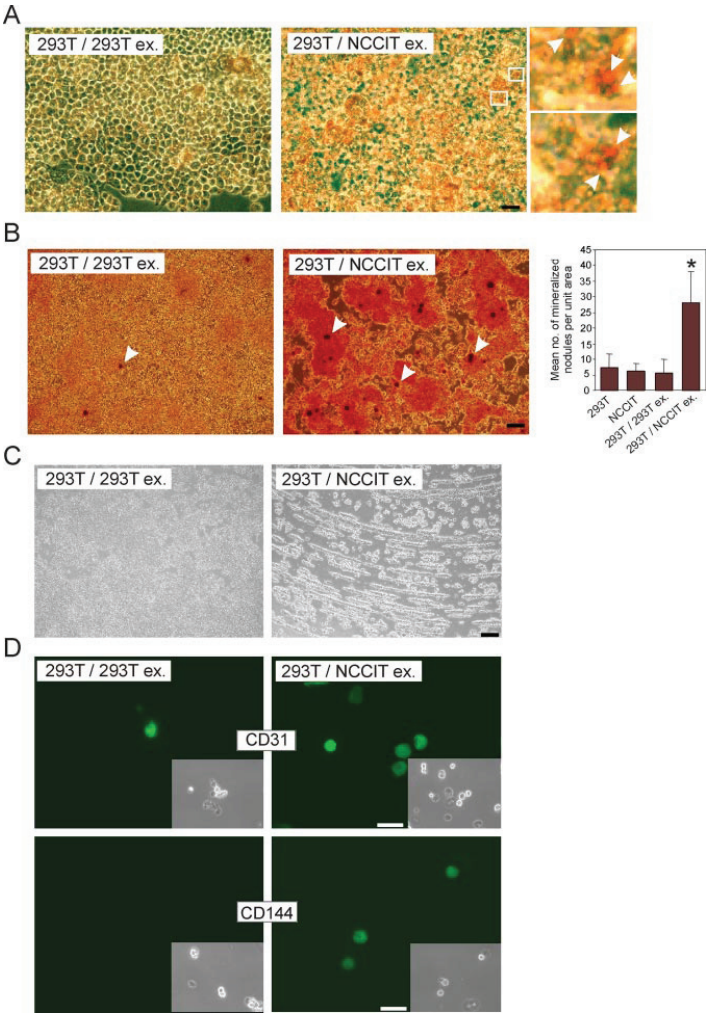


Figure 8. Induction of adipogenic, osteogenic, and endothelial differentiation of 293T cells treated with 293T or NCCIT extract. (A and B) Cells were exposed to 10 μM retinoic acid for 21 d, washed, and cultured in the absence of retinoic acid in adipocyte (A) or osteoblast (B) differentiation medium for 21 d. (A) Cells were stained with Oil-Red-O to reveal lipid droplets. Images on the right are enlargements of two areas framed in white in the adjacent panel (top right-hand quadrant). Arrows point to strongly stained lipid droplets. (B) Cells were stained with Alzarin red to visualize mineralized nodules (arrows). Graph shows mean ± SD number of distinct strongly mineralized nodules (arrows) per unit area (area shown in B). Twenty-four to 27 areas were analyzed within wells of six-well culture plates. *$p < 10^{-6}$ relative to other treatments (ANOVA). (C) 293T and NCCIT extract-treated cells were passaged onto methylcellulose for 7 d to elicit endothelial differentiation. Note the formation of a track phenotype characteristic of cultured endothelial cells (right). (D) Direct immunofluorescence labeling of CD31 and CD144 surface antigens in extract-treated cells induced to differentiate as described in C. After differentiation, cells were loosened from the methylcellulose semisolid substrate by dilution with PBS and thus lost their elongated phenotype. Bars, 40 μm (A), 200 μm (B and C), and 40 μm (D).

ers of pluripotency, as with the NCCIT extract. Within 4–9 d after ESC extract treatment, a proportion 3T3 cells formed distinct colonies of small round cells that lifted from the surface to form embryoid-like bodies (Figure 9A). Fifty to 100 embryoid-like bodies were identified in 100-mm culture plates, whereas most remaining cells retained a fibroblast morphology. Smaller aggregates of round (nonmitotic) cells were also detected. In contrast, 3T3 cells exposed to their own extract grew as fibroblasts and were not distinguishable from untreated cells (Figure 9A). The ESC-like phenotype was maintained after passaging cells weekly for at least 10 wk. RT-PCR analysis of the embryoid body-like structures clearly revealed *Oct4* transcripts (see below), whereas *Lmna*, but not *Lmnb1*, was strongly down-regulated (Figure 9B).

Embryoid-like bodies derived from ESC extract-treated cells expressed ALP, another embryonic and ESC marker, after 8 d of culture (Figure 9, C and D; 1 wk after extract exposure). ALP expression was inhibited by a 24-h exposure to 25 μM RNA PolII inhibitor 5,6-dichloro-1-D-ribofuranosyl benzimidazole (DRB) or to 10 μg/ml protein synthesis inhibitor cycloheximide (CHX) (Figure 9D). Thus, ALP expression was endogenous to the extract-treated cells. No expression was detected in control cells (Figure 9, C and D). Furthermore, immunofluorescence analysis showed that ~90% of embryoid-like body-derived cells expressed intranuclear Oct-4, whereas in the same cells lamin A/C expression was reduced to undetectable levels (our unpublished data). Immunoblots confirmed this observation (Figure 9E) and indicated that ESC extract-treated cells expressed Oct4 to levels similar to ESCs. Oct4 not was detected in 3T3 cells incubated in their own extract or in intact (nonpermeabilized) 3T3 cells treated with ESC extract (Figure 9E). This ruled out the detection of an unspecific anti-Oct4 immunoreactive product and of any extract-derived Oct4 protein that would stick to the cell surface. Detection of Oct4 protein in ESC extract-treated cells required a threshold

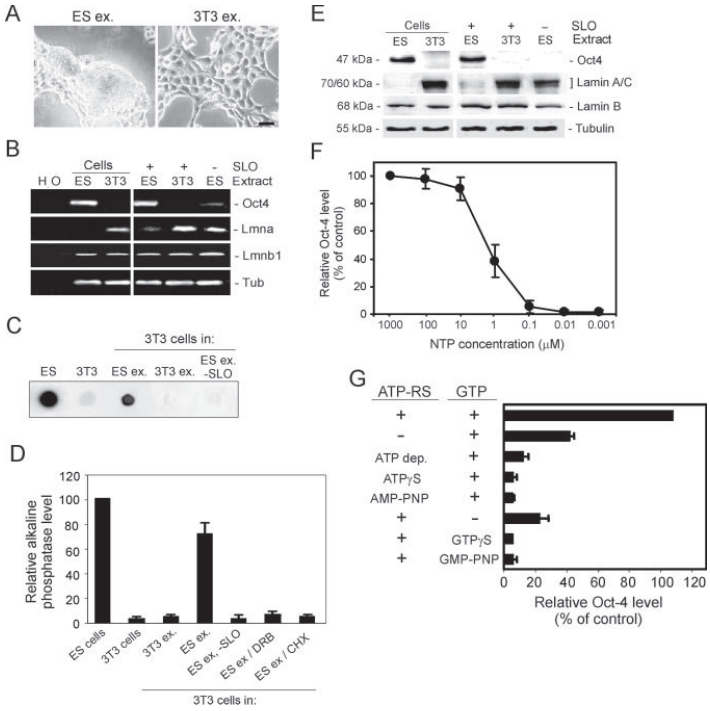


Figure 9. Induction of Oct4 and ALP expression in 3T3 cells exposed to mouse ESC extract. (A) 3T3 cells 10 d after treatment with ESC extract (ES ex.) or 3T3 extract (3T3 ex.). Bar, 20 μm. (B) RT-PCR analysis of expression of indicated genes in ESCs, 3T3 cells and 3T3 cells at 2 wk after treatment with ESC extract. (C) ALP expression in cells treated as described in B (2 wk after extract treatment). (D) Relative ALP level in ESCs, 3T3 cells, and in 3T3 cells treated in 3T3 extract or in ESC extract alone or with 25 μM DRB or 10 μg/ml CHX, as indicated. Cells were analyzed 2 wk after extract treatment. (E) Indicated cell types (as in B) were analyzed by Western blotting for expression of Oct4, lamin A/C, B-type lamins, and γ-tubulin, 2 wk after extract treatment. (F) Relative Oct4 level in 3T3 cells treated with ESC extract containing decreasing concentrations of NTPs (1 wk after extract treatment). (G) Relative Oct4 level in 3T3 cells exposed to ESC extract containing indicated ATP or GTP analogues. ATP-RS, ATP-regenerating system (2 wk after extract treatment).

(10 μM) concentration of NTPs in the extract (Figure 9F), providing additional evidence for the lack of unspecific detection of Oct4 in these cells.

Closer examination of the need for exogenous nucleotides to promote Oct4 detection indicated a requirement for ATP and GTP hydrolysis (Figure 9G). Indeed, ATP depletion from the extract with glucose and hexokinase, replacement of exogenous ATP with adenosine-5′-*O*-(3-thio)triphosphate or adenyl-5′-yl imidodiphosphate, or GTP removal or substitution with guanosine 5′-*O*-(3-thio)triphosphate or guanosine 5′-[β,γ-imido]triphosphate abrogated Oct4 detection (Figure 9G). This is consistent with a role of active nuclear import for transcription in cell extracts (Håkelien *et al*., 2002). Furthermore, heat treatment (95°C; 5 min), trypsinization, or proteinase K treatment of the ESC extract abolished Oct4 detection in 3T3 cells (Figure 10). However, DNAse I (100 mg/ml) or RNAse A (50 mg/ml) did not affect Oct4 levels in extract-treated cells (see below; Figure 10), ruling out a significant contribution of mRNA or DNA of extract origin.

The ESC Extract Induces a Biphasic Wave of Oct4 Transcription and Translation

To gain insight on the dynamics of Oct4 induction in ESC extract-treated cells and evaluate any putative contribution of extract-derived Oct4 protein, we assessed intracellular Oct4 protein levels over time after extract treatment and determined the effect of PolII and protein synthesis inhibitors on Oct4 induction. Relative Oct4 levels in ESC extract-treated cells are shown in Figure 11A. A biphasic response to extract exposure was observed. First, Oct4 was detected as early as 1 h after recovery of the cells from the extract, and the level peaked at 24 h (Figure 11A). This peak was followed by a marked reduction of Oct4 by 36 h to a level barely detectable by 48 h. By 72 h, however, a second wave of Oct4 was detected of amplitude similar to or higher than the first wave, and it persisted for at least 5 d (Figure 11A). Oct4 protein was also detected in these cells 5 and 10 wk after extract treatment (our unpublished data); thus, we anticipate that the second elevation of Oct4 in these cells is long-lasting. Of note, the biphasic Oct4 protein elevation paralleled fluctuations in *Oct4* transcripts, as determined by real-time RT-.PCR (Figure 11D), suggesting a short half-life (a few hours) of Oct4 RNA and protein.

Culture of ESC extract-treated cells with 25 μM DRB from 1 to 24 h after extract treatment dramatically reduced Oct4 levels by 1 h (Figure 11B, top), suggesting that Oct4 detection at this time point resulted primarily from transcription but also to a minor extent from uptake of Oct4 protein from the extract. This hypothesis was verified by a complete double immunodepletion of Oct4 from the ESC extract (Figure 12A), which resulted in a reduced Oct4 level by 1 h in non-DRB-treated 3T3 cells, without affecting subsequent levels (Figure 12, B and 1C). DRB exposure of cells treated with Oct4-depleted extract completely abolished Oct4 detection by 1 h (our unpublished data). We concluded, therefore, that Oct4 detected by the first hour of culture after extract exposure originated from the extract and from RNA PolII-mediated transcription and translation.

The effect of PolII inhibition on the dynamics of Oct4 expression in ESC extract-treated cells differed with timing of drug exposure after extract treatment. A 1- to 24-h DRB treatment almost completely blocked the fist Oct4 elevation

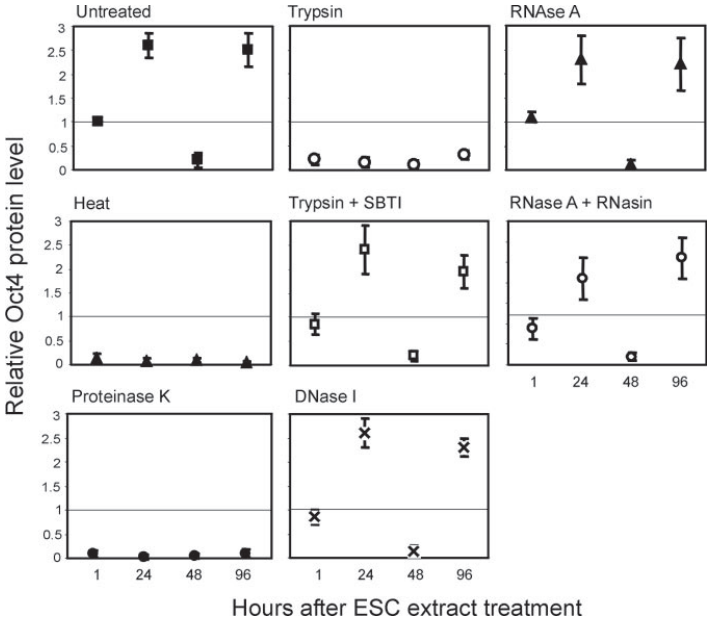


Figure 10. Relative Oct4 protein levels in 3T3 cells exposed to ESC extract pretreated as indicated in each panel. Cells were cultured for 1–96 h after extract treatment, lysed, and analyzed by Western blotting. Oct4 protein levels were determined by densitometric analysis of duplicate blots for each time point under each condition. Level 1 indicates Oct4 level at 1 h after removal of cells from an untreated ESC extract (top left).

(Figure 11B, top), supporting a role of RNA PolII in this process. In addition, and unexpectedly, the second wave of Oct4 induction was also dramatically impaired (Figure 11B, top). Again, these changes paralleled *Oct4* transcript levels: the first up-regulation of *Oct4* mRNA was inhibited, the second rise in *Oct4* transcription was also severely compromised, albeit not fully blocked, such that *Oct4* transcripts were up-regulated five- to sevenfold above the 48-h baseline

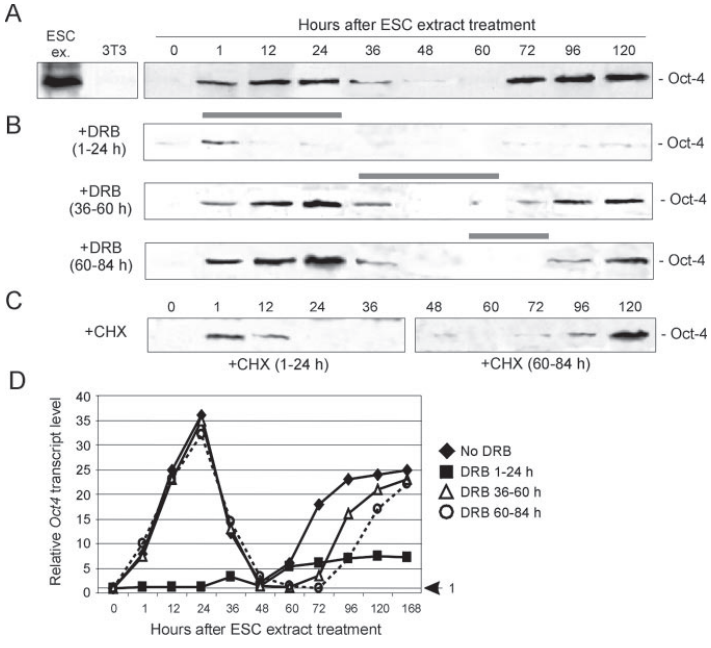


Figure 11. Oct4 expression in ESC extract-treated cells is biphasic and RNA PolII dependent. (A) Immunoblotting analysis of intracellular Oct4 levels in 3T3 cells treated with ESC extract and cultured for indicated time periods. (B) Cells were exposed to 25 μM DRB at 1–24 h (top), 36–60 h (middle), and 60–84 h (bottom) of culture. (C) Cells were exposed to 10 μg/ml CHX at 1–24 h (left) or 60–84 h (right) of culture before immunoblotting. (D) Real-time RT-PCR analysis of *Oct4* expression in 3T3 cells exposed to ESC extract. Cells were cultured with 0 or 25 μM DRB at indicated time periods as described in B. Reference level (level 1) is *Oct4* mRNA level in 3T3 cells immediately upon plating cells after recovery from extract. Data show a representative set from two experiments.

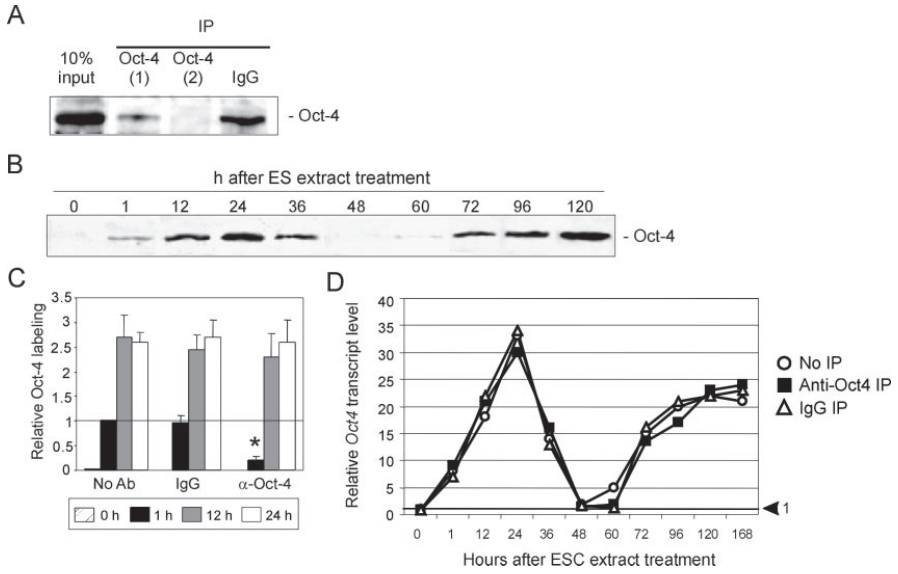


Figure 12. Immunodepletion of Oct4 from ESC extract maintains Oct4 expression profile. (A) Immunoprecipitation of Oct4 from ESC extract. Numbers indicate rounds of immunoprecipitation (IP). Resulting extracts were immunoblotted using anti-Oct4 antibodies. (B) 3T3 cells were exposed to immunodepleted ESC extract, cultured for indicated time periods, and blotted using anti-Oct4 antibodies. (C) Relative intracellular Oct4 protein level in 3T3 cells exposed to control (no antibody), mock-depleted (IgG), or anti-Oct4-depleted ESC extract and cultured for indicated time periods. *$p < 0.001$ relative to the 1-h time point in other treatments (ANOVA). Data from two experiments. (D) Real-time RT-PCR analysis of *Oct4* expression in 3T3 cells exposed to intact, mock-depleted, or Oct4-depleted ESC extract. Reference level (level 1) is Oct4 transcript level in 3T3 cells immediately upon plating cells after recovery from extract. Data show a representative set of two experiments.

instead of the ~25-fold normally observed (Figure 11D). This suggests that the second, long-lasting induction of *Oct4* up-regulation is dependent on an early, short-term boost of *Oct4* transcription and/or translation.

DRB exposure from 36 to 60 h after extract treatment, during the dip in Oct4 mRNA and protein levels, did not affect early Oct4 transcript and protein levels and only slightly delayed the second rise of Oct4 (Figure 11B, middle). This delay was also noticed at the transcript level, but it did not significantly affect Oct4 mRNA or protein level at 7 d (168 h; Figure 11D). Moreover, DRB applied from 55 to 84 h after extract treatment completely abrogated Oct4 transcription and translation by 72 h, but removal of the drug ultimately restored control Oct4 levels (Figure 11B, bottom, and D). Thus, there is a requirement for PolII activity for de novo transcription of *Oct4* 3 d after extract treatment. Last, both Oct4 elevations were abolished upon incubation with 10 μg/ml CHX, a protein synthesis inhibitor (Figure 11C). We concluded that whereas uptake of limited amounts of short-lived extract-derived Oct4 protein probably occurs, the two phases of Oct4 induction result from transcriptional and translational activity in extract-treated cells.

In summary, this study provides transcriptional and functional evidence that an extract of undifferentiated EC or ES cells can induce markers of dedifferentiation and signs of differentiation plasticity in an otherwise more developmentally restricted cell type. Furthermore, the NCCIT extract induces DNA demethylation of *OCT4*, indicative of an epigenetic reprogramming event at this locus.

DISCUSSION

Target Cell Type-specific Programming of Gene Expression

The induction of a transcriptional profile of, according to our data, uncertain stability, suggests the establishment of a program of gene expression, as opposed to a complete functional reprogramming such as that occurring after nuclear transfer. Of the hundreds of NCCIT-specific genes up- or down-regulated on any given week after NCCIT extract treatment, only 5–7% are altered by Jurkat extract, and most of these genes overlap with genes altered (statistically by chance) in cells exposed to their own extract. This argues for some cell-type specificity in the nature of genes modulated by extract treatment. Despite some stability in the expression profile of specific genes after extract exposure, not all changes seem to be heritable. Genes with unstable expression pattern may include "passive bystanders" that generate a transcriptional noise (Paulsson, 2004) and result from more specific alterations in the transcriptional network. Perturbation in the network, however, would be expected to lead to changes trickling down the network until a transcriptional equilibrium is reached (Miklos and Maleszka, 2004). Fluctuations in the gene expression profile therefore may result from incomplete reprogramming and from heterogeneity in the transcriptional response to extract treatment. Nevertheless, the dynamics of gene expression may also illustrate a temporal compartmentalization, in terms of timing, duration, and periodicity of gene activity required to establish a heritable transcriptional network (Klevecz *et al*., 2004).

Evidence of Induction of Potential for Pluripotency

The gene expression program elicited by NCCIT extract suggests the establishment of a potential for multilineage differentiation in otherwise more developmentally restricted cells. An indicator of dedifferentiation is the down-regulation of genes indicative of a differentiated state. This is exemplified by the down-regulation of many 293T cell genes, including the kidney natriuretic peptide receptor C (*NPR3*), and the repression of lamin A (*LMNA*), a marker of differentiated cells (Hutchison and Worman, 2004). *LMNA* down-regulation seems specific for extracts of undifferentiated cells that do not express lamin A/C. In contrast, cardiomyocyte extracts can up-regulate *LMNA* expression in adipose stem cells, an event that correlates with differentiation toward a cardiomyocyte phenotype (Gaustad *et al*., 2004), and *LMNA* is reactivated upon retinoic acid-mediated differentiation of NCCIT extract-treated cells. Thus, the transcriptional status of *LMNA* provides a direct assessment of (de)differentiation transitions mediated by cell extracts. The mechanism of gene inactivation in extracts is unclear. However, evidence for the down-regulation of many genes by single small interfering RNAs (Mathieu and Bender, 2004), possibly through a control of DNA methylation (Matzke and Birchler, 2005), raises the hypothesis of a contribution of small RNAs in extract-based nuclear (re)programming.

An indicator of pluripotency is the up-regulation of genes characteristic of undifferentiated EC (NCCIT) or ES cells. Several embryonic, germ cell, and stem cell genes are activated to levels similar to those of NCCIT cells. Of note, *Oct4* is expressed in ESCs to maintain pluripotency and acts in cooperation with *SOX2* (Avilion *et al*., 2003). The latter is also expressed in extract-treated cells. The Oct4 transcription factor acts on a subset of target genes, including *UTF1*, *REX1*, *OCT2*, and *NANOG* (see Hoffman and Carpenter, 2005 for an updated review of human embryonic and ESC genes). These were found to be up-regulated by NCCIT extract. Furthermore, because *UTF1* expression requires synergistic activities of Oct4 and Sox2 (Nishimoto *et al*., 1999), our results suggest the formation of a functional transcriptional complex between these factors.

Another feature of NCCIT extract-treated cells is the expression of genes suggestive of a potential for multiple lineage differentiation and acquisition of neurogenic, adipogenic, osteogenic, and endothelial differentiation ability. Differentiation potential toward other lineages was not investigated. Multilineage priming is a hallmark of hematopoietic stem cells (Akashi *et al*., 2003) and mesenchymal stem cells from bone marrow (Woodbury *et al*., 2002) and adipose tissue (Boquest *et al*., 2005). It may reflect their ability to promptly differentiate into a specific cell type in the tissue in which they reside, in response to simulation. Thus, similarly to somatic stem cells, the transcriptional signature of NCCIT extract-treated cells extends across germ layer boundaries. Additionally, because they also express embryonic and ESC markers, these cells display characteristics of a perhaps more precursor cell than the starting epithelial cell type.

Chromatin Remodeling Associated with Nuclear Reprogramming

The NCCIT extract retains the ability to elicit epigenetic reprogramming of *OCT4* in 293T cells. Our data illustrate the demethylation of six of eight cytosines in CpG dinucleotides between CR2 and CR3 in the *OCT4* promoter (Nordhoff *et al*., 2001). *Oct4* DNA demethylation in thymocyte nuclei has been reported after fusion with EG or ES cells (Tada *et al*., 1997, 2001) and is required for *Oct4* transcription after nuclear transplantation into *Xenopus* oocytes (Simonsson and Gurdon, 2004). The process driving *OCT4* DNA demethylation remains unclear but seems to require deproteinization (Simonsson and Gurdon, 2004), and it may involve cleavage of methyl groups (Ramchandani *et al*., 1999) or cytosine deamination (Morgan *et al*., 2004). The ability to induce DNA demethylation in bulk cells or nuclei incubated in extracts raises the possibility of isolating the DNA demethylation activity involved.

A transient induction of *Oct4* transcription and translation—independent of uptake of residual Oct4 protein from the extract—is triggered within the first hours after extract exposure. This early *Oct4* up-regulation may be explained by nuclear uptake of extract-derived transcription factors and chromatin remodelers that target the Oct4 promoter (Nordhoff *et al*., 2001). This possibility is supported by the inhibitory effect of removing proteins from the extract (Figure 10) and of immunodepleting BRG1 from mouse ESC extracts on *Oct4* transcription in 3T3 cells (our unpublished data). The transient (24- to 48-h) nature of this first wave of *Oct4* activation presumably results from depletion of factors (most transcription factors have a half-life of hours). This suggests that transcription factor synthesis and targeting are not optimally sustained during the first hours after extract treatment. The second wave of *Oct4* up-regulation, however, is sustained for several days and weeks. Long-term *Oct4* expression is consistent with DNA demethylation taking place in our system. Timing of long-term *Oct4* activation by ESC extract is consistent with the time interval observed between introduction of nuclei into oocytes and *Oct4* demethylation in *Xenopus* oocytes (Simonsson and Gurdon, 2004). How early Oct4 demethylation occurs after extract exposure, however, remains undetermined, Nevertheless, if *Oct4* demethylation is required for expression of the gene (Simonsson and Gurdon, 2004), the rapid induction of RNA PolII-dependent *Oct4* transcription in ESC extract (Figure 11) also suggests that demethylation (at least of *Oct4*) is very rapidly triggered upon extract treatment.

Alteration of gene expression in extract-treated cells implies a global and locus-specific remodeling of chromatin. Remodeling of mammalian chromatin by *Xenopus* egg extract depends on ATPase activity of a chromatin remodeling complex (Kikyo *et al*., 2000), and in a similar system BRG1 was shown to be involved in *OCT4* activation (Hansis *et al*., 2004). We have to date no evidence for ATP-dependent chromatin remodeling in our system. However, *OCT4* activation requires ATP hydrolysis, and immunodepletion of BRG1 from mouse ESC extracts abolishes *OCT4* transcription (our unpublished data). *OCT4* promoter DNA demethylation (this study), and hyperacetylation of histone H4 at the *IL2* locus in cells treated with Jurkat extract (Håkelien *et al*., 2002) provide evidence that locus-specific chromatin remodeling takes place in our system. Conceivably, controlled manipulations of epigenetic alterations may enhance the heritability of gene expression in (re)programmed cells and may prove beneficial for reprogramming cell fate in a therapeutic context.

ACKNOWLEDGMENTS

We are grateful to A. S. Burnside for input on the ESC system, E. Ormerud for karyotyping analysis, and O. Sigurjonsson for antibodies. This work was

supported by grants from Kirin Brewery (Tokyo, Japan), the Research Council of Norway, a Functional Genomics (FUGE) initiative from the Research Council of Norway, the Norwegian Center for Stem Cell Research, the Norwegian Cancer Society and The University of Oslo.

REFERENCES

Akashi, K., He, X., Chen, J., Iwasaki, H., Niu, C., Steenhard, B., Zhang, J., Haug, J., and Li, L. (2003). Transcriptional accessibility for genes of multiple tissues and hematopoietic lineages is hierarchically controlled during early hematopoiesis. Blood *101*, 383–389.

Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. *17*, 126–140.

Blelloch, R. H., Hochedlinger, K., Yamada, Y., Brennan, C., Kim, M., Mintz, B., Chin, L., and Jaenisch, R. (2004). Nuclear cloning of embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA *101*, 13985–13990.

Boquest, A. C., Shahdadfar, A., Fronsdal, K., Sigurjonsson, O., Tunheim, S. H., Collas, P., and Brinchmann, J. E. (2005). Isolation and transcription profiling of purified uncultured human stromal stem cells: alteration of gene expression following in vitro cell culture. Mol. Biol. Cell *16*, 1131–1141.

Byrne, J. A., Simonsson, S., Western, P. S., and Gurdon, J. B. (2003). Nuclei of adult mammalian somatic cells are directly reprogrammed to oct-4 stem cell gene expression by amphibian oocytes. Curr. Biol. *13*, 1206–1213.

Cattaneo, E., and McKay, R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature *347*, 762–765.

Chambers, I., and Smith, A. (2004). Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene *23*, 7150–7160.

Chaudhary, N., and Courvalin, J.-C. (1993). Stepwise reassembly of the nuclear envelope at the end of mitosis. J. Cell Biol. *122*, 295–306.

Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce, d. L. F., and Robl, J. M. (1998). Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. Nat. Biotechnol. *16*, 642–646.

Cowan, C. A., Atienza, J., Melton, D. A., and Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science *309*, 1369–1373.

Damjanov, I., Horvat, B., and Gibas, Z. (1993). Retinoic acid-induced differentiation of the developmentally pluripotent human germ cell tumor-derived cell line, NCCIT. Lab. Investig. *68*, 220–232.

Flasza, M., Shering, A. F., Smith, K., Andrews, P. W., Talley, P., and Johnson, P. A. (2003). Reprogramming in inter-species embryonal carcinoma-somatic cell hybrids induces expression of pluripotency and differentiation markers. Cloning Stem Cells *5*, 339–354.

Fortunel, N. O., *et al.* (2003). Comment on "'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". Science *302*, 393

Gaustad, K. G., Boquest, A. C., Anderson, B. E., Gerdes, A. M., and Collas, P. (2004). Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes. Biochem. Biophys. Res. Commun. *314*, 420–427.

Gurdon, J. B., and Byrne, J. A. (2003). The first half-century of nuclear transplantation. Proc. Natl. Acad. Sci. USA *100*, 8048–8052.

Håkelien, A. M., Gaustad, K. G., and Collas, P. (2004). Transient alteration of cell fate using a nuclear and cytoplasmic extract of an insulinoma cell line. Biochem. Biophys. Res. Commun. *316*, 834–841.

Håkelien, A. M., Gaustad, K. G., Taranger, C. K., Skålhegg, B. S., Kuntziger, T., and Collas, P. (2005). Long-term in vitro, cell-type-specific genome-wide reprogramming of gene expression. Exp. Cell Res. *309*, 32–47.

Håkelien, A. M., Landsverk, H. B., Robl, J. M., Skålhegg, B. S., and Collas, P. (2002). Reprogramming fibroblasts to express T-cell functions using cell extracts. Nat. Biotechnol. *20*, 460–466.

Hansis, C., Barreto, G., Maltry, N., and Niehrs, C. (2004). Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. Curr. Biol. *14*, 1475–1480.

Hoffman, L. M., and Carpenter, M. K. (2005). Characterization and culture of human embryonic stem cells. Nat. Biotechnol. *23*, 699–708.

Hosler, B. A., LaRosa, G. J., Grippo, J. F., and Gudas, L. J. (1989). Expression of REX-1, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. Mol. Cell Biol. *9*, 5623–5629.

Hutchison, C. J., and Worman, H. J. (2004). A-type lamins: guardians of the soma? Nat. Cell Biol. *6*, 1062–1067.

Hwang, W. S., *et al*. (2004). Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. Science *303*, 1669–1674.

Ji, W., Zhou, W., Gregg, K., Lindpaintner, K., Davis, S., and Davis, S. (2004). A method for gene expression analysis by oligonucleotide arrays from minute biological materials. Anal. Biochem. *331*, 329–339.

Kikyo, N., Wade, P. A., Guschin, D., Ge, H., and Wolffe, A. P. (2000). Active remodeling of somatic nuclei in egg cytoplasm by the nucleosomal ATPase ISWI. Science *289*, 2360–2362.

Klevecz, R. R., Bolen, J., Forrest, G., and Murray, D. B. (2004). A genomewide oscillation in transcription gates DNA replication and cell cycle. Proc. Natl. Acad. Sci. USA *101*, 1200–1205.

Landsverk, H. B., Håkelien, A. M., Küntziger, T., Robl, J. M., Skålhegg, B. S., and Collas, P. (2002). Reprogrammed gene expression in a somatic cell-free extract. EMBO Rep. *3*, 384–389.

Mathieu, O., and Bender, J. (2004). RNA-directed DNA methylation. J. Cell Sci. *117*, 4881–4888.

Matzke, M. A., and Birchler, J. A. (2005). RNAi-mediated pathways in the nucleus. Nat. Rev. Genet. *6*, 24–35.

McGann, C. J., Odelberg, S. J., and Keating, M. T. (2001). Mammalian myotube dedifferentiation induced by newt regeneration extract. Proc. Natl. Acad. Sci. USA *98*, 13699–13704.

Miklos, G. L., and Maleszka, R. (2004). Microarray reality checks in the context of a complex disease. Nat. Biotechnol. *22*, 615–621.

Morgan, H. D., Dean, W., Coker, H. A., Reik, W., and Petersen-Mahrt, S. K. (2004). Activation-induced cytidine deaminase deaminates 5-methylcytosine in DNA and is expressed in pluripotent tissues: implications for epigenetic reprogramming. J. Biol. Chem. *279*, 52353–52360.

Munsie, M. J., Michalska, A. E., O'Brien, C. M., Trounson, A. O., Pera, M. F., and Mountford, P. S. (2000). Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. Curr. Biol. *10*, 989–992.

Nishimoto, M., Fukushima, A., Okuda, A., and Muramatsu, M. (1999). The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Mol. Cell. Biol. *19*, 5453–5465.

Nordhoff, V., Hubner, K., Bauer, A., Orlova, I., Malapetsa, A., and Scholer, H. R. (2001). Comparative analysis of human, bovine, and murine Oct-4 upstream promoter sequences. Mamm. Genome *12*, 309–317.

Okuda, A., *et al.* (1998). UTF1, a novel transcriptional coactivator expressed in pluripotent embryonic stem cells and extra-embryonic cells. EMBO J *17*, 2019–2032.

Paulsson, J. (2004). Summing up the noise in gene networks. Nature *427*, 415–418.

Pells, S., Di Domenico, A .I., Callagher, E. J., and McWhir, J. (2002). Multipotentiality of neuronal cells after spontaneous fusion with embryonic stem cells and nuclear reprogramming in vitro. Cloning Stem Cells *4*, 331–338.

Planat-Benard, V., *et al*. (2004). Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation *109*, 656–663.

Qin, M., Tai, G., Collas, P., Polak, J. M., and Bishop, A. E. (2005). Cell extract-derived differentiation of embryonic stem cells. Stem Cells *23*, 712–718.

Ramchandani, S., Bhattacharya, S. K., Cervoni, N., and Szyf, M. (1999). DNA methylation is a reversible biological signal. Proc. Natl. Acad. Sci. USA *96*, 6107–6112.

Simonsson, S., and Gurdon, J. (2004). DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. Nat. Cell Biol. *6*, 984–990.

Stewart, R., Christie, V. B., and Przyborski, S. A. (2003). Manipulation of human pluripotent embryonal carcinoma stem cells and the development of neural subtypes. Stem Cells *21*, 248–256.

Tada, M., Tada, T., Lefebvre, L., Barton, S. C., and Surani, M. A. (1997). Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. EMBO J. *16*, 6510–6520.

Tada, M., Takahama, Y., Abe, K., Nakastuji, N., and Tada, T. (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr. Biol. *11*, 1553–1558.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E., and Scott, E. W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416*, 542–545.

Teshima, S., Shimosato, Y., Hirohashi, S., Tome, Y., Hayashi, I., Kanazawa, H., and Kakizoe, T. (1988). Four new human germ cell tumor cell lines. Lab. Invest. *59*, 328–336.

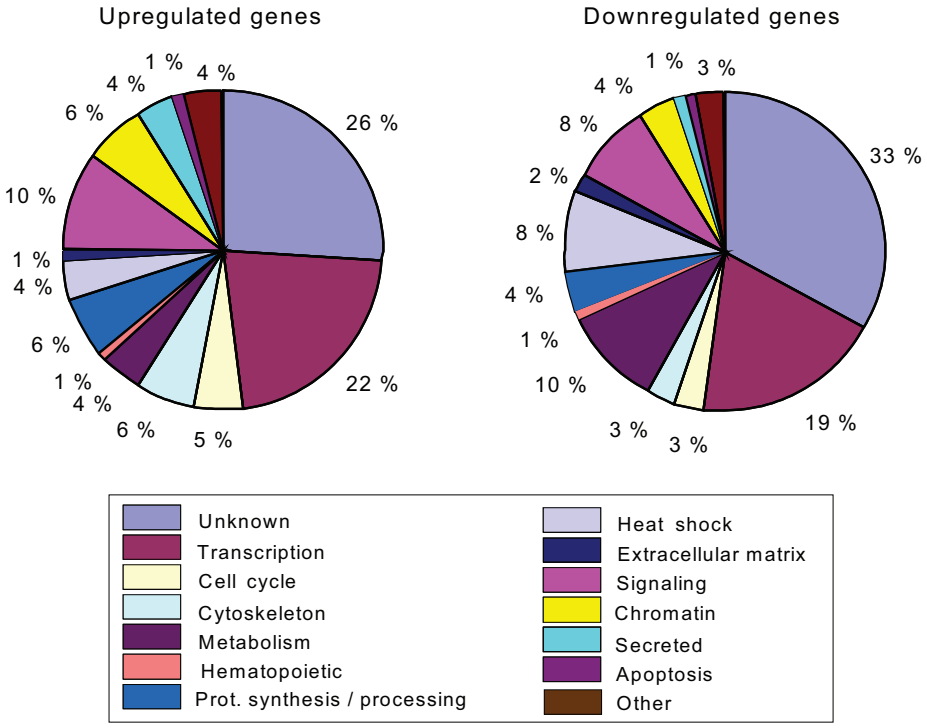
Wakayama, T., Tabar, V., Rodriguez, I., Perry, A. C., Studer, L., and Mombaerts, P. (2001). Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science *292*, 740–743.

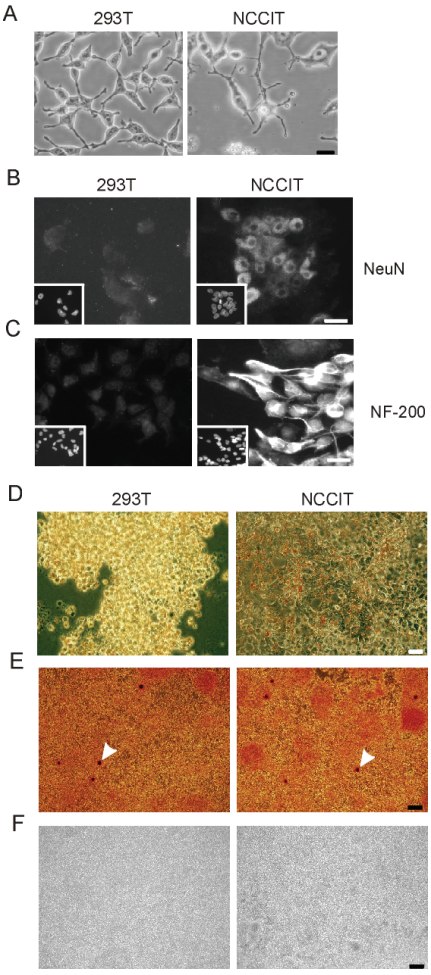
Wilmut, I., Beaujean, N., De Sousa, P. A., Dinnyes, A., King, T. J., Paterson, L. A., Wells, D. N., and Young, L. E. (2002). Somatic cell nuclear transfer. Nature *419*, 583–586.

Woodbury, D., Reynolds, K., and Black, I. B. (2002). Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J. Neurosci. Res. *69*, 908–917.

Wu, X., Avni, D., Chiba, T., Yan, F., Zhao, Q., Lin, Y., Heng, H., and Livingston, D. (2004). SV40 T antigen interacts with Nbs1 to disrupt DNA replication control. Genes Dev. *18*, 1305–1316.

Ying, Q. L., Nichols, J., Evans, E. P., and Smith, A. G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545–548.


Upregulated genes
26 %
22 %
5 %
6 %
4 %
1 %
6 %
4 %
1 %
10 %
6 %
4 %
1 %
4 %
Downregulated genes
33 %
19 %
3 %
3 %
10 %
1 %
4 %
8 %
2 %
8 %
4 %
1 %
Unknown
Transcription
Cell cycle
Cytoskeleton
Metabolism
Hematopoietic
Prot. synthesis / processing
Heat shock
Extracellular matrix
Signaling
Chromatin
Secreted
Apoptosis
Other


A
293T
NCCIT
B
293T
NCCIT
NeuN
C
NF-200
D
293T
NCCIT
E
F

Supplementary Table S1. Real-time RT-PCR primers used in this study

Gene name	Forward primer (F) 5'→3' Reverse primer (R) 5'→3' SYBR® Green or Probe no. (ProbeLibrary)	Product size (bp)
AC133	F: GGGAGAACAATAATAGGATATTTTGAA R: CGATGCCACTTTCTCACTGAT Probe 86	75
ACTB	F: CCAACCGCGAGAAGATGA R: TTGTCACCAGGATCAATGACA Probe 64	97
Actb (mouse)	F: TGACAGGATGCAGAAGGAGA R: CGCTCAGGAGGAGCAATG Not real time PCR	75
APL	F: CCTACCAGCTCATGCATAACATC R: TGGCTTTCTCGTCACTCTCATAC SYBR® Green	114
APOA2	F: AGGTCAAGAGCCCAGAGCTT R: CCTTCTTGATCAGGGGTGTC Probe 68	84
CD9	F: TCGGATTTAACTTCATCTTCTGG R: GTCGAATCGGAGCCATAGTC Probe 56	71
CD31	F: CCACTGCAGAGTACCAGGTG R: TGGCCTCTTTCTTGTCCAGT Probe 12	72
CD144	F: GCAGTCCAACGGAACAGAA R: CATCTTCCCAGGAGGAACAG Probe 30	65
DNMT3B	F: CTGCTTTGCAGCAACACG R: CAGCACCTCCAGGCACTC Probe 74	60
DNMT3L	F: CTCTCAAGCTCCGTTTCACC R: GTACAGGAAGAGGGCATCCA SYBR® Green	189
DSPG3	F: CAGGAGCCTGAATTCACAGG R: CCAAAGACAGGTTGGAAAGTCT Probe 57	66
GAPDH	F: TCGGAGTCAACGGATTTGGT R: TTGCCATGGGTGGAATCATA SYBR® Green	148
LMNA	F: CTGTGGTTGAGGACGACGAG R: TGCGGTAGCTGCGAGTGA SYBR® Green	240
Lmna (mouse)	F: AGCAAGTTGCGTGAGGAGTT R: ACAAGTCCCCCTCCTTCTTG Not real time PCR	64
LMNB1	F: AAGGCGAAGAAGAGAGGTTGAAG R: GCGGAATGAGAGATGCTAACACT	163

	SYBR® Green	
Lmnb1 (mouse)	F: GACCACCATACCCGAGGAGG R: AATGGCACAGCTTTATTCCA Not real time PCR	118
MBP	F: GGGCACGCTTTCCAAAAT R: CCATGGGTGATCCAGAGC Probe 33	62
NANOG	F: CAAAGGCAAACAACCCACTT R: TCTGCTGGAGGCTGAGGTAT SYBR® Green	158
NES	F: CACCTGTGCCAGCCTTTCTTA R: TTTCCTCCCACCCTGTGTCT SYBR® Green	170
NRG1	F: GATCAGCAAATTAGGAAATGACAG R: GGCATACCAGTGATGATCTCG Probe 53	78
NTS	F: AGCTCCTGGAGTCTGTGCTC R: GGTCAAGAAATCTGCTTCTAATGC Probe 35	66
OTX2	F: GGTACCCAGACATCTTCATGC R: CTTAGCTCTTCGATTCTTAAACCATAC Probe 10	95
POU5F1	F: AAGCGATCAAGCAGCGACTAT R: GGAAAGGGACCGAGGAGTACA SYBR® Green	127
Pou5f1 (mouse)	F: GTTGGAGAAGGTGGAACCAA R: CTCCTTCTGCAGGGCTTTC SYBR® Green	61
REX1	F: CAGAACAGAAGAGGCCTTCAC R: TCTGAGTAAGCTGTCTTCAGCAA Probe 62	73
SOX2	F: GCGCCCTGCAGTACAACTC R: GCTGGCCTCGGACTTGAC SYBR® Green	140
STELLA	F: GACCAACAAACAAGGAGCCTAAG R: AGAAGGATCCATCCATTAGACA SYBR® Green	95
Tubb (mouse)	F: CAATGTATACTACAATGAAGCAACTGG R: CCAGACCTGACTGAGTCCATT Not real time PCR	96
UTF1	F: ACCAGCTGCTGACCTTGAAC R: TTGAACGTACCCAAGAACGA SYBR® Green	230
VWF	F: AGTGCAGACCCAACTTCACC R: GTGGGGACACTCTTTTGCAC Probe 04	60

Table shows primers to human transcripts. Primers to mouse transcripts are specified in parentheses.

Supplementary Table S2. NCCIT genes up- or downregulated >3-fold at week eight after treatment with control 293T and Jurkat extracts

Genebank Accession No.	Description
293T extract-treated cells	
Upregulated genes (n=112)	
NM_005345.3	Heat shock 70kD protein 1A (HSPA1A)
NM_004039.1	Annexin A2 (ANXA2)
BC002666.1	Guanylate binding protein 1, interferon-inducible
AW117368 [a]	KIAA0942 protein
NM_012200.2	Beta-1,3-glucuronyltransferase 3 (glucuronosyltransferase I) (B3GAT3)
NM_000227.1	Laminin, alpha 3
BE791251	Claudin 3
NM_001218.2	Carbonic anhydrase XII (CA12)
NM_023037.1 [a]	Putative gene product (13CDNA73)
AF280546.1	Neuropilin-2 soluble isoform 9 (NRP2)
AA502912 [a]	KIAA0906 protein
AK026815.1 [a]	KIAA1102 protein
AW043713 [a]	KIAA1077 protein
AL045513 [a]	KIAA0180 protein
AI282485	HLA-B associated transcript-1
BE901081 [a]	H2A histone family, member X
AL023584 [a]	Contains the HIVEP2 (Schnurri-2)
AK026529.1	Highly similar to transducin (beta)-like 2
AL569804 [a]	KIAA1095 protein
BF968960	Glucose phosphate isomerase
AI401612 [a]	DKFZP434M154 protein
R68573 [a]	Mitochondrial ribosomal protein S12
T90013	C1orf20 gene, partial sequence
AU117487	cAMP responsive element binding protein-like 1
NM_019009.1	TOLLIP protein (LOC54472)
AW189430 [a]	KIAA0244 protein
NM_024952.1 [a]	Hypothetical protein FLJ20950
NM_016234.2	Long-chain fatty acid coenzyme A ligase 5 (FACL5)
NM_016046.1 [a]	Homolog of yeast exosomal core protein CSL4 (CSL4)
NM_024118.1 [a]	Hypothetical protein MGC4692
NM_017842.1 [a]	Hypothetical protein FLJ20489
NM_024105.1 [a]	Hypothetical protein MGC3136
NM_018095.1 [a]	Hypothetical protein FLJ10450
NM_020672.1	S100-type calcium binding protein A14
NM_004422.1	Dishevelled 2 (homologous to Drosophila dsh) (DVL2)
NM_022752.1 [a]	Hypothetical protein FLJ22059
NM_024956.1	Hypothetical protein FLJ23375
NM_022777.1 [a]	Hypothetical protein FLJ14117
AW304174 [a]	Chitobiase, di-N-acetyl
NM_017631.1	Hypothetical protein FLJ20035
NM_014322.1	Opsin 3 (encephalopsin) (OPN3)
NM_023923.1	Hypothetical protein FLJ13171
NM_017912.1 [a]	Hypothetical protein FLJ20637
NM_018534.1 [a]	Hypothetical protein PRO2714
NM_018190.1 [a]	Hypothetical protein FLJ10715
NM_018219.1	Hypothetical protein FLJ10786
NM_014421.1	Dickkopf (Xenopus laevis) homolog 2 (DKK2)
NM_005021.1	Ectonucleotide pyrophosphatase phosphodiesterase 3 (ENPP3)
NM_007167.1	Zinc finger protein 258 (ZNF258)
NM_022741.1 [a]	Hypothetical protein FLJ11850
NM_024708.1 [a]	Hypothetical protein FLJ2255
NM_012282.1	Potassium voltage-gated channel, Isk-related family, 1-like (KCNE1L)
NM_016644.1	Mesenchymal stem cell protein DSC54
NM_017805.1 [a]	Hypothetical protein FLJ20401
NM_024923.1	Hypothetical protein FLJ22389

NM_018001.1 [a]	Hypothetical protein FLJ10120
NM_024907.1	Hypothetical protein FLJ11798
NM_017658.1 [a]	Hypothetical protein FLJ20081
NM_024795.1 [a]	Hypothetical protein FLJ22800
NM_014137.1 [a]	PRO0650 protein
NM_024873.1 [a]	Hypothetical protein FLJ21162
NM_021208.1	EST-YD1 protein (EST-YD1)
NM_018068.1 [a]	Hypothetical protein FLJ10351
NM_014147.1 [a]	HSPC047 protein (HSPC047)
NM_024720.1	Hypothetical protein FLJ23510
NM_013348.1	Potassium inwardly-rectifying channel,subfamily J, 14 (KCNJ14)
NM_018277.1 [a]	Hypothetical protein FLJ10932
NM_025093.1 [a]	Hypothetical protein FLJ11827
NM_013453.1	Sperm protein associated with nucleus, X chromosome, member A1 (SPANXA1)
NM_030900.1 [a]	KIAA0948 protein
NM_030974.1 [a]	Hypothetical protein DKFZp434N1923
NM_024734.1 [a]	Hypothetical protein FLJ12383
NM_018485.1 [a]	G protein-coupled receptor C5L2
NM_020351.1	Macrophage conditioned medium-induced protein smag-64
NM_005417.1	v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (SRC)
NM_001279.1 [a]	Cell death-inducing DFFA-like effector a (CIDEA)
NM_005092.1	Tumor necrosis factor (ligand) 18 (TNFSF18)
NM_031275.1	Testis expressed sequence 12 (TEX12)
AF181985.1	Serinethreonine kinase (KDS)
AY009401.1	WNT6 precursor (WNT6)
AA456973	Activated RNA polymerase II transcription cofactor 4
NM_000393.1	Collagen, type V, alpha 2 (COL5A2)
AL540260 [a]	Human PAC clone RP3-515N1 from 22q11.2-q22
AL571723 [a]	Weakly similar to human tyrosine kinase receptor Tie-1 precursor
AI138993	Uncharacterized hematopoietic stem cells protein MDS026
AI089655 [a]	Hypothetical protein DKFZp547M136
AI828531 [a]	Hypothetical protein DKFZp547M136
AB037823.1 [a]	KIAA1402 protein
AL566528	Neurofilament, light polypeptide
AK025059.1	KIAA1332 protein
AK024432.1	FLJ00022 protein
AL121845 [a]	Contains TNFRSF6B, an ADP-ribosylation factor family protein
AL035588 [a]	Contains genes for TFEB, NPM1 (Nucleophosmin) pseudogene and the MDFI gene
AI279819 [a]	DKFZp564O1763
AW026481	Similar to A41784 tumor necrosis factor-alpha-induced protein B12
AI809961 [a]	Hypothetical protein from BCRA2 region
AI806793	Collagen, type VIII, alpha 2
BF513089	Thioredoxin reductase 3 (TRXR3)
BE881590 [a]	Homo sapiens clone 24421 mRNA
AK023140.1 [a]	Hypothetical protein FLJ13078
AA906578	CLONE=IMAGE:1524202
BF732879 [a]	FLJ14307
AI478455	Empty spiracles (Drosophila) homolog 2
CAI933199	Neurexophilin 4
AW006750 [a]	Hypothetical protein FLJ20059
AI199589 [a]	Hypothetical protein DKFZp434E1723
AK026747.1	FLJ23094 hypothetical protein
AC003982 [a]	Sirtuin (silent mating type information regulation 2, S. cerevisiae, homolog) 4
AK026947.1	3-phosphoinositide dependent protein kinase-1
AA129909 [a]	Moderately similar to ALU7_HUMAN ALU SUBFAMILY SQ
AW301937 [a]	CLONE=IMAGE:2766893
AW514038	Olfactory receptor, family 7, subfamily E, member 47 pseudogene

Downregulated genes (n=36)

L13852	Ubiquitin-activating enzyme E1 related protein
S72904 [b]	APK1 antigen=MAb KI recognized
AB011174 [b]	KIAA0602 protein
L06147 [b]	Human (clone SY11) golgin-95 mRNA
D87470 [b]	KIAA0280 protein
U46024 [b]	Myotubularin (MTM1)
AB029343	HCR (alpha-helix coiled-coil rod homologue)

X74496 [b]	Prolyl oligopeptidase
AI984221 [b]	Clone=IMAGE-2562160
N92501 [b]	Spectrin, beta, non-erythrocytic 1
NM_001387.1	dihydropyrimidinase-like 3 (DPYSL3)
D13891.1 [b]	Id-2H
AI937543 [b]	DC12 protein
NM_002345.1 [b]	Lumican (LUM)
AF324888.1	Myosin phosphatase target subunit 2
NM_005228.1	Epidermal growth factor receptor (EGFR)
AV757675 [b]	Tumor necrosis factor alpha-inducible
NM_003367.1 [b]	Upstream transcription factor 2, c-fos interacting (USF2)
BG434168 [b]	KIAA0254 gene product
X98405.1 [b]	Myelin associated glycoprotein, S-MAG
M90355 [b]	BTF3 protein homologue, basic transcription factor 3, like 2
BG290532 [b]	Moderately similar to Z137_HUMAN ZINC FINGER PROTEIN 13
AW007368	Heme-regulated initiation factor 2-alpha kinase
NM_012141.1 [b]	Deleted in cancer 1; RNA helicase HDBDICE1 (DDX26)
NM_018048.1 [b]	Hypothetical protein FLJ10292
NM_020379.1	1,2-alpha-mannosidase IC (HMIC)
NM_014650.1 [b]	KIAA0798 gene product
NM_019618.1 [b]	Interleukin-1 homolog 1 (IL-1H1)
NM_020358.1 [b]	Ring finger protein 18 (RNF18)
NM_018603.1 [b]	Hypothetical protein PRO1496
NM_005712.1 [b]	HERV-H LTR-associating 1 (HHLA1)
NM_025042.1 [b]	Hypothetical protein FLJ22367
AI948472	Paired box gene 8
AK021672.1 [b]	Hypothetical protein FLJ21820
AW979196 [b]	Moderately similar to ALU1_HUMAN ALU SUBFAMILY J
BF573849 [b]	Weakly similar to ALUC_HUMAN
NCCIT genes specifically upregulated in Jurkat extract-treated cells (n=12/70)	
AA772285 [c]	Vitamin D (1,25- dihydroxyvitamin D3) receptor
AF208043.1 [c]	IFI16b (IFI16b)
NM_000954.1 [c]	Prostaglandin D2 synthase (21kD, brain) (PTGDS)
BG484069 [c]	FANCA gene, exon 10a
AL031228 [c]	Contains BING5, exons 11-15 of BING4, GalT3, RPS18, SACM2L
AW085172 [c]	Highly similar to KPCM_HUMAN PROTEIN KINASE C, MU TYPE
NM_017699.1 [c]	Hypothetical protein FLJ20174
NM_017713.1 [c]	Hypothetical protein FLJ20211
NM_002420.2 [c]	Melastatin 1 (MLSN1)
U46010.1 [c]	Hepatocyte growth factor agonist-antagonist
AI803302 [c]	Z-band alternatively spliced PDZ-motif
NM_024123.1 [c]	Putative Ly-6 superfamily member (G6E)

[a] Gene also upregulated in 293T cells treated with Jurkat extract (n=58).

[b] Gene also downregulated in 293T cells treated with Jurkat extract (n=28).

[c] Gene not detected in 293T extract-treated cells. Total numbers of NCCIT genes up- or downregulated in Jurkat extract-treated cells are 70 (58 annoted [a] + 12 annotated [c]) and 28 (annoted [b]), respectively.

Supplementary Table S3. NCCIT genes consistently up- or downregulated over eight weeks after treatment with NCCIT extract

Upregulated genes (n=686)[a]
Cluster Incl. AB006533:Homo sapiens RecQ5 mRNA for DNA helicase, complete cds
Cluster Incl. M24899:Human triiodothyronine (ear7) mRNA, complete cds
Cluster Incl. AB015331:Homo sapiens HRIHFB2017 mRNA, partial cds
Cluster Incl. M96789:Homo sapiens connexin 37 (GJA4) mRNA, complete cds
Cluster Incl. AI003763:ou91e02.x1 Homo sapiens cDNA, 3' end
Cluster Incl. AL047020:DKFZp586N1517_s1 Homo sapiens cDNA, 3' end
Cluster Incl. AA402435:zt60g10.r1 Homo sapiens cDNA, 5' end
Cluster Incl. W72694:zd68f10.s1 Homo sapiens cDNA, 3' end
Cluster Incl. AI161338:qb80a04.x1 Homo sapiens cDNA, 3' end
gb:BC001643.1 /DEF=Homo sapiens, tumor necrosis factor, alpha-induced protein 1 (endothelial)
gb:AF119897.1 /DEF=Homo sapiens PRO2760 mRNA, complete cds.
gb:NM_022844.1 /DEF=Homo sapiens myosin, heavy polypeptide 11, smooth muscle (MYH11), transcript variant SM2, mRNA.
gb:NM_000552.2 /DEF=Homo sapiens von Willebrand factor (VWF), mRNA.
gb:NM_005562.1 /DEF=Homo sapiens laminin, gamma 2, mRNA.
Consensus includes gb:BC000023.1 /DEF=Homo sapiens, ribosomal protein S19, clone MGC:1630, mRNA, complete cds.
Consensus includes gb:AI467916 AXL receptor tyrosine kinase
gb:NM_006129.2 /DEF=Homo sapiens bone morphogenetic protein 1 (BMP1), transcript variant BMP1-3, mRNA.
gb:NM_014672.1 /DEF=Homo sapiens KIAA0391 gene product (KIAA0391), mRNA.
Consensus includes gb:AW117498 forkhead box O1A (rhabdomyosarcoma)
Consensus includes gb:W72082 complement component C1q receptor
gb:NM_002342.1 /DEF=Homo sapiens lymphotoxin beta receptor (TNFR superfamily, member 3 (LTBR), mRNA.
gb:AF097493.1 /DEF=Homo sapiens glutaminase kidney isoform mRNA, complete cds.
Consensus includes gb:AB007869.1 /DEF=Homo sapiens KIAA0409 mRNA, partial cds.
Consensus includes gb:AW139152 Notch (Drosophila) homolog 3
gb:NM_000702.1 /DEF=Homo sapiens ATPase, Na+K+ transporting, alpha 2 (+) polypeptide (ATP1A2), mRNA.
gb:NM_000129.2 /DEF=Homo sapiens coagulation factor XIII, A1 polypeptide (F13A1), mRNA.
gb:NM_014782.1 /DEF=Homo sapiens KIAA0512 gene product (KIAA0512), mRNA.
Consensus includes gb:T79216 KIAA1046 protein
gb:NM_012306.1 /DEF=Homo sapiens lifeguard (KIAA0950), mRNA.
gb:NM_004244.1 /DEF=Homo sapiens CD163 antigen (CD163), mRNA.
gb:NM_002996.1 /DEF=Homo sapiens small inducible cytokine subfamily D (Cys-X3-Cys), member 1 (SCYD1), mRNA.
gb:NM_014961.1 /DEF=Homo sapiens KIAA0871 protein (KIAA0871), mRNA.
gb:NM_020991.2 /DEF=Homo sapiens chorionic somatomammotropin hormone 2 (CSH2), transcript variant 1, mRNA.
gb:NM_005781.2 /DEF=Homo sapiens activated p21cdc42Hs kinase (ACK1), mRNA.
gb:NM_001998.1 /DEF=Homo sapiens fibulin 2 (FBLN2), mRNA.
gb:NM_000361.1 /DEF=Homo sapiens thrombomodulin (THBD), mRNA.
gb:NM_000397.2 /DEF=Homo sapiens cytochrome b-245, beta polypeptide (chronic granulomatous disease) (CYBB), mRNA.
gb:J02694.1 /DEF=Human myeloperoxidase mRNA, complete cds.
gb:NM_004688.1 Homo sapiens N-myc (and STAT) interactor (NMI), mRNA.
gb:NM_000570.1 Fc fragment of IgG, low affinity IIIb, receptor for (CD16) (FCGR3B), mRNA.
gb:NM_000314.1 /DEF=Homo sapiens phosphatase and tensin homolog (mutated in multiple advanced cancers 1) (PTEN), mRNA
gb:NM_014963.1 /DEF=Homo sapiens KIAA0963 protein (KIAA0963), mRNA.
Consensus includes gb:AI655714 KIAA1052 protein
Consensus includes gb:AA772285 vitamin D (1,25- dihydroxyvitamin D3) receptor
gb:NM_006017.1 /DEF=Homo sapiens prominin (mouse)-like 1 (PROML1), mRNA.
Consensus includes gb:BF305380 G-2 and S-phase expressed 1 /FL=gb:AF223408.1 gb:NM_016426.1
Consensus includes gb:N46430 zinc finger protein 202 /FL=gb:NM_003455.1 gb:AF027218.1 gb:AF027219.1
gb:BC000737.1 /DEF=Homo sapiens, regulator of G-protein signalling 4, clone MGC:2124, mRNA, complete cds.

gb:NM_005980.1 /DEF=Homo sapiens S100 calcium-binding protein P (S100P), mRNA.
Consensus includes gb:BG474736 galactokinase 1
gb:NM_003657.1 /DEF=Homo sapiens breast carcinoma amplified sequence 1 (BCAS1), mRNA.
gb:BC005248.1 /DEF=Homo sapiens, clone MGC:12282, mRNA, complete cds.
gb:NM_000184.1 /DEF=Homo sapiens hemoglobin, gamma G (HBG2), mRNA.
gb:NM_000610.1 /DEF=Homo sapiens CD44 antigen (homing function and Indian blood group system) (CD44), mRNA.
gb:NM_014800.1 /DEF=Homo sapiens KIAA0281 gene product (KIAA0281), mRNA.
gb:NM_000862.1 /DEF=Homo sapiens hydroxy-delta-5-steroid dehydrogenase, 3 beta 1 (HSD3B1), mRNA.
gb:NM_000638.1 /DEF=Homo sapiens vitronectin (VTN), mRNA.
gb:U38321.1 /DEF=Homo sapiens clone rasi-11 matrix metalloproteinase RASI-1 mRNA, complete cds.
gb:NM_004496.1 /DEF=Homo sapiens hepatocyte nuclear factor 3, alpha (HNF3A), mRNA.
gb:NM_004809.1 /DEF=Homo sapiens stomatin-like 1 (STOML1), mRNA.
gb:NM_000035.1 /DEF=Homo sapiens aldolase B, fructose-bisphosphate (ALDOB), mRNA.
gb:NM_000130.2 /DEF=Homo sapiens coagulation factor V (proaccelerin, labile factor) (F5), mRNA.
gb:NM_001714.1 /DEF=Homo sapiens Bicaudal D (Drosophila) homolog 1 (BICD1), mRNA.
gb:NM_014714.1 /DEF=Homo sapiens KIAA0590 gene product (KIAA0590), mRNA.
gb:NM_003716.1 /DEF=Homo sapiens Ca2+-dependent activator protein for secretion (CADPS), mRNA.
gb:NM_006994.2 /DEF=Homo sapiens butyrophilin, subfamily 3, member A3 (BTN3A3), mRNA.
gb:NM_014381.1 /DEF=Homo sapiens mutL (E. coli) homolog 3 (MLH3), mRNA.
gb:NM_002832.1 /DEF=Homo sapiens protein tyrosine phosphatase, non-receptor type 7 (PTPN7), mRNA.
gb:NM_002594.1 /DEF=Homo sapiens proprotein convertase subtilisinkexin type 2 (PCSK2), mRNA.
gb:NM_014882.1 /DEF=Homo sapiens KIAA0053 gene product (KIAA0053), mRNA.
gb:NM_004507.1 /DEF=Homo sapiens HUS1 (S. pombe) checkpoint homolog (HUS1), mRNA.
gb:NM_002060.1 /DEF=Homo sapiens gap junction protein, alpha 4, 37kD (connexin 37) (GJA4), mRNA.
Consensus includes gb:AI419307 ring finger protein 22
gb:NM_002667.1 /DEF=Homo sapiens phospholamban (PLN), mRNA.
gb:NM_002846.1 /DEF=Homo sapiens protein tyrosine phosphatase, receptor type, N (PTPRN), mRNA.
gb:NM_014400.1 /DEF=Homo sapiens GPI-anchored metastasis-associated protein homolog (C4.4A), mRNA.
gb:NM_005213.1 /DEF=Homo sapiens cystatin A (stefin A) (CSTA), mRNA.
gb:NM_002216.1 /DEF=Homo sapiens inter-alpha (globulin) inhibitor, H2 polypeptide (ITIH2), mRNA.
gb:NM_014622.1 /DEF=Homo sapiens loss of heterozygosity, 11, chromosomal region 2, gene A (LOH11CR2A), mRNA.
gb:NM_005130.1 /DEF=Homo sapiens heparin-binding growth factor binding protein (HBP17), mRNA.
gb:NM_012448.1 /DEF=Homo sapiens signal transducer and activator of transcription 5B (STAT5B), mRNA.
Consensus includes gb:BG540504 zinc finger protein, subfamily 1A, 1 (Ikaros)
gb:NM_014221.1 /DEF=Homo sapiens mature T-cell proliferation 1 (MTCP1), mRNA.
gb:NM_000232.1 /DEF=Homo sapiens sarcoglycan, beta (43kD dystrophin-associated glycoprotein) (SGCB), mRNA.
gb:NM_002975.1 /DEF=Homo sapiens stem cell growth factor; lymphocyte secreted C-type lectin (SCGF), mRNA.
gb:NM_006946.1 /DEF=Homo sapiens spectrin, beta, non-erythrocytic 2 (SPTBN2), mRNA.
gb:NM_022829.1 /DEF=Homo sapiens sodium-dependent high-affinity dicarboxylate transporter 3 (NADC3), mRNA.
gb:NM_000399.2 /DEF=Homo sapiens early growth response 2 (Krox-20 (Drosophila) homolog) (EGR2), mRNA.
gb:NM_000238.1 /DEF=Homo sapiens potassium voltage-gated channel, subfamily H (eag-related), member 2 (KCNH2), mRNA.
gb:NM_002309.2 /DEF=Homo sapiens leukemia inhibitory factor (cholinergic differentiation factor) (LIF), mRNA.
gb:NM_001200.1 /DEF=Homo sapiens bone morphogenetic protein 2 (BMP2), mRNA.
gb:NM_002854.1 /DEF=Homo sapiens parvalbumin (PVALB), mRNA. /FEA=mRNA
Consensus includes gb:AL139318 Contains DCT gene for dopachrome tautomerase, gene for DTDP-D-glucose 4,6-dehydratase
Consensus includes gb:AA527340 homeo box B6 /FL=gb:NM_018952.1
gb:NM_014690.1 /DEF=Homo sapiens KIAA0773 gene product (KIAA0773), mRNA.
gb:M28880.1 /DEF=Human erythroid ankyrin mRNA, complete cds.
gb:NM_002005.2 /DEF=Homo sapiens feline sarcoma viral (v-fes) (PRCII) viral (v-fps) oncogene homolog (FES), mRNA.
gb:NM_000854.2 /DEF=Homo sapiens glutathione S-transferase theta 2 (GSTT2), mRNA.
gb:NM_001230.1 /DEF=Homo sapiens caspase 10, apoptosis-related cysteine protease (CASP10), mRNA.
gb:NM_006144.2 /DEF=Homo sapiens granzyme (GZMA), mRNA.
gb:NM_024009.1 /DEF=Homo sapiens gap junction protein, beta 3, 31kD (connexin 31) (GJB3), mRNA.
gb:NM_005401.1 /DEF=Homo sapiens protein tyrosine phosphatase, non-receptor type 14 (PTPN14), mRNA.

gb:NM_001490.1 /DEF=Homo sapiens glucosaminyl (N-acetyl) transferase 1, core 2 (GCNT1), mRNA.
gb:NM_006200.1 /DEF=Homo sapiens proprotein convertase subtilisinkexin type 5 (PCSK5), mRNA.
gb:NM_020980.2 /DEF=Homo sapiens aquaporin 9 (AQP9), mRNA.
gb:NM_000603.1 /DEF=Homo sapiens nitric oxide synthase 3 (endothelial cell) (NOS3), mRNA.
Consensus includes gb:NM_007045.1 /DEF=Homo sapiens FGFR1 oncogene partner (FOP), mRNA
gb:NM_005658.1 /DEF=Homo sapiens TNF receptor-associated factor 1 (TRAF1), mRNA.
gb:NM_002336.1 /DEF=Homo sapiens low density lipoprotein receptor-related protein 6 (LRP6), mRNA.
gb:NM_001870.1 /DEF=Homo sapiens carboxypeptidase A3 (mast cell) (CPA3), mRNA.
gb:NM_000756.1 /DEF=Homo sapiens corticotropin releasing hormone (CRH), mRNA.
gb:NM_018843.1 /DEF=Homo sapiens mitochondrial carrier family protein (LOC55972), mRNA.
gb:NM_000499.2 /DEF=Homo sapiens cytochrome P450, subfamily I, polypeptide 1 (CYP1A1), mRNA.
gb:NM_001461.1 /DEF=Homo sapiens flavin containing monooxygenase 5 (FMO5), mRNA.
gb:NM_025228.1 /DEF=Homo sapiens hypothetical protein dJ434O14.3 (DJ434O14.3), mRNA.
gb:NM_005012.1 /DEF=Homo sapiens receptor tyrosine kinase-like orphan receptor 1 (ROR1), mRNA.
gb:NM_002214.1 /DEF=Homo sapiens integrin, beta 8 (ITGB8), mRNA.
gb:NM_001541.1 /DEF=Homo sapiens heat shock 27kD protein 2 (HSPB2), mRNA.
Consensus includes gb:AW975818 hypothetical protein FLJ21940 /FL=gb:NM_022828.1
Consensus includes gb:AI269290 solute carrier family 18 (vesicular monoamine), member 2
gb:NM_002220.1 /DEF=Homo sapiens inositol 1,4,5-trisphosphate 3-kinase A (ITPKA), mRNA.
gb:NM_016381.1 /DEF=Homo sapiens hypothetical protein (DKFZp434J0310), mRNA.
gb:NM_002960.1 /DEF=Homo sapiens S100 calcium-binding protein A3 (S100A3), mRNA.
gb:NM_002908.1 /DEF=Homo sapiens v-rel avian reticuloendotheliosis viral oncogene homolog (REL), mRNA.
gb:NM_003298.1 Homo sapiens nuclear receptor subfamily 2, group C, member 2 (NR2C2), mRNA.
gb:NM_001821.1 /DEF=Homo sapiens choroideremia-like (Rab escort protein 2) (CHML), mRNA.
gb:NM_006674.1 /DEF=Homo sapiens MHC class I region ORF (P5-1), mRNA.
gb:NM_007105.1 /DEF=Homo sapiens solute carrier family 22, member 1-like antisense (SLC22A1LS), mRNA.
gb:NM_000366.1 /DEF=Homo sapiens tropomyosin 1 (alpha) (TPM1), mRNA.
gb:NM_004742.1 /DEF=Homo sapiens BAI1-associated protein 1 (BAIAP1), mRNA.
gb:NM_001174.2 /DEF=Homo sapiens Rho GTPase activating protein 6 (ARHGAP6), transcript variant 2, mRNA.
gb:NM_025013.1 /DEF=Homo sapiens KIAA1031 protein (KIAA1031), mRNA.
gb:NM_007368.1 /DEF=Homo sapiens RAS p21 protein activator 3 (Ins(1,3,4,5)P4-binding protein) (RASA3), mRNA.
gb:NM_002312.1 /DEF=Homo sapiens ligase IV, DNA, ATP-dependent (LIG4), mRNA.
gb:NM_006183.2 /DEF=Homo sapiens neurotensin (NTS), mRNA.
gb:NM_021114.1 /DEF=Homo sapiens serine protease inhibitor, Kazal type, 2 (acrosin-trypsin inhibitor) (SPINK2), mRNA.
gb:NM_000928.1 /DEF=Homo sapiens phospholipase A2, group IB (pancreas) (PLA2G1B), mRNA.
gb:NM_018651.1 /DEF=Homo sapiens zinc finger protein (ZFP), mRNA
gb:NM_004778.1 /DEF=Homo sapiens G protein-coupled receptor 44 (GPR44), mRNA.
gb:NM_002411.1 /DEF=Homo sapiens mammaglobin 1 (MGB1), mRNA.
gb:NM_007314.1 /DEF=Homo sapiens v-abl Abelson murine leukemia viral oncogene homolog 2 (ABL2), variant b, mRNA.
Consensus includes gb:AI769310 tolloid-like 1 /FL=gb:U91963.1 gb:NM_012464.1 gb:AF282732.1
gb:NM_003456.1 /DEF=Homo sapiens zinc finger protein 205 (ZNF205), mRNA.
gb:NM_005849.1 /DEF=Homo sapiens immunoglobulin superfamily, member 6 (IGSF6), mRNA.
Consensus includes gb:BE856376 very long-chain acyl-CoA synthetase; lipidosin /FL=gb:NM_015162.1 gb:AF179481.1
gb:NM_002420.2 /DEF=Homo sapiens melastatin 1 (MLSN1), mRNA.
gb:NM_002652.1 /DEF=Homo sapiens prolactin-induced protein (PIP), mRNA.
gb:NM_004062.1 /DEF=Homo sapiens cadherin 16, KSP-cadherin (CDH16), mRNA.
gb:NM_003381.1 /DEF=Homo sapiens vasoactive intestinal peptide (VIP), mRNA.
gb:NM_000448.1 /DEF=Homo sapiens recombination activating gene 1 (RAG1), mRNA.
gb:BC005124.1 /DEF=Homo sapiens, homeo box D3, clone MGC:10470, mRNA, complete cds.
gb:NM_006898.2 /DEF=Homo sapiens homeo box D3 (HOXD3), mRNA.
gb:NM_000236.1 /DEF=Homo sapiens lipase, hepatic (LIPC), mRNA.
gb:NM_000128.2 /DEF=Homo sapiens coagulation factor XI (plasma thromboplastin antecedent) (F11), variant 1, mRNA.
gb:NM_002910.4 Homo sapiens renin-binding protein (RENBP), mRNA.
gb:NM_000372.1 /DEF=Homo sapiens tyrosinase (oculocutaneous albinism IA) (TYR), mRNA.

gb:NM_016384.1 /DEF=Homo sapiens hypothetical protein (HSPC050), mRNA.
gb:NM_002104.1 /DEF=Homo sapiens granzyme K (serine protease, granzyme 3; tryptase II) (GZMK), mRNA.
gb:NM_007223.1 /DEF=Homo sapiens putative G protein coupled receptor (GPR), mRNA.
gb:NM_002277.1 /DEF=Homo sapiens keratin, hair, acidic,1 (KRTHA1), mRNA. /
gb:NM_006344.1 /DEF=Homo sapiens macrophage lectin 2 (calcium dependent) (HML2), mRNA.
gb:NM_006229.1 /DEF=Homo sapiens pancreatic lipase-related protein 1 (PNLIPRP1), mRNA.
gb:NM_014907.1 /DEF=Homo sapiens KIAA0967 protein (KIAA0967), mRNA.
gb:NM_004101.1 /DEF=Homo sapiens coagulation factor II (thrombin) receptor-like 2 (F2RL2), mRNA.
gb:NM_005106.2 /DEF=Homo sapiens deleted in lung and esophageal cancer 1 (DLEC1), transcript variant DLEC1-N1, mRNA.
gb:NM_014274.1 /DEF=Homo sapiens Alu-binding protein with zinc finger domain (ABPZF), mRNA.
gb:NM_004186.1 /DEF=Homo sapiens sema domain, immunoglobulin domain 3F (SEMA3F), mRNA.
gb:NM_015370.1 /DEF=Homo sapiens hypothetical protein (HS747E2A), mRNA.
gb:NM_001794.1 /DEF=Homo sapiens cadherin 4, type 1, R-cadherin (retinal) (CDH4), mRNA.
gb:NM_001104.1 /DEF=Homo sapiens actinin, alpha 3 (ACTN3), mRNA.
gb:NM_005728.1 /DEF=Homo sapiens endonuclease G-like 2 (ENDOGL2), mRNA.
gb:NM_005666.1 /DEF=Homo sapiens H factor (complement)-like 3 (HFL3), mRNA.
gb:NM_005838.1 /DEF=Homo sapiens putative glycine-N-acyltransferase (GAT), mRNA.
gb:NM_006065.1 /DEF=Homo sapiens signal regulatory protein, beta, 1 (SIRP-BETA-1), mRNA.
gb:NM_003126.1 /DEF=Homo sapiens spectrin, alpha, erythrocytic 1 (elliptocytosis 2) (SPTA1), mRNA.
gb:NM_014120.1 /DEF=Homo sapiens PRO0214 protein (PRO0214), mRNA.
gb:NM_000197.1 /DEF=Homo sapiens hydroxysteroid (17-beta) dehydrogenase 3 (HSD17B3), mRNA.
gb:NM_001899.1 /DEF=Homo sapiens cystatin S (CST4), mRNA.
gb:NM_005605.1 /DEF=Homo sapiens protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform (PPP3CC), mRNA.
gb:NM_030379.1 /DEF=Homo sapiens GLI-Kruppel family member GLI2 (GLI2), transcript variant 1, mRNA.
gb:NM_006140.1 /DEF=Homo sapiens colony stimulating factor 2 receptor, alpha, low-affinity (CSF2RA), mRNA.
gb:AF005213.1 /DEF=Homo sapiens ankyrin 1 (ANK1) mRNA, complete cds.
gb:NM_002039.1 /DEF=Homo sapiens GRB2-associated binding protein 1 (GAB1), mRNA.
gb:NM_003833.2 /DEF=Homo sapiens matrilin 4 (MATN4), transcript variant 1, mRNA.
gb:NM_018634.1 /DEF=Homo sapiens hypothetical protein PRO2893 (PRO2893), mRNA.
gb:NM_001118.1 /DEF=Homo sapiens adenylate cyclase activating polypeptide 1 receptor type I (ADCYAP1R1), mRNA.
gb:NM_004258.1 /DEF=Homo sapiens immunoglobulin superfamily, member 2 (IGSF2), mRNA.
gb:NM_003561.1 /DEF=Homo sapiens phospholipase A2, group X (PLA2G10), mRNA.
gb:NM_000762.2 /DEF=Homo sapiens cytochrome P450, subfamily IIA, polypeptide 6 (CYP2A6), mRNA.
gb:NM_004212.1 /DEF=Homo sapiens solute carrier family 28, member 2 (SLC28A2), mRNA.
gb:NM_006678.1 Homo sapiens CMRF35 leukocyte immunoglobulin-like receptor (CMRF35), mRNA.
gb:NM_024325.1 /DEF=Homo sapiens hypothetical protein MGC10715 (MGC10715), mRNA.
gb:NM_001523.1 /DEF=Homo sapiens hyaluronan synthase 1 (HAS1), mRNA.
gb:NM_001729.1 /DEF=Homo sapiens betacellulin (BTC), mRNA.
gb:NM_016343.1 /DEF=Homo sapiens centromere protein F (350400kD, mitosin) (CENPF), mRNA.
gb:NM_002777.2 /DEF=Homo sapiens proteinase 3 (PRTN3), mRNA.
gb:NM_017513.1 /DEF=Homo sapiens metaphase chromosome protein 1 (HSMCR30), mRNA.
gb:NM_000335.1 /DEF=Homo sapiens sodium channel, voltage-gated, type V, alpha polypeptide (SCN5A), mRNA.
gb:NM_005593.1 /DEF=Homo sapiens myogenic factor 5 (MYF5), mRNA.
gb:NM_006640.1 /DEF=Homo sapiens MLL septin-like fusion (MSF), mRNA.
gb:NM_004256.1 /DEF=Homo sapiens organic cationic transporter-like 3 (ORCTL3), mRNA.
gb:NM_018546.1 /DEF=Homo sapiens hypothetical protein PRO2958 (PRO2958), mRNA.
gb:NM_002363.1 /DEF=Homo sapiens melanoma antigen, family B, 1 (MAGEB1), mRNA.
gb:NM_003744.1 /DEF=Homo sapiens numb (Drosophila) homolog (NUMB), mRNA.
gb:NM_001531.1 /DEF=Homo sapiens major histocompatibility complex, class I-like sequence (HLALS), mRNA.
gb:NM_000451.2 /DEF=Homo sapiens short stature homeobox (SHOX), transcript variant SHOXa, mRNA.
gb:NM_024716.1 /DEF=Homo sapiens hypothetical protein FLJ23505 (FLJ23505), mRNA.
gb:NM_003508.1 /DEF=Homo sapiens frizzled (Drosophila) homolog 9 (FZD9), mRNA.
gb:NM_004680.1 /DEF=Homo sapiens chromodomain protein, Y chromosome, 1 (CDY1), mRNA.
gb:NM_013308.1 /DEF=Homo sapiens platelet activating receptor homolog (H963), mRNA.

gb:NM_013416.1 /DEF=Homo sapiens neutrophil cytosolic factor 4 (40kD) (NCF4), transcript variant 2, mRNA.
gb:NM_001187.1 /DEF=Homo sapiens B melanoma antigen (BAGE), mRNA.
gb:NM_021981.1 /DEF=Homo sapiens pre-TNK cell associated protein (1D12A), mRNA.
gb:NM_025068.1 /DEF=Homo sapiens hypothetical protein FLJ13381 (FLJ13381), mRNA.
gb:NM_004857.1 /DEF=Homo sapiens A kinase (PRKA) anchor protein 5 (AKAP5), mRNA.
gb:NM_002188.1 /DEF=Homo sapiens interleukin 13 (IL13), mRNA.
gb:NM_006866.1 /DEF=Homo sapiens leukocyte immunoglobulin-like receptor, subfamily A, member 2 (LILRA2), mRNA.
gb:NM_018896.1 /DEF=Homo sapiens calcium channel, voltage-dependent, alpha 1G subunit (CACNA1G), mRNA.
gb:NM_002390.2 /DEF=Homo sapiens a disintegrin and metalloproteinase domain 11 (ADAM11), transcript variant 1, mRNA.
gb:NM_005468.1 /DEF=Homo sapiens N-acetylated alpha-linked acidic dipeptidase-like (NAALADASEL), mRNA.
gb:NM_005575.1 /DEF=Homo sapiens leucylcystinyl aminopeptidase (LNPEP), mRNA.
gb:NM_001989.1 /DEF=Homo sapiens even-skipped homeo box 1 (homolog of Drosophila) (EVX1), mRNA.
gb:NM_001840.1 /DEF=Homo sapiens cannabinoid receptor 1 (brain) (CNR1), mRNA.
gb:NM_006664.1 /DEF=Homo sapiens small inducible cytokine subfamily A (Cys-Cys), member 27 (SCYA27), mRNA.
gb:NM_002738.1 /DEF=Homo sapiens protein kinase C, beta 1 (PRKCB1), mRNA.
gb:NM_000825.1 /DEF=Homo sapiens gonadotropin-releasing hormone 1 (leutinizing-releasing hormone) (GNRH1), mRNA.
gb:NM_006885.1 /DEF=Homo sapiens AT-binding transcription factor 1 (ATBF1), mRNA.
gb:NM_003891.1 /DEF=Homo sapiens protein Z, vitamin K-dependent plasma glycoprotein (PROZ), mRNA.
gb:NM_015601.1 /DEF=Homo sapiens DKFZP564G092 protein (DKFZP564G092), mRNA.
gb:NM_015879.1 /DEF=Homo sapiens sialyltransferase 8C (SIAT8C), mRNA.
gb:NM_000888.3 /DEF=Homo sapiens integrin, beta 6 (ITGB6), mRNA.
gb:AF338730.1 /DEF=Homo sapiens potassium voltage-gated channel, Shab-related subfamily, member 2 (KCNB2), mRNA.
gb:NM_031269.1 /DEF=Homo sapiens PRO1386 protein (PRO1386), mRNA.
gb:NM_017959.1 /DEF=Homo sapiens hypothetical protein FLJ20802 (FLJ20802), mRNA.
gb:NM_001447.1 /DEF=Homo sapiens FAT tumor suppressor (Drosophila) homolog 2 (FAT2), mRNA.
gb:NM_002176.1 /DEF=Homo sapiens interferon, beta 1, fibroblast (IFNB1), mRNA.
gb:NM_000716.1 /DEF=Homo sapiens complement component 4-binding protein, beta (C4BPB), mRNA.
gb:NM_020328.1 /DEF=Homo sapiens activin A receptor, type IB (ACVR1B), transcript variant 3, mRNA.
gb:NM_002144.1 /DEF=Homo sapiens homeo box B1 (HOXB1), mRNA.
gb:NM_022975.1 /DEF=Homo sapiens fibroblast growth factor receptor 2 (FGFR2), transcript variant 8, mRNA.
gb:NM_022976.1 /DEF=Homo sapiens fibroblast growth factor receptor 2 (FGFR2), transcript variant 9, mRNA.
gb:NM_004495.1 /DEF=Homo sapiens neuregulin 1 (NRG1), transcript variant HRG-gamma, mRNA.
gb:NM_021777.1 /DEF=Homo sapiens a disintegrin and metalloproteinase domain 28 (ADAM28), transcript variant 3, mRNA.
gb:NM_022375.1 /DEF=Homo sapiens oculomedin (OCLM), mRNA.
gb:NM_005058.1 /DEF=Homo sapiens RNA binding motif protein, Y chromosome, family 1, member A1 (RBMY1A1), mRNA.
gb:NM_002036.1 /DEF=Homo sapiens Duffy blood group (FY), mRNA.
gb:NM_020479.1 /DEF=Homo sapiens ankyrin 1, erythrocytic (ANK1), transcript variant 6, mRNA.
gb:NM_020480.1 /DEF=Homo sapiens ankyrin 1, erythrocytic (ANK1), transcript variant 7, mRNA.
gb:NM_004510.1 /DEF=Homo sapiens interferon-induced protein 75, 52kD (IFI75), mRNA.
gb:U01157.1 /DEF=Human glucagon-like peptide-1 receptor mRNA with CA dinucleotide repeat, complete cds.
gb:NM_004991.1 /DEF=Homo sapiens myelodysplasia syndrome 1 (MDS1), mRNA.
gb:NM_004976.1 /DEF=Homo sapiens potassium voltage-gated channel, Shaw-related subfamily, member 1 (KCNC1), mRNA.
gb:NM_001049.1 /DEF=Homo sapiens somatostatin receptor 1 (SSTR1), mRNA.
gb:NM_006538.1 /DEF=Homo sapiens BCL2-like 11 (apoptosis facilitator) (BCL2L11), mRNA.
gb:NM_020297.1 /DEF=Homo sapiens ATP-binding cassette, sub-family C (CFTRMRP), member 9 (ABCC9), mRNA.
gb:NM_003493.1 /DEF=Homo sapiens H3 histone family, member T (H3FT), mRNA.
gb:NM_004189.1 /DEF=Homo sapiens SRY (sex determining region Y)-box 14 (SOX14), mRNA.
gb:NM_003537.1 /DEF=Homo sapiens H3 histone family, member L (H3FL), mRNA.
gb:NM_020389.1 /DEF=Homo sapiens putative capacitative calcium channel (trp7), mRNA.
gb:NM_019093.1 /DEF=Homo sapiens UDP glycosyltransferase 1 family, polypeptide A3 (UGT1A3), mRNA.
gb:NM_000614.1 /DEF=Homo sapiens ciliary neurotrophic factor (CNTF), mRNA.
gb:NM_016431.1 /DEF=Homo sapiens mitogen-activated protein kinase 8 interacting protein 2 (MAPK8IP2), mRNA.
Consensus includes gb:BG327863 CD24 antigen (small cell lung carcinoma cluster 4 antigen)
Consensus includes gb:AI424923 adaptor-related protein complex 3, delta 1 subunit /FL=gb:AF002163.1

gb:J02923.1 /DEF=Human 65-kilodalton phosphoprotein (p65) mRNA, complete cds.
gb:BC003143.1 /DEF=Homo sapiens, dual specificity phosphatase 6, clone MGC:3789, mRNA, complete cds.
gb:AF074000.1 /DEF=Homo sapiens Po66 carbohydrate binding protein mRNA, complete cds.
Consensus includes gb:BG165833 fatty acid desaturase 1
gb:AF208043.1 /DEF=Homo sapiens IFI16b (IFI16b) mRNA, complete cds.
gb:M13577.1 /DEF=Human myelin basic protein (MBP) mRNA, complete cds.
gb:M25079.1 /DEF=Human sickle cell beta-globin mRNA, complete cds.
gb:AF068266.1 /DEF=Homo sapiens EHT protein mRNA, complete cds.
gb:BC001283.1 /DEF=Homo sapiens, Similar to nuclear factor IB, clone MGC:5146, mRNA, complete cds.
gb:BC002690.1 /DEF=Homo sapiens, keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner), mRNA.
gb:BC001743.1 /DEF=Homo sapiens, Similar to hypothetical protein FLJ10803, clone MGC:933, mRNA, complete cds.
gb:U48437.1 /DEF=Human amyloid precursor-like protein 1 mRNA, complete cds.
gb:M29644.1 /DEF=Human insulin-like growth factor I mRNA, complete cds.
gb:BC001060.1 /DEF=Homo sapiens, paired box gene 8, clone MGC:2141, mRNA, complete cds.
gb:AF072132.1 /DEF=Homo sapiens integrin alpha-7 mRNA, complete cds.
Consensus includes gb:BC004864.1 /DEF=Homo sapiens, clone MGC:10576, mRNA, complete cds.
Consensus includes gb:AI860917 glycoprotein Ib (platelet), beta polypeptide
Consensus includes gb:AI628464 MAD (mothers against decapentaplegic, Drosophila) homolog 6
gb:AF274863.1 /DEF=Homo sapiens secretory pathway component Sec31B-1 mRNA, alternatively spliced, complete cds.
gb:AF083068.1 /DEF=Homo sapiens NAD+ ADP-ribosyltransferase 3 (ADPRT3) mRNA, complete cds.
gb:BC004300.1 /DEF=Homo sapiens, Similar to villin-like, clone MGC:10896, mRNA, complete cds.
gb:M34986.1 /DEF=Human erythropoietin receptor mRNA, complete cds.
gb:D14826.1 /DEF=Human mRNA for hCREM (cyclic AMP-responsive element modulator) type 2 protein, complete cds.
gb:U63041.1 /DEF=Human neural cell adhesion molecule CD56 mRNA, complete cds.
gb:AF097419.1 /DEF=Homo sapiens 10IHW 9022 IkBL protein (NFKBIL1) mRNA, NFKBIL1-+738T allele, complete cds.
Consensus includes gb:AA608820 KIAA0921 protein
Consensus includes gb:AW205153 CLONE=IMAGE:2720104 /UG=Hs.57973 hypothetical protein
Consensus includes gb:AA582460 ribosomal protein L5 /FL=gb:U66589.1
gb:AF064484.1 /DEF=Homo sapiens natural resistance-associated macrophage protein 2 non-IRE form (NRAMP2) mRNA.
gb:U06935.1 /DEF=Human thyrotroph embryonic factor (TEF) mRNA, complete cds.
gb:AB013452.1 /DEF=Homo sapiens mRNA for ATPaseII, complete cds.
gb:D28114.1 /DEF=Human mRNA for MOBP (myelin-associated oligodendrocytic basic protein), complete cds, clone hOPRP2.
gb:U66584.1 /DEF=Human alphaA-crystallin (CRYAA) mRNA, complete cds.
Consensus includes gb:R64001 CCR4-NOT transcription complex, subunit 4
gb:AF009635.1 /DEF=Homo sapiens clone 17.7 immunoglobulin-like transcript 5 protein mRNA, complete cds.
gb:M11734.1 /DEF=Human granulocytemacrophage colony-stimulating factor mRNA, complete cds.
gb:BC001265.1 /DEF=Homo sapiens, Similar to hypothetical protein dJ462O23.2, clone MGC:5034, mRNA, complete cds.
gb:AB033823.1 /DEF=Homo sapiens mRNA for h-TEKTIN-t, complete cds.
gb:BC005196.1 /DEF=Homo sapiens, kallikrein 2, prostatic, clone MGC:12201, mRNA, complete cds.
gb:AF323729.1 /DEF=Homo sapiens OSBP-related protein 7 mRNA, complete cds.
gb:D38300.1 /DEF=Homo sapiens mRNA for prostaglandin E receotor EP3 subtype 4 isoform, complete cds.
gb:U11287.1 /DEF=Human N-methyl-D-aspartate receptor subunit NR3 (hNR3) mRNA, complete cds.
gb:J04948.1 /DEF=Human alkaline phosphatase (ALP-1) mRNA, complete cds.
gb:BC000582.1 /DEF=Homo sapiens, Similar to KIAA0180 protein, clone MGC:2482, mRNA, complete cds.
gb:AB033605.1 /DEF=Homo sapiens mRNA for pUb-R5, complete cds.
gb:BC003408.1 /DEF=Homo sapiens, melanoma antigen, family A, 12, clone MGC:4914, mRNA, complete cds.
gb:U71087.1 /DEF=Human MAP kinase kinase MEK5b mRNA, complete cds.
gb:BC001279.1 /DEF=Homo sapiens, Similar to KIAA0626 gene product, clone MGC:5129, mRNA, complete cds.
gb:AF214738.1 /DEF=Homo sapiens C9orf10b mRNA, complete cds, alternatively spliced.
gb:AF241788.1 /DEF=Homo sapiens NPD011 (NPD011) mRNA, complete cds.
gb:AF007748.1 /DEF=Homo sapiens karyopherin beta2b homolog mRNA, complete cds.
gb:BC004552.1 /DEF=Homo sapiens, clone MGC:10646, mRNA, complete cds.
gb:U26744.1 /DEF=Human dystrobrevin-gamma mRNA, complete cds.
gb:BC001372.1 /DEF=Homo sapiens, Similar to ORF, clone MGC:2274, mRNA, complete cds.

gb:J04449.1 /DEF=Homo sapiens (clone NF 10) cytochrome P-450 nifedipine oxidase mRNA, complete cds.
gb:AF064103.1 /DEF=Homo sapiens Cdc14A3 phosphatase mRNA, complete cds.
gb:U46010.1 /DEF=Human HGF agonistantagonist mRNA, complete cds.
gb:BC000052.1 /DEF=Homo sapiens, Similar to peroxisome proliferative activated receptor, alpha, clone MGC:2237, mRNA.
gb:D86586.1 /DEF=Homo sapiens mRNA for SCGF-beta, complete cds.
gb:M81590.1 /DEF=Homo sapiens serotonin 1D receptor (5-HT1D~) mRNA, complete cds.
gb:U03891.2 /DEF=Homo sapiens phorbolin I mRNA, complete cds.
gb:AF130071.1 /DEF=Homo sapiens clone FLB9023 PRO2425 mRNA, complete cds.
gb:AF293342.1 /DEF=Homo sapiens RNF6 protein (RNF6) mRNA, complete cds, alternatively spliced.
gb:BC006333.1 /DEF=Homo sapiens, clone MGC:12564, mRNA, complete cds.
gb:AF122827.1 /DEF=Homo sapiens neurofibromatosis type 2 protein isoform Mer162 (NF2) mRNA, alternatively spliced.
gb:AF130066.1 /DEF=Homo sapiens clone FLB8124 PRO2179 mRNA, complete cds.
gb:M30894.1 /DEF=Human T-cell receptor Ti rearranged gamma-chain mRNA V-J-C region, complete cds.
gb:AB008913.1 /DEF=Homo sapiens mRNA for Pax-4, complete cds.
gb:D89788.1 /DEF=Homo sapiens mRNA for AML1, complete cds (acute myeloid leukemia 1; aml1 oncogene)
gb:AF312386.1 /DEF=Homo sapiens clone L4 AML1AMP19 fusion protein (AML1AMP19 fusion) mRNA, complete cds.
gb:AF116771.1 /DEF=Homo sapiens p51 delta mRNA, complete cds.
gb:AF149096.1 /DEF=Homo sapiens transforming growth factor-alpha variant I mRNA.
gb:AB012043.1 /DEF=Homo sapiens mRNA for NBR13, complete cds.
gb:U85943.1 /DEF=Homo sapiens mRNA-associated protein mrnp41 mRNA, complete cds.
gb:AF009007.1 /DEF=Homo sapiens immunoglobulin-like transcript 2c mRNA, complete cds.
gb:M33653.1 /DEF=Human (clones HT-125,133) alpha-2 type IV collagen (COL4A2) mRNA, complete cds.
gb:AB016900.1 /DEF=Homo sapiens HGC6.1.2 mRNA, complete cds.
gb:U52913.1 /DEF=Human B219OB receptor isoform HuB219.2 precursor mRNA, complete cds.
gb:AF004291.1 /DEF=Homo sapiens germ cell nuclear factor (GCNF) mRNA, complete cds.
gb:AF117899.1 /DEF=Homo sapiens LDLR-FUT fusion protein (LDLR-FUT) mRNA, complete cds.
gb:AF229067.1 /DEF=Homo sapiens PADI-H protein mRNA, complete cds.
gb:D50479.1 /DEF=Homo sapiens mRNA for protein-tyrosine kinase, complete cds.
gb:U70862.1 /DEF=Human nuclear factor I B3 mRNA, complete cds.
gb:AB042825.1 /DEF=Homo sapiens RECQL5 gamma mRNA for DNA helicase recQ5 gamma, complete cds.
gb:U73531.1 /DEF=Human G protein-coupled receptor STRL33.3 (STRL33) mRNA, complete cds.
gb:AF081924.1 /DEF=Homo sapiens calciumcalmodulin-dependent protein kinase II beta 6 subunit (CAMKB) mRNA.
gb:AF172449.1 /DEF=Homo sapiens clone 127 opioid growth factor receptor mRNA, complete cds.
gb:AF000424.1 /DEF=Homo sapiens LST1 mRNA, cLST1C splice variant, complete cds.
gb:U43279.1 /DEF=Human nucleoporin nup 36 mRNA, complete cds.
gb:M13077.1 /DEF=Human placental alkaline phosphatase mRNA, complete cds
gb:L23515.1 /DEF=Human Ig rearranged gamma-chain, V-DXP4-JH4b, complete cds.
gb:L14458.1 /DEF=Human Ig rearranged kappa-chain gene V-J-region, complete cds.
gb:L14455.1 /DEF=Human Ig rearranged mu-chain gene V-N-D-N-J-reion, complete cds.
gb:K03226.1 /DEF=Human preprourokinase mRNA, complete cds.
gb:AF349571.1 /DEF=Homo sapiens hemoglobin alpha-1 globin chain (HBA1) mRNA, complete cds.
gb:BC005856.1 /DEF=Homo sapiens, clone MGC:2889, mRNA, complete cds.
gb:BC005926.1 /DEF=Homo sapiens, ecotropic viral integration site 2B, clone MGC:14529, mRNA, complete cds.
gb:BC006196.1 /DEF=Homo sapiens, tumor necrosis factor receptor superfamily, member 9, clone MGC:2172, mRNA.
gb:AF319573.1 /DEF=Homo sapiens clone T2P4 3-5 exonuclease TREX2 (TREX2) mRNA, complete cds.
gb:AB001733.1 /DEF=Homo sapiens mRNA for single-chain antibody, complete cds.
gb:U88712.1 /DEF=Human phosphodiesterase 4C mRNA, complete cds.
gb:AF187964.1 /DEF=Homo sapiens voltage gated potassium channel Kv4.3 short splice variant (Kv4.3) mRNA, complete cds.
gb:AF110314.1 /DEF=Homo sapiens herpesvirus immunoglobulin-like receptor HIgR mRNA, complete cds.
gb:AF222341.1 /DEF=Homo sapiens T-cell specific surface glycoprotein CD28 isoform 1 (CD28) gene, complete cds.
gb:U27331.1 /DEF=Human alpha (1,3) fucosyltransferase (FUT6) mRNA, isoform I, complete cds.
gb:AF138302.1 /DEF=Homo sapiens decorin variant C mRNA, complete cds.
Consensus includes gb:AW277253 adenylate kinase 2
Consensus includes gb:NM_000954.1 /DEF=Homo sapiens prostaglandin D2 synthase (21kD, brain) (PTGDS), mRNA.

Consensus includes gb:AL516854 putative translation initiation factor
Consensus includes gb:AL575403 KIAA0620 protein
Consensus includes gb:AW138902 highly similar to AF052178 Homo sapiens clone 24523
Consensus includes gb:H65865 hypothetical protein FLJ13910
gb:AB028998.1 /DEF=Homo sapiens mRNA for KIAA1075 protein, partial cds.
Consensus includes gb:AI814660 protein kinase, cAMP-dependent, regulatory, type I, beta
Consensus includes gb:AB014558.1 cryptochrome 2 (photolyase-like)
Consensus includes gb:AA923354 monoamine oxidase A
Consensus includes gb:AI703074 transcription factor 7-like 2 (T-cell specific, HMG-box)
gb:AB002304.1 KIAA0306 protein /DEF=Human mRNA for KIAA0306 gene, partial cds.
Consensus includes gb:AL080169.1 hypothetical protein DKFZP434C171
Consensus includes gb:AI935123 CLONE=IMAGE:2464769 /UG=Hs.57548 ESTs
Consensus includes gb:AL033377 Contains an exon similar to parts of BMP and Tolloid genes.
Consensus includes gb:AI818736 similar to S. cerevisiae RER1
Consensus includes gb:AW007573 DKFZP586L151 protein
Consensus includes gb:AI640861 dynein, cytoplasmic, light intermediate polypeptide 2
Consensus includes gb:N30342 KIAA0339 gene product
Consensus includes gb:BE966372 hepatitis delta antigen-interacting protein A
Consensus includes gb:AL531750 collagen, type VI, alpha 2
Consensus includes gb:AI803302 Z-band alternatively spliced PDZ-motif
Consensus includes gb:AI697108 mucin 5, subtype B, tracheobronchial
Consensus includes gb:BE044614 tenascin XB
Consensus includes gb:AI022387 heterogeneous nuclear ribonucleoprotein H1 (H)
Consensus includes gb:BE673445 Homo sapiens chromosome 19, cosmid R28379
Consensus includes gb:AW182892 guanylate kinase 1
Consensus includes gb:NM_018957.1 /DEF=Homo sapiens SH3-domain binding protein 1 (SH3BP1), mRNA.
Consensus includes gb:AK022846.1 highly similar to Human inositol polyphosphate 5-phosphatase (5ptase) mRNA.
Consensus includes gb:BF671400 LIM protein (similar to rat protein kinase C-binding enigma)
Consensus includes gb:AV686235 mannan-binding lectin serine protease 1 (C4C2 activating component of Ra-reactive factor)
Consensus includes gb:AA971768 kinase suppressor of ras
Consensus includes gb:AA741303 syntrophin, beta 2 (dystrophin-associated protein A1, 59kD, basic component 2)
Consensus includes gb:AL524520 G protein-coupled receptor 49
Consensus includes gb:AL050204.1 /DEF=Homo sapiens mRNA; cDNA DKFZp586F1223
Consensus includes gb:AI732381 cytokeratin 20
Consensus includes gb:AI307586 DKFZp566H0124
Consensus includes gb:AI885290 spondin 1, (f-spondin) extracellular matrix protein
Consensus includes gb:AI922937 hypothetical protein FLJ11282
Consensus includes gb:AI435954 /FEA=ESThypothetical protein R31240_1
Consensus includes gb:AA017721 DKFZp564N1662
Consensus includes gb:AI829961 CD7 antigen (p41)
Consensus includes gb:AA865601 Homo sapiens Chromosome 16 BAC clone CIT987SK-A-923A4
Consensus includes gb:AF070526.1 /DEF=Homo sapiens clone 24787 mRNA sequence.
Consensus includes gb:AB029030.1 /DEF=Homo sapiens mRNA for KIAA1107 protein, partial cds. /
Consensus includes gb:AF070577.1 /DEF=Homo sapiens clone 24461 mRNA sequence.
Consensus includes gb:X91817.1 /DEF=H.sapiens mRNA for transketolase-like protein (2418 bp).
Consensus includes gb:BF432795 guanine nucleotide binding protein (G protein), gamma 7
Consensus includes gb:AI478172 homogentisate 1,2-dioxygenase (homogentisate oxidase)
Consensus includes gb:AW474434 Moderately similar to unknown H.sapiens
Consensus includes gb:AF020774.1 Hair and skin epidermal-type 12-lipoxygenase-related protein (ALOX12E) mRNA
Consensus includes gb:AI017382 KIAA1218 protein
Consensus includes gb:BF058643 EGF-like repeats and discoidin I-like domains 3
Consensus includes gb:AA772023 SWISNF related, actin dependent regulator of chromatin, subfamily a, member 4
Consensus includes gb:AI263044 Homo sapiens clone 24626 mRNA sequence
Consensus includes gb:AA725078 paired box gene 1

Consensus includes gb:AI656822 Homo sapiens mRNA; cDNA DKFZp434D024
Consensus includes gb:NM_000608.1 /DEF=Homo sapiens orosomucoid 2 (ORM2), mRNA.
Consensus includes gb:NM_005293.1 /DEF=Homo sapiens G protein-coupled receptor 20 (GPR20), mRNA.
Consensus includes gb:NM_002169.1 /DEF=Homo sapiens interferon, alpha 5 (IFNA5), mRNA.
Consensus includes gb:R99037acetyl-Coenzyme A carboxylase beta /FL=gb:U89344.1 gb:NM_001093.1
Consensus includes gb:BE877796 collagen, type VIII, alpha 1 /FL=gb:NM_001850.1
Consensus includes gb:BF215673 Drosophila Kelch like protein /FL=gb:NM_019117.1
Consensus includes gb:U82671 Homo sapiens chromosome Xq28 melanoma antigen families A2a (MAGEA2A), A12 (MAGEA12), A2b (MAGEA2B), A3 (MAGEA3)
Consensus includes gb:U18549 /DEF=Human GPR6 G protein-coupled receptor gene, complete cds
Consensus includes gb:AA004579 TATA box binding protein (TBP)-associated factor, RNA polymerase I, B, 63kD
Consensus includes gb:AF007146.1 Homo sapiens clone 23686 and 23885 mRNA sequences
Consensus includes gb:AU118874 Homo sapiens PAR5 gene, complete sequence
Consensus includes gb:BG111168 chromosome 6 open reading frame 9
Consensus includes gb:BF434424 spectrin, beta, non-erythrocytic 1
Consensus includes gb:AL022165 Contains a probable Zinc Finger protein (pseudo)gene
Consensus includes gb:BE794962 hypothetical protein
Consensus includes gb:BF348061 neural cell adhesion molecule 1
Consensus includes gb:AJ275469 /DEF=Homo sapiens partial IGVH3 gene for immunoglobulin heavy chain V region
Consensus includes gb:AF070571.1 /DEF=Homo sapiens clone 24739 mRNA sequence.
Consensus includes gb:AJ006701.1 /DEF=Homo sapiens mRNA for putative serinethreonine protein kinase, partial.
Consensus includes gb:AK023845.1 weakly similar to PROBABLE UBIQUITIN CARBOXYL-TERMINAL HYDROLASE FAF
Consensus includes gb:AI123471 hypothetical protein MGC3178
Consensus includes gb:X83301.1 /DEF=H.sapiens SMA5 mRNA.
Consensus includes gb:AF035294.1 KIAA1024 protein
Consensus includes gb:AC002550 G protein-coupled receptor, family C, group 5, member B
Consensus includes gb:AL080134.1 /DEF=Homo sapiens mRNA; cDNA DKFZp434G043
Consensus includes gb:AL021026 Contains FMO2 and FMO3 genes for Flavin-containing Monooxygenase 2 and 3
Consensus includes gb:AV733308 integrin, alpha 6
Consensus includes gb:AF090886.1 /DEF=Homo sapiens clone HQ0072.
Consensus includes gb:AF339785.1 /DEF=Homo sapiens clone IMAGE:1963178, mRNA sequence.
Consensus includes gb:S83390.1 /DEF=T3 receptor-associating cofactor-1 human, fetal liver, mRNA, 2930 nt.
Consensus includes gb:AK021571.1 /DEF=Homo sapiens cDNA FLJ11509
Consensus includes gb:AF054994.1 /DEF=Homo sapiens clone 23832 mRNA sequence.
Consensus includes gb:AW301235 Homo Sapiens mRNA, partial cDNA sequence from cDNA selection, DCR1-16.0
Consensus includes gb:AI189839 integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
Consensus includes gb:AI561253 9 similar to Homo sapiens gene for glycosylphosphatidylinositol anchor attachment 1 (GPAA1)
Consensus includes gb:AK021571.1 Homo sapiens cDNA FLJ11509 fis, clone HEMBA1002166
Consensus includes gb:U79300.1 /DEF=Human clone 23629 mRNA sequence.
Consensus includes gb:AU150691 Homo sapiens cDNA FLJ10577 fis, clone NT2RP2003367
Consensus includes gb:AU146646 Homo sapiens cDNA FLJ10270 fis, clone HEMBB1001096
Consensus includes gb:AK026980.1 highly similar to HSZNF37 Homo sapiens ZNF37A mRNA for zinc finger protein.
Consensus includes gb:AU145354 Homo sapiens cDNA FLJ11396 fis, clone HEMBA1000604
Consensus includes gb:AU147194 Homo sapiens cDNA FLJ12102 fis, clone HEMBB1002684
Consensus includes gb:AF038194.1 /DEF=Homo sapiens clone 23821 mRNA sequence.
Consensus includes gb:AU146952 Homo sapiens cDNA FLJ12046 fis, clone HEMBB1001962
Consensus includes gb:BE740743 thyroid stimulating hormone receptor
Consensus includes gb:AF035314.1 /DEF=Homo sapiens clone 23651 mRNA sequence.
Consensus includes gb:AL080207.1 /DEF=Homo sapiens mRNA, DKFZP434G232 protein
Consensus includes gb:AK022094.1 /DEF=Homo sapiens cDNA FLJ12032 fis, clone HEMBB1001880.
Consensus includes gb:BG484069 Homo sapiens FANCA gene, exon 10a
Consensus includes gb:AI073549 Contains a novel gene and an exon of the ESR1 gene for estrogen receptor 1 (NR3A1)
Consensus includes gb:AK023515.1 Homo sapiens cDNA FLJ13453 fis, clone PLACE1003205
Consensus includes gb:AK022322.1 /DEF=Homo sapiens cDNA FLJ12260 fis, clone MAMMA1001551.

Consensus includes gb:AK000861.1 /DEF=Homo sapiens cDNA FLJ20854 fis, clone ADKA01341.
Consensus includes gb:L34409.1 Homo Sapiens (clone B3B3E13) chromosome 4p16.3 DNA fragment
Consensus includes gb:AK021614.1 /DEF=Homo sapiens cDNA FLJ11552 fis, clone HEMBA1003021.
Consensus includes gb:AA835004 hypothetical protein
Consensus includes gb:N53959 Rhesus blood group, CcEe antigens
Consensus includes gb:AK022363.1 /DEF=Homo sapiens cDNA FLJ12301 fis, clone MAMMA1001858.
Consensus includes gb:AK025077.1 /DEF=Homo sapiens cDNA: FLJ21424 fis, clone COL04157.
Consensus includes gb:AL158172 Contains the PLA2G5 gene for two isoforms of phospholiapse A2 group V
Consensus includes gb:AK026820.1 /DEF=Homo sapiens cDNA: FLJ23167 fis, clone LNG09902.
Consensus includes gb:AF009267.1 Homo sapiens clone FBA1 Cri-du-chat region mRNA
Consensus includes gb:AC002544 KIAA0220 protein
Consensus includes gb:AL049259.1 /DEF=Homo sapiens mRNA; cDNA DKFZp564E193
Consensus includes gb:AU148005 /FEA=EST /DB_XREF=gi:11009526 /DB_XREF=est:AU148005 /CLONE=MAMMA1002355
Consensus includes gb:AU147200 Homo sapiens cDNA FLJ12105 fis, clone HEMBB1002699
Consensus includes gb:AK026040.1 /DEF=Homo sapiens cDNA: FLJ22387 fis, clone HRC07655.
Consensus includes gb:AK023621.1 highly similar to Homo sapiens mRNA for KIAA0878 protein.
Consensus includes gb:AF115765 /DEF=Homo sapiens Artemin gene, alternative forms, complete cds
Consensus includes gb:U02309.1 /DEF=Human PAX-3 mRNA, partial cds.
Consensus includes gb:AL031228 Contains BING5, exons 11 to 15 of BING4, GalT3 RPS18,SACM2L
Consensus includes gb:AK022450.1highly similar to Homo sapiens mRNA for ganglioside sialidase.
Consensus includes gb:AL109682.1 Homo sapiens mRNA full length insert cDNA clone EUROIMAGE 35394
Consensus includes gb:AL137285.1 /DEF=Homo sapiens mRNA; cDNA DKFZp434D2416
Consensus includes gb:R37427 Homo sapiens clone IMAGE 25997
Consensus includes gb:Z22970.1 CD163 antigen
Consensus includes gb:AK022215.1 /DEF=Homo sapiens cDNA FLJ12153 fis, clone MAMMA1000458.
Consensus includes gb:X78931.1 /DEF=H.sapiens HZF8 mRNA for zinc finger protein.
Consensus includes gb:X64116 poliovirus receptor
Consensus includes gb:AL137713.1 /DEF=Homo sapiens mRNA; cDNA DKFZp434I0523
Consensus includes gb:AU159276 Homo sapiens cDNA FLJ13867 fis, clone THYRO1001262
Consensus includes gb:X95238.1 /DEF=H.sapiens mRNA for cysteine-rich secretory protein-1 delta.
Consensus includes gb:AF086641 /DEF=Homo sapiens truncated tenascin XB (TNXB) gene, partial cds
Consensus includes gb:U02520.1 /DEF=Human collagen type IV alpha 3 mRNA, partial cds.
Consensus includes gb:Z49258 transketolase-like 1
Consensus includes gb:AF103295.1 immunoglobulin heavy chain variable region
Consensus includes gb:AK024949.1 Homo sapiens cDNA: FLJ21296 fis, clone COL02029
Consensus includes gb:AK025363.1 /DEF=Homo sapiens cDNA: FLJ21710 fis, clone COL10087.
Consensus includes gb:AK024949.1 /DEF=Homo sapiens cDNA: FLJ21296 fis, clone COL02029.
Consensus includes gb:D38024 /DEF=Human facioscapulohumeral muscular dystrophy (FSHD) gene region
Consensus includes gb:AL049252.1 /DEF=Homo sapiens mRNA; cDNA DKFZp564D193
Consensus includes gb:X65232.1 /DEF=H.sapiens mRNA for Zinc-finger protein (ZNFpT7).
Consensus includes gb:U25801.1 /DEF=Human Tax1 binding protein mRNA, partial cds.
Consensus includes gb:AL049963.1 /DEF=Homo sapiens mRNA; cDNA DKFZp564A132 (from clone DKFZp564A132).
Consensus includes gb:AL050219.1 /DEF=Homo sapiens mRNA; cDNA DKFZp586J1623 (from clone DKFZp586J1623).
Consensus includes gb:AL121890 Contains a novel gene, a 40S ribosomal protein S21 pseudogene, 2 CpG islands
Consensus includes gb:AF009660 /DEF=Homo sapiens T cell receptor beta locus, TCRBV7S3A2 to TCRBV12S2 region
Consensus includes gb:AL022068 Contains a KRT18 (Cytokeratin 18, CK18)) pseudogene
Consensus includes gb:AL022152 Contains two exons similar to MAGE gene family
Consensus includes gb:AB022847.1 /DEF=Homo sapiens mRNA for norepinephrine transporter isoform 2, partial cds.
Consensus includes gb:AL117447.1 /DEF=Homo sapiens mRNA; cDNA DKFZp586A0617
Consensus includes gb:U58994 /DEF=Human ladinin (LAD) gene, complete cds
Consensus includes gb:AL110190.1 /DEF=Homo sapiens mRNA; cDNA DKFZp564J2116
Consensus includes gb:AK000185.1 /DEF=Homo sapiens cDNA FLJ20178 fis, clone COL09990.
Consensus includes gb:AL133618.1 /DEF=Homo sapiens mRNA; cDNA DKFZp434C2021
Consensus includes gb:U06641.1 /DEF=Human UDP glucuronosyltransferase mRNA, partial cds.

Consensus includes gb:AL353949.1 /DEF=Homo sapiens mRNA; cDNA DKFZp761P1114
Consensus includes gb:AF164963.1 /DEF=Homo sapiens tumor antigen NA88-A pseudogene, complete sequence.
Consensus includes gb:AK025325.1 /DEF=Homo sapiens cDNA: FLJ21672 fis, clone COL09025.
Consensus includes gb:AK026856.1 /DEF=Homo sapiens cDNA: FLJ23203 fis, clone ADKA02487.
Consensus includes gb:AL390857 Contains an HNRPA1 (heterogeneous nuclear ribonucleoprotein A1) pseudogene
Consensus includes gb:U40372.1 /DEF=Human 3,5 cyclic nucleotide phosphodiesterase (HSPDE1C3A) mRNA, partial cds.
Consensus includes gb:X91103.1 /DEF=H.sapiens mRNA for Hr44 protein.
Consensus includes gb:AJ133768.1 /DEF=Homo sapiens mRNA for ZASP protein, partial, varient 2.
Consensus includes gb:L23852.1 /DEF=Homo sapiens (clone Z146) retinal mRNA, 3 end and repeat region.
Consensus includes gb:AL049545 Contains an RPL7 (60S Ribosomal Protein L7) pseudogene, a RAB1 pseudogene
Consensus includes gb:AF098114.1 /DEF=Homo sapiens truncated alpha IIb protein mRNA, partial cds.
Consensus includes gb:Y18284.1 /DEF=Homo sapiens mRNA for mannose binding lectin-associated serine protease-2
Consensus includes gb:U52428 /DEF=Human fatty acid synthase gene, partial cds
Consensus includes gb:AF007194.1 /DEF=Homo sapiens mucin (MUC3) mRNA, partial cds
Consensus includes gb:X97875 H.sapiens EP4 prostaglandin receptor pseudogene
Consensus includes gb:AC003079 Human BAC clone GS1-303P24 from 7q21-22
Consensus includes gb:L37198.1 /DEF=Homo sapiens (clone B3B3E13) Huntingtons disease candidate region mRNA fragment.
Consensus includes gb:AF135564.1 /DEF=Homo sapiens p50 killer cell activating receptor KAR-K1d mRNA.
Consensus includes gb:AC006033 Homo sapiens BAC clone RP11-121A8 from 7p14-p13
Consensus includes gb:X69383 /DEF=H.sapiens gene for T cell receptor gamma V region 5
Consensus includes gb:AJ000388.1 /DEF=Homo sapiens mRNA for calpain-like protease CANPX.
Consensus includes gb:AK000918.1 highly similar to Homo sapiens VAMP-associated protein of 33 kDa mRNA.
Consensus includes gb:D25272.1 /DEF=Homo sapiens mRNA, clone:RES4-16.
Consensus includes gb:AL034450 Contains high mobility group protein 2a
Consensus includes gb:AW613387 Moderately similar to TYPH_HUMAN THYMIDINE PHOSPHORYLASE PRECURSOR
Consensus includes gb:AI346187 Weakly similar to ALUE_HUMAN
Consensus includes gb:AA741028 Moderately similar to ALUA_HUMAN
Consensus includes gb:AI088162 Moderately similar to ALU3_HUMAN ALU SUBFAMILY SB1
Consensus includes gb:AL583687 CLONE=CS0DJ008YL09 (5 prime)
Consensus includes gb:BF448596 CLONE=IMAGE:3571902
Consensus includes gb:AA457019 Weakly similar to ALU7_HUMAN ALU SUBFAMILY SQ
Consensus includes gb:AW451230 Highly similar to KIAA0311 H.sapiens
Consensus includes gb:BG151284 CLONE=IMAGE:4262274 /UG=Hs.322737 ESTs
Consensus includes gb:BG281679 Highly similar to YXHUT thymidylate synthase H.sapiens
Consensus includes gb:BF942161 CLONE=IMAGE:4118994
Consensus includes gb:AW295066 CLONE=IMAGE:2730209
Consensus includes gb:AW085172 Highly similar to KPCM_HUMAN PROTEIN KINASE C, MU TYPE H.sapiens
Consensus includes gb:AA479678 Moderately similar to ALU8_HUMAN ALU SUBFAMILY SX
gb:NM_003498.1 /DEF=Homo sapiens stannin (SNN), mRNA.
Consensus includes gb:BC001080.1 hypothetical protein MGC2749
gb:NM_015894.1 /DEF=Homo sapiens SCG10-like-protein (SCLIP), mRNA.
gb:NM_022552.2 /DEF=Homo sapiens DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3A), mRNA.
gb:NM_024894.1 /DEF=Homo sapiens hypothetical protein FLJ14075 (FLJ14075), mRNA.
gb:NM_017734.1 /DEF=Homo sapiens hypothetical protein FLJ20271 (FLJ20271), mRNA.
gb:NM_024663.1 /DEF=Homo sapiens hypothetical protein FLJ11583 (FLJ11583), mRNA.
gb:NM_024095.1 /DEF=Homo sapiens hypothetical protein MGC5540 (MGC5540), mRNA.
gb:NM_020353.1 /DEF=Homo sapiens phospholipid scramblase 4 (LOC57088), mRNA.
gb:NM_014154.1 /DEF=Homo sapiens HSPC056 protein (HSPC056), mRNA.
gb:NM_017629.1 /DEF=Homo sapiens hypothetical protein FLJ20033 (FLJ20033), mRNA.
gb:NM_024630.1 /DEF=Homo sapiens hypothetical protein FLJ20984 (FLJ20984), mRNA.
gb:NM_024648.1 /DEF=Homo sapiens hypothetical protein FLJ22222 (FLJ22222), mRNA.
Consensus includes gb:AB020675.1 /DEF=Homo sapiens mRNA for KIAA0868 protein, partial cds.
gb:NM_018696.1 /DEF=Homo sapiens elaC (E.coli) homolog 1 (ELAC1), mRNA.
gb:NM_024526.1 /DEF=Homo sapiens hypothetical protein FLJ21522 (FLJ21522), mRNA.

gb:NM_024712.1 /DEF=Homo sapiens hypothetical protein FLJ13824 (FLJ13824), mRNA.
gb:NM_020659.1 /DEF=Homo sapiens tweety (Drosophila) homolog 1 (TTYH1), mRNA.
gb:NM_001643.1 /DEF=Homo sapiens apolipoprotein A-II (APOA2), mRNA.
gb:NM_021197.1 /DEF=Homo sapiens WAP four-disulfide core domain 1 (WFDC1), mRNA.
gb:NM_013246.1 /DEF=Homo sapiens cardiotrophin-like cytokine; neurotrophin-1B-cell stimulating factor-3 (CLC), mRNA.
gb:NM_004669.1 /DEF=Homo sapiens chloride intracellular channel 3 (CLIC3), mRNA.
gb:NM_024500.1 /DEF=Homo sapiens likely ortholog of mouse polydom (POLYDOM), mRNA.
gb:NM_024515.1 /DEF=Homo sapiens hypothetical protein MGC4645 (MGC4645), mRNA.
gb:NM_001231.1 /DEF=Homo sapiens calsequestrin 1 (fast-twitch, skeletal muscle) (CASQ1) mRNA.
gb:NM_024689.1 /DEF=Homo sapiens hypothetical protein FLJ14103 (FLJ14103), mRNA.
gb:NM_017699.1 /DEF=Homo sapiens hypothetical protein FLJ20174 (FLJ20174), mRNA
gb:NM_016511.1 /DEF=Homo sapiens C-type lectin-like receptor-1 (LOC51267), mRNA.
gb:NM_014332.1 /DEF=Homo sapiens small muscle protein, X-linked (SMPX), mRNA.
gb:NM_016931.1 /DEF=Homo sapiens NADPH oxidase 4 (NOX4), mRNA.
gb:NM_024758.1 /DEF=Homo sapiens hypothetical protein FLJ23384 (FLJ23384), mRNA. /
gb:NM_021924.1 /DEF=Homo sapiens mucin and cadherin-like (MUCDHL), mRNA.
gb:NM_024838.1 /DEF=Homo sapiens hypothetical protein FLJ22002 (FLJ22002), mRNA.
gb:NM_016613.1 /DEF=Homo sapiens AD021 protein (LOC51313), mRNA.
gb:NM_015995.1 /DEF=Homo sapiens Kruppel-like factor 13 (KLF13), mRNA.
gb:NM_021804.1 /DEF=Homo sapiens angiotensin I converting enzyme (peptidyl-dipeptidase A) 2 (ACE2), mRNA.
gb:NM_017888.1 /DEF=Homo sapiens hypothetical protein FLJ20581 (FLJ20581), mRNA.
gb:NM_024753.1 /DEF=Homo sapiens hypothetical protein FLJ11457 (FLJ11457), mRNA.
gb:NM_023067.1 /DEF=Homo sapiens forkhead transcription factor FOXL2 (BPES), mRNA.
gb:NM_000396.1 /DEF=Homo sapiens cathepsin K, mRNA.
gb:NM_005209.1 /DEF=Homo sapiens crystallin, beta A2 (CRYBA2), mRNA.
gb:NM_022352.1 /DEF=Homo sapiens caspase recruitment domain protein 9 (LOC64170), mRNA.
gb:NM_016298.1 /DEF=Homo sapiens muscle disease-related protein (LOC51725), mRNA.
gb:NM_025214.1 /DEF=Homo sapiens CTCL tumor antigen se57-1 (SE57-1), mRNA.
gb:NM_020655.1 /DEF=Homo sapiens junctophilin 3 (JPH3), mRNA.
gb:NM_024677.1 /DEF=Homo sapiens hypothetical protein FLJ14001 (FLJ14001), mRNA.
gb:NM_024791.1 /DEF=Homo sapiens hypothetical protein FLJ22756 (FLJ22756), mRNA.
gb:NM_021827.1 /DEF=Homo sapiens hypothetical protein FLJ23514 (FLJ23514), mRNA.
gb:NM_012269.1 /DEF=Homo sapiens hyaluronoglucosaminidase 4 (HYAL4), mRNA.
gb:NM_017790.1 /DEF=Homo sapiens homolog of mouse C2PA (FLJ20370), mRNA.
gb:NM_024796.1 /DEF=Homo sapiens hypothetical protein FLJ22639 (FLJ22639), mRNA.
gb:NM_014579.1 /DEF=Homo sapiens zinc transporter (ZIP2), mRNA.
gb:NM_018961.1 /DEF=Homo sapiens ubiquitin associated and SH3 domain containing, A (UBASH3A), mRNA.
gb:NM_024869.1 /DEF=Homo sapiens hypothetical protein FLJ14050 (FLJ14050), mRNA.
gb:NM_016593.1 /DEF=Homo sapiens oxysterol 7alpha-hydroxylase (CYP39A1), mRNA.
gb:NM_024876.1 /DEF=Homo sapiens hypothetical protein FLJ12229 (FLJ12229), mRNA.
gb:NM_022161.1 /DEF=Homo sapiens livin inhibitor-of-apotosis (LIVIN), mRNA.
gb:NM_012139.1 /DEF=Homo sapiens deafness locus associated putative guanine nucleotide exchange factor (DELGEF), mRNA.
gb:NM_018678.1 /DEF=Homo sapiens lipopolysaccharide specific response-68 protein (LSR68), mRNA.
gb:NM_014406.1 /DEF=Homo sapiens potassium calcium-activated channel, subfamily M, beta 3-like (KCNMB3L), mRNA.
gb:NM_020346.1 /DEF=Homo sapiens differentiation-associated Na-dependent inorganic phosphate cotransporter (DNPI), mRNA.
gb:NM_025008.1 /DEF=Homo sapiens hypothetical protein FLJ13544 (FLJ13544), mRNA.
gb:NM_017713.1 /DEF=Homo sapiens hypothetical protein FLJ20211 (FLJ20211), mRNA.
gb:NM_013227.1/DEF=Homo sapiens chondroitin sulfate proteoglycan 1, mRNA.
gb:NM_018262.1 /DEF=Homo sapiens hypothetical protein FLJ10897 (FLJ10897), mRNA.
gb:NM_024763.1 /DEF=Homo sapiens hypothetical protein FLJ23129 (FLJ23129), mRNA. 1
gb:NM_024888.1 /DEF=Homo sapiens hypothetical protein FLJ11535 (FLJ11535), mRNA.
gb:NM_020377.1 /DEF=Homo sapiens cysteinyl leukotriene CysLT2 receptor; cDNA
gb:NM_016610.1 /DEF=Homo sapiens Toll-like receptor 8 (LOC51311), mRNA.
gb:NM_018065.1 /DEF=Homo sapiens hypothetical protein FLJ10346 (FLJ10346), mRNA.

gb:NM_013359.1 /DEF=Homo sapiens zinc finger protein 221 (ZNF221), mRNA.
gb:NM_014099.1 /DEF=Homo sapiens PRO1768 protein (PRO1768), mRNA.
gb:NM_014127.1 /DEF=Homo sapiens PRO0456 protein (PRO0456), mRNA.
gb:NM_014273.1 /DEF=Homo sapiens disintegrin-like and metalloprotease type 1 motif 6 (ADAMTS6), mRNA.
gb:NM_017965.1 /DEF=Homo sapiens hypothetical protein FLJ20839 (FLJ20839), mRNA.
gb:NM_018601.1 /DEF=Homo sapiens hypothetical protein PRO1446 (PRO1446), mRNA.
gb:NM_018606.1 /DEF=Homo sapiens hypothetical protein PRO1787 (PRO1787), mRNA.
gb:AK026737.1 /DEF=Homo sapiens fibronectin 1, cDNA.
gb:NM_020669.1 /DEF=Homo sapiens uncharacterized gastric protein ZA52P (LOC57399), mRNA.
gb:NM_024980.1 /DEF=Homo sapiens hypothetical protein FLJ12132 (FLJ12132), mRNA.
gb:NM_016109.1 /DEF=Homo sapiens PPAR(gamma) angiopoietin related protein (PGAR), mRNA.
gb:NM_017941.1 /DEF=Homo sapiens hypothetical protein FLJ20721 (FLJ20721), mRNA.
gb:NM_014100.1 /DEF=Homo sapiens PRO1770 protein (PRO1770), mRNA.
gb:NM_006394.1 /DEF=Homo sapiens regulated in glioma (RIG), mRNA.
gb:NM_023038.1 /DEF=Homo sapiens a disintegrin and metalloproteinase domain 19 (meltrin beta) (ADAM19), mRNA.
gb:NM_015977.1 /DEF=Homo sapiens Williams-Beuren syndrome chromosome region 14 (WBSCR14), mRNA.
gb:NM_022139.1 /DEF=Homo sapiens GDNF family receptor alpha 4 (GFRA4), mRNA.
gb:NM_018723.1 /DEF=Homo sapiens ataxin 2-binding protein 1 (A2BP1), mRNA.
gb:NM_016528.1 /DEF=Homo sapiens hydroxyacid oxidase 3 (medium-chain) (HAO3), mRNA.
gb:NM_030764.1 /DEF=Homo sapiens SH2 domain-containing phosphatase anchor protein 1 (SPAP1), mRNA.
gb:NM_030788.1 /DEF=Homo sapiens DC-specific transmembrane protein (LOC81501), mRNA.
gb:NM_014009.1 /DEF=Homo sapiens immunodeficiency, polyendocrinopathy, enteropathy, X-linked (IPEX), mRNA.
gb:NM_024123.1 /DEF=Homo sapiens putative Ly-6 superfamily member (G6E), mRNA.
gb:NM_013941.1 /DEF=Homo sapiens olfactory receptor, family 10, subfamily C, member 1 (OR10C1), mRNA.
gb:NM_013288.1 /DEF=Homo sapiens DNA binding protein for surfactant protein B (HUMBINDC), mRNA.
gb:NM_023919.1 /DEF=Homo sapiens taste receptor, family B, member 4 (TRB4), mRNA.
gb:NM_030772.1 /DEF=Homo sapiens connexin 59 (GJA10), mRNA.
gb:NM_030760.1 /DEF=Homo sapiens endothelial differentiation, sphingolipid G-protein-coupled receptor, 8 (EDG8), mRNA.
gb:NM_030753.1 /DEF=Homo sapiens wingless-type MMTV integration site family, member 3 (WNT3), mRNA.
gb:AL136572.1 /DEF=Homo sapiens mRNA; cDNA DKFZp761I2123 (from clone DKFZp761I2123); complete cds.
gb:AF326591.1 /DEF=Homo sapiens fenestrated-endothelial linked structure protein (FELS) mRNA, complete cds.
Consensus includes gb:BC000794.1 /DEF=Homo sapiens, pre-mRNA splicing factor similar to S. cerevisiae Prp18 mRNA.
gb:BC000122.1 /DEF=Homo sapiens, Similar to nuclear localization signals binding protein 1, clone MGC:3104, mRNA.
Consensus includes gb:AL046979 tensin
Consensus includes gb:BF058465 G-rich RNA sequence binding factor 1
Consensus includes gb:AW086021 hypothetical protein FLJ23407
Consensus includes gb:AI694562 */FEA=EST /DB_XREF=gi:4971902 /DB_XREF=est:wd72g08.x1* /CLONE=IMAGE:2337182
Consensus includes gb:AI191771 wingless-type MMTV integration site family, member 6
Consensus includes gb:NM_000847.1 /DEF=Homo sapiens glutathione S-transferase A3 (GSTA3), mRNA.
Consensus includes gb:AU145025 Homo sapiens cDNA FLJ14025 fis, clone HEMBA1003667
Consensus includes gb:AF216693 Homo sapiens interleukin-1 receptor antagonist homolog 1 (IL1HY1) gene, complete cds
Consensus includes gb:BG387770 Weakly similar to C57785 zinc finger protein ZNF140 H.sapiens
Consensus includes gb:AI742810 Moderately similar to CRGD_HUMAN GAMMA CRYSTALLIN D H.sapiens
Consensus includes gb:BG025063 CLONE=IMAGE:4364304
Consensus includes gb:AW009884 Highly similar to HUMAN PROTEIN PHOSPHATASE 2A, BETA

Downregulated genes (n=161)[b]

gb:M97935.1 /DEF=Homo sapiens transcription factor ISGF-3 mRNA, complete cds.
Cluster Incl. M99436:Human transducin-like enhancer protein (TLE2) mRNA, complete cds
Cluster Incl. AA209463:zq84h11.s1 Homo sapiens cDNA, 3 end /clone=IMAGE-648357
Consensus includes gb:AI439556 upregulated by 1,25-dihydroxyvitamin D-3
gb:NM_006472.1 /DEF=Homo sapiens upregulated by 1,25-dihydroxyvitamin D-3 (VDUP1), mRNA.
gb:NM_021079.1 /DEF=Homo sapiens N-myristoyltransferase 1 (NMT1), mRNA.
gb:NM_003088.1 /DEF=Homo sapiens singed (Drosophila)-like (sea urchin fascin homolog like) (SNL), mRNA.

gb:NM_004740.1 /DEF=Homo sapiens TGFB1-induced anti-apoptotic factor 1 (TIAF1), mRNA.
gb:NM_005526.1 /DEF=Homo sapiens heat shock transcription factor 1 (HSF1), mRNA.
gb:NM_014822.1 /DEF=Homo sapiens SEC24 (S. cerevisiae) related gene family, member D (SEC24D), mRNA.
gb:NM_005474.2 /DEF=Homo sapiens histone deacetylase 5 (HDAC5), mRNA.
Consensus includes gb:U55968/CLONE=26508 /UG=Hs.166318 lipin 2 /FL=gb:D87436.1 gb:NM_014646.1
gb:NM_000937.1 /DEF=Homo sapiens polymerase (RNA) II (DNA directed) polypeptide A (220kD) (POLR2A), mRNA.
gb:NM_006486.1 /DEF=Homo sapiens fibulin 1 (FBLN1), transcript variant D, mRNA.
Consensus includes gb:AI363836 fumarate hydratase
gb:NM_005886.1 /DEF=Homo sapiens katanin p80 (WD40-containing) subunit B 1 (KATNB1), mRNA.
gb:NM_004860.2 /DEF=Homo sapiens fragile X mental retardation, autosomal homolog 2 (FXR2), mRNA.
gb:NM_003164.1 /DEF=Homo sapiens syntaxin 5A (STX5A), mRNA.
gb:NM_000560.1 /DEF=Homo sapiens CD53 antigen (CD53), mRNA.
gb:NM_014921.1 /DEF=Homo sapiens lectomedin-2 (KIAA0821), mRNA.
Consensus includes gb:AI734228 WW domain binding protein 4 (formin binding protein 21)
gb:NM_006297.1 /DEF=Homo sapiens X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1), mRNA.
gb:NM_003199.1 /DEF=Homo sapiens transcription factor 4 (TCF4), mRNA.
gb:NM_016194.1 /DEF=Homo sapiens hypothetical protein (DKFZp586O1922), mRNA.
gb:NM_005044.1 /DEF=Homo sapiens protein kinase, X-linked (PRKX), mRNA.
gb:NM_013279.1 /DEF=Homo sapiens chromosome 11open reading frame 9 (C11ORF9), mRNA.
gb:NM_020309.1 /DEF=Homo sapiens brain-specific Na-dependent inorganic phosphate cotransporter (BNPI), mRNA.
gb:M58581.1 /DEF=Human carnitine palmitoyltransferase (CPT1) mRNA, complete cds.
Consensus includes gb:AI933301 KH-type splicing regulatory protein (FUSE binding protein 2)
gb:NM_013255.1 /DEF=Homo sapiens muskelin 1, intracellular mediator containing kelch motifs (MKLN1), mRNA.
gb:NM_022172.1 /DEF=Homo sapiens pyruvate carboxylase (PC), nuclear gene encoding mitochondrial protein, mRNA
gb:NM_014732.1 /DEF=Homo sapiens KIAA0513 gene product (KIAA0513), mRNA.
gb:NM_006315.1 /DEF=Homo sapiens ring finger protein 3 (RNF3), mRNA.
gb:NM_000048.1 /DEF=Homo sapiens argininosuccinate lyase (ASL), mRNA.
gb:NM_003141.1 /DEF=Homo sapiens Sjogren syndrome antigen A1 (SSA1), mRNA.
gb:NM_003550.1 /DEF=Homo sapiens MAD1 (mitotic arrest deficient, yeast, homolog)-like 1 (MAD1L1), mRNA.
gb:NM_005855.1 /DEF=Homo sapiens receptor (calcitonin) activity modifying protein 1 (RAMP1), mRNA.
gb:NM_005225.1 /DEF=Homo sapiens E2F transcription factor 1 (E2F1), mRNA.
gb:NM_000033.2 /DEF=Homo sapiens ATP-binding cassette, sub-family D (ALD), member 1 (ABCD1), mRNA.
gb:NM_000168.2 /DEF=Homo sapiens GLI-Kruppel family member GLI3 (GLI3), mRNA.
gb:NM_003450.1 /DEF=Homo sapiens zinc finger protein 174 (ZNF174), mRNA.
gb:NM_002557.1 /DEF=Homo sapiens oviductal glycoprotein 1, 120kD (mucin 9, oviductin) (OVGP1), mRNA.
gb:NM_002200.1 /DEF=Homo sapiens interferon regulatory factor 5 (IRF5), mRNA.
gb:NM_003874.1 /DEF=Homo sapiens CD84 antigen (leukocyte antigen) (CD84), mRNA.
Consensus includes gb:BF001594 peptidylprolyl isomerase (cyclophilin)-like 2 /FL=gb:U37219.1 gb:NM_014337.1
gb:NM_005924.1 /DEF=Homo sapiens mesenchyme homeo box 2 (growth arrest-specific homeo box) (MEOX2), mRNA.
gb:AF191653.1 /DEF=Homo sapiens diphosphoinositol polyphosphate phosphohydrolase type 2 beta (NUDT4) mRNA
gb:NM_001492.3 /DEF=Homo sapiens growth differentiation factor 1 (GDF1), mRNA.
gb:NM_003655.1 /DEF=Homo sapiens chromobox homolog 4 (Drosophila Pc class) (CBX4), mRNA.
gb:AF112345.1 /DEF=Homo sapiens integrin alpha 10 subunit (ITGA10) mRNA, complete cds.
gb:NM_001917.1 /DEF=Homo sapiens D-amino-acid oxidase (DAO), mRNA.
gb:NM_004262.1 /DEF=Homo sapiens airway trypsin-like protease (HAT), mRNA.
gb:NM_012301.1 /DEF=Homo sapiens atrophin-1 interacting protein 1; activin receptor interacting protein 1 (KIAA0705), mRNA.
gb:NM_000180.1 /DEF=Homo sapiens guanylate cyclase 2D, membrane (retina-specific) (GUCY2D), mRNA.
gb:NM_006599.1 /DEF=Homo sapiens nuclear factor of activated T-cells 5, tonicity-resonsive (NFAT5), mRNA.
gb:NM_002043.1 /DEF=Homo sapiens gamma-aminobutyric acid (GABA) receptor, rho 2 (GABRR2), mRNA.
gb:NM_012181.1 /DEF=Homo sapiens FK506-binding protein 8 (38kD) (FKBP8), mRNA.
gb:NM_013421.1 /DEF=Homo sapiens gamma-glutamyltransferase 1 (GGT1), transcript variant 2, mRNA.
gb:M73554.1 /DEF=Human bcl-1 mRNA, complete CDs.
Consensus includes gb:BF125756 Homo sapiens mRNA; cDNA DKFZp564N1272
gb:AF092132.1 /DEF=Homo sapiens PAK2 mRNA, complete cds.

Consensus includes gb:AI438999 nuclear receptor coactivator 3
gb:AF001690.1 /DEF=Homo sapiens EXT like protein 3 (EXTL3) mRNA, complete cds
Consensus includes gb:AI753638 KIAA0772 gene product
gb:BC002827.1 /DEF=Homo sapiens, tropomyosin 4, clone MGC:3641, mRNA, complete cds.
gb:BC004153.1 /DEF=Homo sapiens, Similar to poly(rC)-binding protein 4, clone MGC:2386, mRNA, complete cds.
gb:BC004434.1 /DEF=Homo sapiens, clone MGC:3975, mRNA, complete cds.
gb:BC002649.1 /DEF=Homo sapiens, H1 histone family, member 2, clone MGC:3992, mRNA, complete cds.
gb:AL136710.1 /DEF=Homo sapiens mRNA; cDNA DKFZp566P0524 (from clone DKFZp566P0524); complete cds.
gb:U50383.1 /DEF=Human retinoic acid-responsive protein (NN8-4AG) mRNA, complete cds.
gb:BC000723.1 /DEF=Homo sapiens, Similar to carnitine acetyltransferase, clone MGC:1564, mRNA, complete cds.
Consensus includes gb:AA761181 CD24 antigen (small cell lung carcinoma cluster 4 antigen)
Consensus includes gb:AW193656 inhibitor of growth 1 family, member 1
gb:BC002477.1 /DEF=Homo sapiens, clone MGC:3090, mRNA, complete cds.
gb:BC004349.1 /DEF=Homo sapiens, Similar to RAN binding protein 3, clone MGC:1177, mRNA, complete cds.
gb:AF288390.1 /DEF=Homo sapiens B3GALT2 mRNA, complete cds.
gb:BC002557.1 /DEF=Homo sapiens, Similar to GATA-binding protein 2, clone MGC:2306, mRNA, complete cds.
gb:U27336.1 /DEF=Human alpha (1,3) fucosyltransferase (FUT6) mRNA, minor transcript II, complete cds.
gb:AF231056.1 /DEF=Homo sapiens BRG1-Associated Factor 250a (BAF250a) mRNA, complete cds.
gb:M55575.1 /DEF=Human branched chain alpha-keto acid dehydrogenase (BCKDHB) E1-beta subunit mRNA, complete cds.
gb:AF119889.1 /DEF=Homo sapiens PRO2667 mRNA, complete cds.
gb:BC006363.1 /DEF=Homo sapiens, exostoses (multiple)-like 3, clone MGC:12750, mRNA, complete cds.
gb:U80918.1 /DEF=Homo sapiens transcription factor (NF-ATcC) mRNA, complete cds.
gb:AF067524.1 /DEF=Homo sapiens PITSLRE protein kinase beta SV12 isoform (CDC2L2) mRNA, complete cds.
gb:BC003683.1 /DEF=Homo sapiens, Similar to flotillin 2, clone MGC:5052, mRNA, complete cds.
gb:L20492.1 /DEF=Human gamma-glutamyl transpeptidase mRNA, complete cds.
gb:M20206.1 /DEF=Human laminin B1 mRNA, complete cds.
Consensus includes gb:AB014538.1 /DEF=Homo sapiens mRNA for KIAA0638 protein, partial cds.
Consensus includes gb:AA812224 KIAA0770 protein
Consensus includes gb:AI393355 KIAA0630 protein
Consensus includes gb:AI357376 homolog of yeast ubiquitin-protein ligase Rsp5; potential epithelial sodium channel regulator
Consensus includes gb:N30339 collagen, type V, alpha 1
Consensus includes gb:AL536319 KIAA0993 protein
Consensus includes gb:AB014574.1 /DEF=Homo sapiens mRNA for KIAA0674 protein, partial cds.
Consensus includes gb:AW054826 highly similar to AF055023 Homo sapiens clone 24723 mRNA
Consensus includes gb:T62872 KIAA1232 protein
Consensus includes gb:AI567426 KIAA1547 protein
Consensus includes gb:AL583340 KIAA0602 protein
Consensus includes gb:AI992251CLONE=IMAGE:2499767 /UG=Hs.184581 ESTs
Consensus includes gb:AA114166 CLONE=IMAGE:564004 /UG=Hs.23964 sin3-associated polypeptide, 18kD
Consensus includes gb:BG230758 Weakly similar to T31475 hypothetical protein Y62F5A.1b - Caenorhabditis elegans C.elegans
Consensus includes gb:AI934469 KIAA0779 protein
Consensus includes gb:AV705938 neuronal Shc adaptor homolog
Consensus includes gb:AB014573.1 /DEF=Homo sapiens mRNA for KIAA0673 protein, partial cds.
Consensus includes gb:BE858194 Homo sapiens mRNA full length insert cDNA clone EUROIMAGE 26539
Consensus includes gb:N25325 calmodulin 1 (phosphorylase kinase, delta)
Consensus includes gb:BF984434 C-terminal binding protein 1
Consensus includes gb:AV704353 Conserved gene telomeric to alpha globin cluster
Consensus includes gb:BE138647 KIAA0741 gene product
gb:U69268.1 NAD (H)-specific isocitrate dehydrogenase gamma subunit mRNA, alternatively spliced, partial cds.
Consensus includes gb:W84525 DKFZP586B2420 protein
Consensus includes gb:AB007877.1 /DEF=Homo sapiens KIAA0417 mRNA, complete cds.
Consensus includes gb:AF043899.1 /DEF=Homo sapiens amphiphysin IIc1 mRNA, complete cds.
Consensus includes gb:NM_002503.1 /DEF=Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (NFKBIB), mRNA.

Consensus includes gb:AL551046 Human DNA from chromosome 19-specific cosmid F25965, genomic sequence
Consensus includes gb:R39094 KIAA1085 protein
Consensus includes gb:U70544.1 /DEF=Homo sapiens HLA class II DRB4 null antigen (HLA-DRB4) pseudogene mRNA.
Consensus includes gb:S65921.1 /DEF=anti-colorectal carcinoma light chain=glycoprotein CANAG-50 specific IgG1 kappa
Consensus includes gb:AF103295.1 /DEF=Homo sapiens clone N97 immunoglobulin heavy chain variable region mRNA
Consensus includes gb:AK024457.1 /DEF=Homo sapiens mRNA for FLJ00049 protein, partial cds.
Consensus includes gb:AF009205.1 /DEF=Homo sapiens clone L5 unknown mRNA, partial cds.
Consensus includes gb:AL137428.1 /DEF=Homo sapiens mRNA; cDNA DKFZp761N1323
Consensus includes gb:X07024.1 /DEF=Human X chromsome mRNA for CCG1 protein inv. in cell proliferation.
Consensus includes gb:S76475.1 /DEF=neurotrophic tyrosine kinase, receptor, type 3
Consensus includes gb:AL050308 Contains a NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, pseudogene
Consensus includes gb:AI001784 Highly similar to A42735 ribosomal protein L10, cytosolic H.sapiens
gb:NM_016031.1 /DEF=Homo sapiens elongation of very long chain fatty acids-like 1 (ELOVL1), mRNA.
gb:NM_025082.1 /DEF=Homo sapiens hypothetical protein FLJ13111 (FLJ13111), mRNA.
gb:NM_022917.1 /DEF=Homo sapiens hypothetical protein FLJ21959 (FLJ21959), mRNA.
gb:NM_023071.1 /DEF=Homo sapiens hypothetical protein FLJ13117 (FLJ13117), mRNA.
gb:NM_018231.1 /DEF=Homo sapiens hypothetical protein FLJ10815 (FLJ10815), mRNA.
gb:NM_024585.1 /DEF=Homo sapiens hypothetical protein FLJ22160 (FLJ22160), mRNA.
gb:NM_013385.2 /DEF=Homo sapiens pleckstrin homology, Sec7 and coiledcoil domains 4 (PSCD4), mRNA.
gb:NM_017586.1 /DEF=Homo sapiens chromosome 9 open reading frame 7 (C9ORF7), mRNA.
gb:NM_015711.1 /DEF=Homo sapiens glioma tumor suppressor candidate region gene 1 (GLTSCR1), mRNA.
gb:NM_020228.1 /DEF=Homo sapiens PR domain containing 10 (PRDM10), mRNA. /
gb:NM_014353.1 /DEF=Homo sapiens RAB26, member RAS oncogene family (RAB26), mRNA.
gb:NM_020664.1 /DEF=Homo sapiens 2,4-dienoyl CoA reductase 2, peroxisomal (DECR2), mRNA.
gb:NM_017741.1 /DEF=Homo sapiens hypothetical protein FLJ20280 (FLJ20280), mRNA.
Consensus includes gb:AI992095 mesenchymal stem cell protein DSC43
gb:NM_000908.1 /DEF=Homo sapiens natriuretic peptide receptor Cguanylate cyclase C (NPR3), mRNA.
gb:NM_017726.1 /DEF=Homo sapiens hypothetical protein FLJ20251 (FLJ20251), mRNA.
gb:NM_018063.1 /DEF=Homo sapiens hypothetical protein FLJ10339 (FLJ10339), mRNA.
gb:NM_007059.1 /DEF=Homo sapiens kaptin (actin-binding protein) (KPTN), mRNA.
gb:NM_030915.1 /DEF=Homo sapiens hypothetical protein DKFZp566J091 (DKFZP566J091), mRNA.
gb:NM_031301.1 /DEF=Homo sapiens hypothetical protein DKFZp564D0372 (DKFZP564D0372), mRNA.
gb:NM_018482.1 /DEF=Homo sapiens KIAA1249 protein (KIAA1249), mRNA.
gb:NM_023076.1 /DEF=Homo sapiens hypothetical protein FLJ23360 (FLJ23360), mRNA.
gb:NM_022467.1 /DEF=Homo sapiens N-acetylgalactosamine-4-O-sulfotransferase (GALNAC-4-ST1), mRNA.
gb:BC002382.1 /DEF=Homo sapiens, COX15 (yeast) homolog, cytochrome c oxidase assembly protein, mRNA
gb:U15642.1 /DEF=Human transcription factor E2F-5 mRNA, complete cds.
gb:AF282167.1 /DEF=Homo sapiens DRC3 mRNA, complete cds.
Consensus includes gb:AV741657 leucine zipper protein 1
Consensus includes gb:AK024269.1 weakly similar to TROPOMYOSIN 1, FUSION PROTEIN 33.
Consensus includes gb:BF436315 /FEA=EST /DB_XREF=gi:11448630 /DB_XREF=est:7p06b05.x1 /CLONE=IMAGE:3644888
Consensus includes gb:AI057637 Weakly similar to 2109260A B cell growth factor H.sapiens
Consensus includes gb:BF437591 RNA binding motif protein 3
Consensus includes gb:AL117626.1 /DEF=Homo sapiens mRNA; cDNA DKFZp434B105; partial cds.
Consensus includes gb:AF218012.1 /DEF=Homo sapiens clone PP3795 unknown mRNA.
Consensus includes gb:AV700403 /FEA=EST /DB_XREF=gi:10302374 /DB_XREF=est:AV700403 /CLONE=GKBADA01
Consensus includes gb:AW974816 Weakly similar to ALU1_HUMAN ALU SUBFAMILY J

[a] Genes represented in Figure 6G, left graph, week 8, gray bar.

[b] Genes represented in Figure 6G, right graph, week 8, gray bar.

Supplementary Table S4. Changes in expression level of markers of dedifferentiation and multi-lineage differentiation potential in 293T cells treated with Jurkat extract

Name	Jurkat cells (fold upregulation)[a]	Jurkat extract-treated cells (fold upregulation over 8 weeks) [a]
Somatic cell markers		
LMNA	0.9	0.9 – 2.1
LMNB1	0.7	1.2 – 2.2
LMNB2	1.4	0.5 – 1.3
NPR3	1.4	1.4 – 3.4
Embryonic, germ cell and stem cell markers		
OCT4 and Oct4-responsive genes		
POU5F1	0.2	0.05 – 0.2
SOX2	0.25	0.8 – 1.2
UTF1	0.1	0.05 – 0.1
REX1	0.4	0.6 – 2.3
FOXD3	0.75	0.9 – 1.9
Telomerase and telomerase-associated factors		
TERT	0.9	0.6 – 1.8
TERF1	0.3	0.6 – 5.2
TERF2	1.3	1.6 – 3.1
Others		
POU3F1	1.5	0.7 – 1.8
ALP1	1.4	0.7 – 1.5
ALP1	1.3	0.8 – 2.2
CD44	0.8	0.7 – 1.1
LIF	0.2	0.2 – 1.5
FZD9	0.4	0.8 – 1.9
TEF	0.7	0.8 – 2.0
SCGF	1.1	0.8 – 1.0
GCNF	1.3	0.6 – 1.0
SPINK2	3.0	0.9 – 2.7
DKK2	0.8	5.5 – 9.3
INTA6	1.9	0.4 – 1.2
Markers of potential lineage- specific differentiation		
Osteogenic lineage		
BMP1	0.2	0.5 – 1.9
BMP2	0.5	0.5 – 2.5
OGN	1.5	1.4 – 2.7
CTSK	1.1	1.2 – 1.6
TNFRSF11B	0.6	0.3 – 0.5
Endothelial lineage		
VWF	3.5	0.4 – 2.7
NOS3	1.8	0.7 – 1.3
Myogenic lineage		
MYF5	0.9	0.2 – 1.0
TMP1	2.2	0.9 – 3.9
MYH11	0.75	0.7 – 1.0
Neurogenic lineage		
NTS	3.0	1.9 – 2.8
NRG1	1.3	0.8 – 1.1
MBP	2.3	0.8 – 1.3
MOBP	1.4	0.3 – 1.6
NCAM1	0.9	0.5 – 1.0
CD56	0.75	0.5 – 1.5
Adipogenic lineage		

ADRP	0.8	0.6 – 1.0
APOA2	0.6	0.5 – 1.1
APOD	0.3	0.6 – 1.0
APOE	2.1	0.3 – 1.0
APOC1	1.1	0.8 – 1.9
PPARG2	0.2	0.3 – 0.6
FAD1	0.9	0.2 – 2.3
Chondrogenic lineage		
COL4A3	1.2	0.8 – 1.6
COL5A2	1.8	1.0 – 2.0
COL8A1	1.8	0.5 – 1.9
COLL1A1	1.9	0.9 – 2.8
CSPG2	0.2	0.5 – 1.7
AGC1	1.3	0.7 – 1.4
DSPG3	0.1	0 3 – 1.4
CSPG1	2.2	0.9 – 1.2
FNP	0.1	1.2 – 2.5
FN1	0.1	0.1 – 1.8

[a] Range of -fold upregulation over weeks 1-8, relative to 293T cells.

[b] Also upregulated in 293T cells exposed to 293T extract.

Supplementary Table S5. Changes in expression level of housekeeping genes in NCCIT extract-treated cells

Name	Genebank Accession No.	Description	NCCIT cells (fold upregulation)[a]	NCCIT extract-treated cells (fold upregulation)[a]			
				Week 1	Week 2	Week 4	Week 8
Housekeeping genes							
18S	M10098.1	18S ribosomal RNA	1.3	1.3	1.5	1.3	2.5
28S	M11167.1	28S ribosomal RNA	1.3	1.0	0.9	1.1	1.0
GAPDH	M33197.1	Glyceraldehyde-3-phosphate dehydrogenase	2.6	1.4	1.1	1.7	1.5
GAPDH	AL035604	Glyceraldehyde-3-phosphate dehydrogenase	1.0	1.0	1.0	1.0	1.0
GAPDH	AK026525	Glyceraldehyde-3-phosphate dehydrogenase	3.2	1.4	1.2	1.9	1.6
TUBA	L11645.1	Tubulin, alpha	1.2	1.5	1.3	1.4	1.3
TUBA3	AF141347.1	Tubulin, alpha 3	3.8	1.9	1.8	1.3	1.9
TUBAL2	NM_018943.1	Alpha tubulin-like 2	1.0	1.0	1.0	1.0	1.0
TUBB	NM_001069.1	Tubulin, beta	1.7	0.6	0.9	0.6	0.6
TUBB1	NM_030773.1	Tubulin, beta 1	1.0	1.0	0.9	1.0	0.9
TUBB2	BC004188.1	Tubulin, beta 2	0.6	1.1	1.3	0.9	1.0
TUBB4	NM_006086.1	Tubulin, beta 4	0.7	0.8	1.5	0.6	0.7
TUBG2	NM_016437.1	Tubulin, gamma 2	1.1	0.8	0.9	0.9	1.0
HPRT1	NM_000194.1	Hypoxanthine phosphoribosyltransferase 1	1.0	1.7	1.4	1.7	1.4
ACTB	X00351.1	Beta actin	1.4	1.4	1.2	1.3	1.3
Ribosomal protein genes							
RPL0, RPL3, RPL4, RPL6, RPL8, RPL9, RPL10, RPL10A, RPL11, RPL12, RPL13A, RPL17, RPL18, RPL18A, RPL21, RPL22, RPL23. RPL23A, RPL24, RPL27, RPL27A, RPL28, RPL29, RPL31, RPL32, RPL34, RPL35, RPL36A, RPL37, RPL37A, RPL38, RPL41, RPL44, RPLP1, RPLP2			0.8 – 1.2	0.8 – 1.2	0.8 – 1.2	0.8 – 1.2	0.8 – 1.2

[a] Relative to 293T cells.

II

Molecular Biology of the Cell
Vol. 18, 1543–1553, May 2007

Epigenetic Reprogramming of *OCT4* and *NANOG* Regulatory Regions by Embryonal Carcinoma Cell Extract[D]

Christel T. Freberg, John Arne Dahl, Sanna Timoskainen, and Philippe Collas

Department of Biochemistry, Faculty of Medicine, Institute of Basic Medical Sciences, University of Oslo, 0317 Oslo, Norway

Submitted January 16, 2007; Revised February 7, 2007; Accepted February 9, 2007
Monitoring Editor: Carl-Henrik Heldin

Analyses of molecular events associated with reprogramming somatic nuclei to pluripotency are scarce. We previously reported the reprogramming of epithelial cells by extract of undifferentiated embryonal carcinoma (EC) cells. We now demonstrate reprogramming of DNA methylation and histone modifications on regulatory regions of the developmentally regulated *OCT4* and *NANOG* genes by exposure of 293T cells to EC cell extract. *OCT4* and *NANOG* are transcriptionally up-regulated and undergo mosaic cytosine-phosphate-guanosine demethylation. *OCT4* demethylation occurs as early as week 1, is enhanced by week 2, and is most prominent in the proximal promoter and distal enhancer. Targeted *OCT4* and *NANOG* demethylation does not occur in 293T extract-treated cells. Retinoic acid-mediated differentiation of reprogrammed cells elicits *OCT4* promoter remethylation and transcriptional repression. Chromatin immunoprecipitation analyses of lysines K4, K9, and K27 of histone H3 on *OCT4* and *NANOG* indicate that primary chromatin remodeling determinants are acetylation of H3K9 and demethylation of dimethylated H3K9. H3K4 remains di- and trimethylated. Demethylation of trimethylated H3K9 and H3K27 also occurs; however, trimethylation seems more stable than dimethylation. We conclude that a central epigenetic reprogramming event is relaxation of chromatin at loci associated with pluripotency to create a conformation compatible with transcriptional activation.

INTRODUCTION

Reprogramming of a differentiated somatic cell into a pluripotent cell may have applications in regenerative medicine, and as such, several approaches are being examined to produce embryonic stem (ES)-like cells. Nuclear transplantation into oocytes has demonstrated that functional nuclear reprogramming is possible, through the production of nuclear transfer ES cells (Cibelli *et al*., 1998; Munsie *et al*., 2000; Wakayama *et al*., 2001) and cloned animals (Wilmut *et al*., 2002; Gurdon and Byrne, 2003). Fusion of somatic cells with ES or embryonal carcinoma (EC) cells also elicits a reprogramming of the somatic genome within the hybrids, demonstrated by X chromosome reactivation (Tada *et al*., 2001), changes in gene expression profile, and acquisition of ES cell properties, including contribution to all germ layers in teratomas and in aggregation chimeras (Tada *et al*., 1997, 2001; Pells *et al*., 2002; Terada *et al*., 2002; Ying *et al*., 2002; Cowan *et al*., 2005). Recently, retroviral transduction and constitutive expression of four factors (Oct4, Sox2, Klf4, and c-Myc) was also shown to induce an ES cell-like behavior in mouse fibroblasts, similar to that reported by fusion with ES cells (Takahashi and Yamanaka, 2006). A fourth approach to reprogramming entails treatment of reversibly permeabilized somatic cells with an extract of another differentiated cell type (Håkelien *et al*., 2002) or of undifferentiated, pluripotent ES or EC cells (Taranger *et al*., 2005). Notably, epithelial 293T cells treated with extract of undifferentiated human EC (NCCIT) cells induces expression of genes associated with pluripotency, such as *OCT4* and *NANOG*; causes down-regulation of somatic cell-specific genes, such as lamin A (*LMNA*); and enhances in vitro differentiation capacity (Taranger *et al*., 2005). From these observations, it is increasingly clear that exposure of a somatic genome to factors derived from pluripotent cells or eggs is sufficient to elicit partial or complete reprogramming of nuclear function.

All reprogramming approaches investigated to date seem to involve modifications of the epigenome. Methylation in the 5-position of a cytosine in a cytosine-phosphate-guanosine (CpG) dinucleotide is a heritable modification that favors genomic integrity, ensures proper regulation of gene expression, and is essential for long-term gene silencing (Antequera, 2003). Partial DNA demethylation in restricted areas in the *Oct4/OCT4* regulatory region has been reported previously (Tada *et al*., 1997; Simonsson and Gurdon, 2004; Cowan *et al*., 2005; Takahashi and Yamanaka, 2006), and it is proposed be required for activation of the gene (Simonsson and Gurdon, 2004). These studies have been extended with the demonstration that the *Nanog* promoter is also demethylated in nuclear transfer ES cells (Blelloch *et al*., 2006), in fibroblast–ES cell hybrids (Cowan *et al*., 2005), and in transduced cells (Takahashi and Yamanaka, 2006). Additionally, acetylation and methylation of lysine (K) residues in the amino-terminal tail of histones H3 and H4, which regulate chromatin assembly on promoters and thereby promoter activation (Lachner and Jenuwein, 2002), have been shown in mouse

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07–01–0029) on February 21, 2007.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

Address correspondence to: Philippe Collas (philippe.collas@medisin.uio.no).

Abbreviations used: EC, embryonal carcinoma; ES, embryonic stem; HBSS, Hanks' balanced salt solution; ChIP, chromatin immunoprecipitation; RA, retinoic acid; RT-PCR, reverse transcription-polymerase chain reaction; SLO, Streptolysin O; TSS, transcription start site.

thymocyte–ES cell hybrids (Kimura *et al*., 2004). A limitation of cell fusion or transduction approaches to nuclear reprogramming, however, is the mixing of genomic sequences, making epigenetic analyses of the reprogrammed cells dependent on single nucleotide polymorphism or species specificity of the sequences examined. In the cell extract system, only a limited nonquantitative assessment of demethylation has been reported on *OCT4* (Taranger *et al*., 2005), and no quantifiable indications exist to date of epigenetic reprogramming of the human *OCT4* and *NANOG* loci.

Here, we provide evidence of reprogramming of DNA methylation and histone modifications on the *NANOG* promoter and throughout the *OCT4* regulatory region in human epithelial cells as a result of transient exposure to EC cell extract. Bisulfite sequencing analysis of *OCT4* and *NANOG* regulatory regions reveals mosaic DNA demethylation over time. Assessment of six modifications of histone H3 by using a novel quick and quantitative chromatin immunoprecipitation (Q^2ChIP) assay indicates that chromatin remodeling also takes place on *OCT4* and *NANOG* to establish a conformation compatible with transcriptional activation. Subsequent stimulation of extract-treated cells with retinoic acid (RA) promotes a remethylation of *OCT4*, arguing for specificity of the methylation changes elicited by the extract. Because only the somatic cell genome is present in the extract, the approach constitutes a useful tool for investigating the molecular processes behind nuclear reprogramming.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies against H3K9ac (catalog no. 06-942), H3K9m2 (07-441), H3K9m3 (07-442), and H3K27m3 (05-851) were from Upstate Biotechnology (Lake Placid, NY). Antibodies against H3K4m2 (Ab7766) and H3K4m3 (Ab8580) were from Abcam (Cambridge, United Kingdom). Other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Cells

293T cells and undifferentiated human EC cells (NCCIT) were cultured in RPMI 1640 medium containing 10% fetal calf serum (complete RPMI medium) (Taranger *et al*., 2005). Cells treated with extracts were seeded at 100,000 cells/well in a 48-well plate and cultured in 250 μl of complete RMPI medium after membrane resealing. In some experiments, cells were also induced to differentiate with 10 μM RA in bacterial culture plates for up to 3 wk as described previously (Taranger *et al*., 2005).

Reprogramming Extracts

Extracts of NCCIT cells or control 293T cells were prepared as described (Taranger *et al*., 2005). In short, cells were washed in phosphate-buffered saline (PBS) and in cell lysis buffer, and then they were sedimented and resuspended in cell lysis buffer. Cells were sonicated on ice; the lysate was sedimented at 15,000 $\times$ *g* for 15 min; and the supernatant was aliquoted, frozen in liquid nitrogen, and stored at -80°C. Extracts were diluted with H_2O before use to adjust osmolarity to ~300 mOsm.

Plasma Membrane Permeabilization and Extract Treatment

The procedure was as reported previously (Taranger *et al*., 2005) with minor modifications. In short, 500,000 293T cells were washed in 500 μl of cold Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) and resuspended in 490 μl of ice-cold HBSS. Tubes were placed in a H_2O bath at 37°C for 2 min, and 10 μl of Streptolysin O (SLO; 100 μg/ml stock diluted 1:10 in cold HBSS; Sigma-Aldrich) was added (final concentration, 200 ng/ml). Samples were incubated horizontally in a H_2O bath for 30 min at 37°C with occasional agitation and placed on ice. Note that optimal SLO concentration and time of incubation need to be adjusted for each SLO batch. Samples were diluted with 1 ml of cold HBSS, and cells were sedimented at 120 $\times$ *g* for 5 min at 4°C. Permeabilization was assessed by uptake of a fluorescent dextran in separate samples 24 h after resealing and replating the cells (Taranger *et al*., 2005).

Permeabilized cells (500,000) were suspended in 500 μl of NCCIT or 293T extract (control) containing an ATP-regenerating system and 1 mM of each nucleotide triphosphate. Tubes were incubated horizontally for 1 h at 37°C in a H_2O bath with occasional agitation. To reseal membranes, the extract was diluted with complete RPMI medium containing 2 mM $CaCl_2$, and cells were seeded at 100,000 cells/well in a 48-well plate. After ~4 h, floating cells were removed, and plated cells were cultured in complete RPMI medium.

Bisulfite Sequencing

DNA was purified by two phenol chloroform isoamyalcohol extractions, followed by one extraction with chloroform isoamylalcohol, and then the DNA was ethanol-precipitated. DNA was dissolved indifferently in H_2O or TE buffer (10 mM Tris-HCl, pH 8.0, and 10 mM EDTA). Bisulfite conversion (Warnecke *et al*., 2002) was performed using the MethylEasy DNA bisulfite modification kit as described by the manufacturer (Human Genetic Signatures, Sydney, Australia). Converted DNA was used fresh or stored at -20°C. Converted DNA was amplified by polymerase chain reaction (PCR) by using primers published previously (Deb-Rinker *et al*., 2005) or designed with MethPrimer (www.urogene.org/methprimer/index1.html) (Supplemental Table 1). PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 1 min, 50/55/58°C for 1 min (temperature was primer-dependent; see Supplemental Table 1), and 72°C for 1 min, followed by 10 min at 72°C. PCR products were purified with the GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich) and then cloned into bacteria by TOPO TA cloning (Invitrogen, Carlsbad, CA) and reverse-sequenced using M13 primers (MWG Biotech, High Point, NC). Sequences of 10 bacterial clones per genomic region examined are represented as rows of circles, with each circle symbolizing the methylation state of one CpG. Chi-square tests were done to compare percentages of methylation between cell types or treatments. Unpaired *t* tests were performed to compare 1) the extent of methylation of a specific CpG deducted from 10 sequences, and 2) numbers of methylated CpGs in a given sequence, between cell populations. The *t* test results are provided in Tables 1 and 2 and throughout the text.

Chromatin Immunoprecipitation (Q^2ChIP)

To minimize sample loss during the ChIP procedure and maximize ChIP specificity, we recently developed and validated a quick and quantitative Q^2ChIP assay (Dahl and Collas, 2007) also used in this study. To prepare antibody–bead complexes, paramagnetic beads (Dynabeads protein A; Dynal Biotech, Oslo, Norway) were washed twice in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, and 140 mM NaCl), and then they were resuspended in 1 volume of RIPA buffer. Beads (10 μl) were added to 90 μl of RIPA buffer containing 2.4 μg of primary antibody in a 0.2-ml PCR tube, and then they were incubated on a rotator for 2 h at 4°C. For DNA–protein cross-linking, 20 mM of the histone deacetylase inhibitor sodium butyrate was added to cells immediately before harvesting (and to all solutions thereafter). Cells were fixed in suspension with 1% formaldehyde for 8 min in PBS at 1–2 $\times$ 10^6 cells/ml, and fixation was stopped with 125 mM glycine for 5 min. All subsequent steps were performed on ice or at 4°C. Cross-linked cells were washed twice in PBS/20 mM butyrate and lysed by a sixfold dilution in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, and protease inhibitors) containing 20 mM butyrate. Aliquots of 200 μl were sonicated each for 10 $\times$ 30 s on ice to generate chromatin fragments of ~500 base pairs. The lysate was sedimented at 10,000 $\times$ *g* for 10 min, the supernatant was collected, and chromatin concentration was determined by A_{260} from an aliquot diluted 100-fold.

Chromatin diluted (2 A_{260} units) in RIPA buffer/20 mM butyrate was transferred to a 0.2-ml tube containing antibody–bead complexes (see above), and the sample was rotated at 40 rpm for 2 h at 4°C. Immune complexes were washed three times in RIPA buffer and once in TE buffer, each for 4 min at 4°C on a rotator set at 40 rpm. The ChIP material was transferred to a new tube, and TE was replaced with 150 μl of elution buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM butyrate, and 50 mM NaCl) containing 1% SDS and 50 μg/ml proteinase K. Samples were incubated for 2 h at 68°C on a Thermomixer at 1300 rpm (Eppendorf, Hamburg, Germany). Elution buffer was recovered, the ChIP material was reextracted for 5 min, and both supernatants were pooled. Another 200-μl elution buffer was added to the eluted material, and DNA was extracted once with phenol-chloroform isoamyl alcohol, once with chloroform isoamyl alcohol, and then ethanol precipitated.

Immunoprecipitated DNA was analyzed in triplicates by real-time PCR starting from 5 μl of DNA (from a total of 150 μl). PCR conditions were 95°C for 3 min and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. ChIP PCR primers are listed in Supplemental Table 1. Data are presented as -fold enrichment of precipitated DNA associated with a given histone modification, relative to a 1/100 dilution of input chromatin (Feldman *et al*., 2006; Dahl and Collas, 2007). ChIPs were performed in two separate experiments as well as from 293T extract-treated cells, 293T cells, and NCCIT cells.

Real-Time Reverse Transcription (RT)-PCR

RT-PCR was carried from 1 μg of total RNA by using the Iscript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA (20 μl) was diluted 1:10, and 5 μl was used in each of triplicate quantitative PCRs on a MyiQ real-time PCR detection system with IQ SYBR Green (Bio-Rad). Primers used are listed in Supplemental Table 2. PCR conditions were 95°C for 3 min and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Data were analyzed using

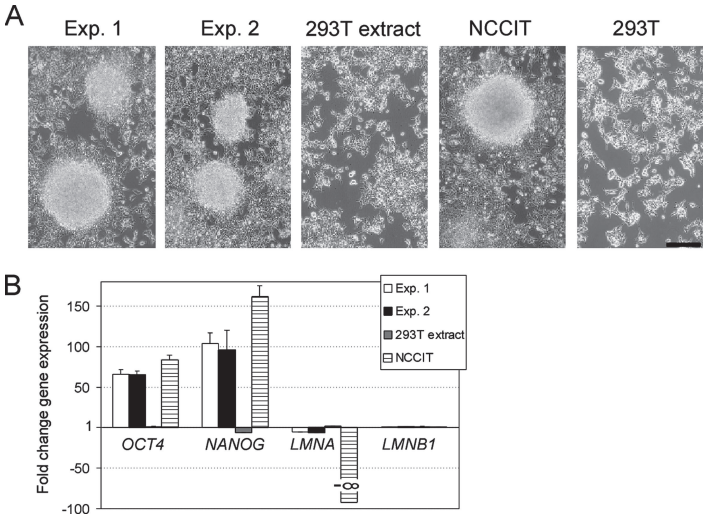


Figure 1. Morphological changes of 293T cells elicited by EC extract treatment are associated with transcriptional up-regulation of pluripotency genes. (A) Morphology of 293T cells, NCCIT cells, and 293T cells 4 wk after treatment with two different EC extracts (experiments 1 and 2) or with a 293T cell extract. Bar, 200 μm. (B) Quantitative RT-PCR analysis of expression of *OCT4*, *NANOG*, *LMNA*, and *LMNB1* in each cell group relative to mRNA level in 293T cells (mean ± SD from 2 separate analyses in each experiment, each with triplicate RT-PCRs). mRNA levels in NCCIT cells are also shown.

formulas of (Pfaffl, 2001) with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as normalization control.

RESULTS

Up-Regulation of OCT4 and NANOG Expression in EC Extract-treated Cells

Permeabilized epithelial 293T cells exposed for 1 h to a whole-cell postchromosomal supernatant of undifferentiated NCCIT cells, resealed and cultured, formed colonies of tightly packed cells over time with a morphology characteristic of NCCIT cells (Figure 1A). Colony formation was independent of cell density, and it did not occur among cells treated with a control 293T cell extract. Quantitative RT-PCR analysis of gene expression in these cultures 4 wk after extract treatment, in two separate experiments, indicates that *OCT4* and *NANOG* were strongly up-regulated from levels barely detectable by real-time RT-PCR (Figure 1B). Moderate down-regulation of *LMNA* expression also occurred, supporting the absence of detection of lamins A and C in the nuclear envelope of EC extract-treated cells (Taranger *et al.*, 2005). None of these changes were detected in 293T extract-treated cells (Figure 1B). As expected, expression of the constitutively expressed lamin B1 (*LMNB1*) gene was not altered by extract treatment (Figure 1B).

OCT4 and NANOG Promoters Undergo Partial DNA Demethylation in EC Extract-treated Cells

To determine whether the EC extract was capable of eliciting epigenetic modifications on exogenous chromatin templates, we first examined DNA methylation changes in the *OCT4*, *NANOG*, and *LMNA* promoter regions. Bisulfite sequencing analysis was carried out to establish 5′-3′ CpG methylation profiles across the *OCT4* proximal promoter (PP, which included the transcription start site, or TSS), the proximal enhancer (PE), and the distal enhancer (DE). Nine amplicons (referred to as *OCT4* regions 1–9) were examined, collectively covering 47 potentially methylated CpG dinucleotides within nucleotides −2995 to +66 relative to the TSS (Figure 2, bisulfite sequencing [BiS] primers; see Supplemental Figure 1 for sequence information). Three regions were also examined in the *NANOG* promoter, encompassing a total of 14 CpGs within nucleotides −1503 to −163 relative to the TSS (Figure 2 and Supplemental Figure 1). The proximal *LMNA* promoter region examined encompassed nine CpGs within nucleotides −277 to +92 relative to the TSS (Figure 2 and Supplemental Figure 1).

The *OCT4* region examined was methylated in 293T cells and largely unmethylated in NCCIT cells ($p < 10^{-4}$; Figure 3A; see Table 1 for statistical analysis). This methylation pattern was consistent with the pattern of expression of

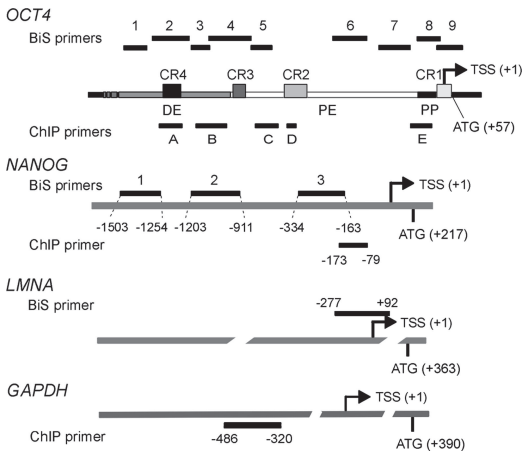


Figure 2. *OCT4*, *NANOG*, *LMNA*, and *GAPDH* regulatory regions examined by bisulfite sequencing and by ChIP in this study. Localization of amplicons generated with BiS and ChIP primers are shown. Numbers are nucleotide numbers in relation to the transcription start site (TSS; +1). Sequence coverage in the *OCT4* locus is shown in Supplemental Table 1. CR1-CR4 on *OCT4* refer to conserved regions 1–4; DE, distal enhancer; PE, proximal enhancer; and PP, proximal promoter.

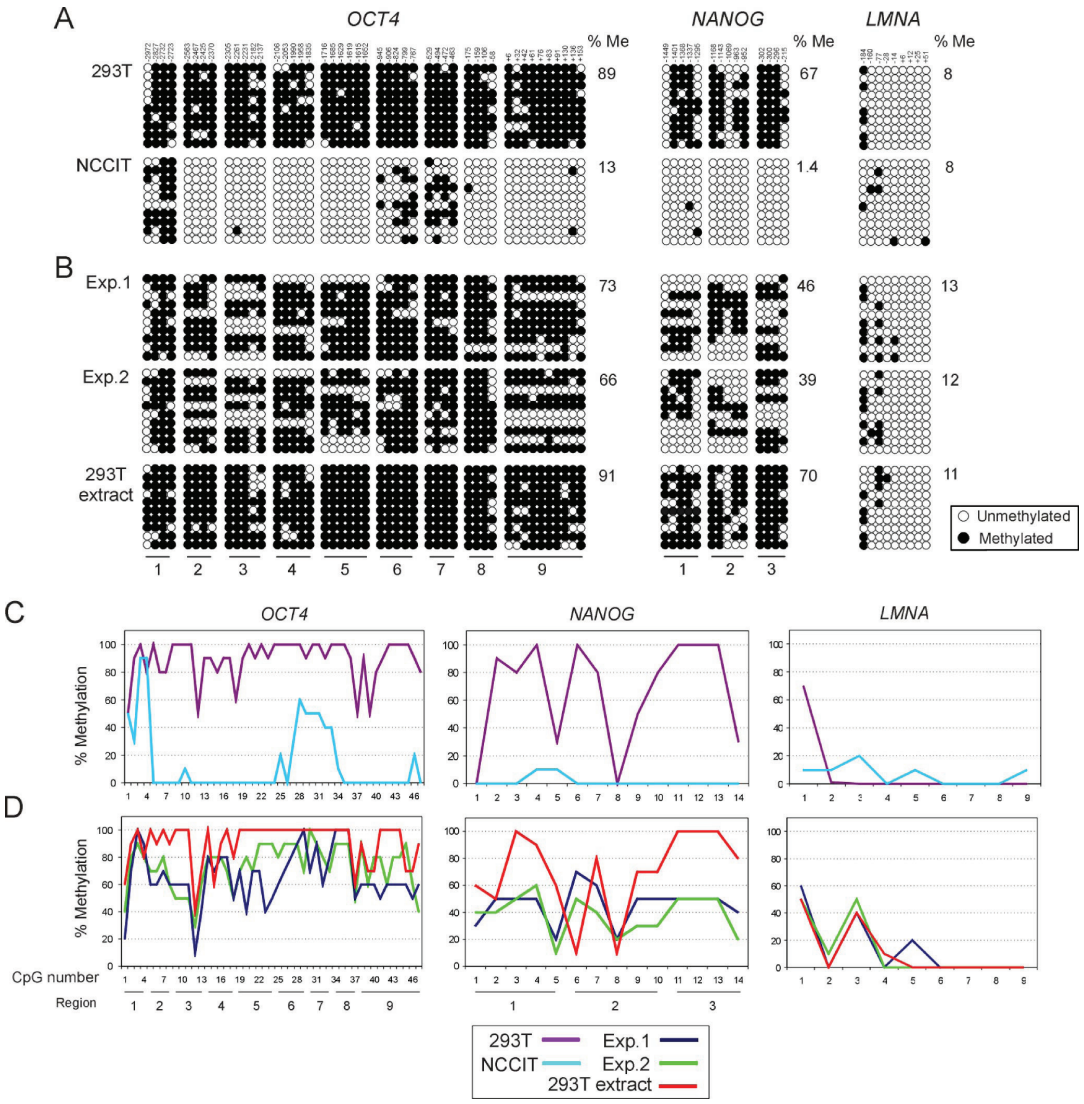


Figure 3. Transient exposure of 293T cells to EC extract elicits DNA demethylation of *OCT4* and *NANOG*. (A) Bisulfite sequencing analysis of *OCT4*, *NANOG*, and *LMNA* methylation in 293T and NCCIT cells. Top numbers indicate CpG number relative to the TSS. Global percentages of methylated cytosines (%Me) are shown. Each row of circles for a given amplicon represents the methylation status of each CpG in one bacterial clone for that region. Series of 10 clones are shown. Bottom numbers (under B) refer to amplicon number, i.e., the *OCT4* and *NANOG* regions examined (see Figure 2). (B) 293T cells treated with EC extract (2 experiments, experiments 1 and 2) or with 293T extract were analyzed as described in A. (C and D) Percentages of methylated cytosines in each position in *OCT4*, *NANOG*, and *LMNA* determined from data shown in A and B. On the *x*-axes, CpG no. 1 is the 5′-most cytosine examined in each region. Positions of genomic regions examined are shown. For *t* test analysis, see Table 1 and text.

OCT4 in NCCIT and 293T cells. Nevertheless, in NCCIT cells, the 5′ end of the DE (region 1) was overall methylated, whereas regions 6 and 7 in the PE were more, and mosaically, methylated than the rest of the region (Figure 3A). This observation was consistent with *OCT4* methylation profiles reported in the undifferentiated EC cell line, NT2 (Deb-Rinker *et al.*, 2005). Likewise, the *NANOG* promoter was 67% methylated in 293T cells but unmethylated in NCCIT cells ($p < 10^{-4}$; Figure 3A; Table 1). Thus, the *OCT4* and *NANOG* regulatory regions examined display sufficiently distinct methylation patterns to be analyzed in extract-treated cells. Last, we found that the *LMNA* promoter was hypomethylated both in 293T and NCCIT cells, with, however, CpG no. 1 being more methylated in 293T cells ($p < 0.001$; Figure 3A).

Table 1. *t* test analysis of the numbers of methylated CpGs between treatments or cell types at wk 4 after EC or 293T extract treatment

	Comparison of nos. of methylated CpGs between cell types (p values)[a]				
Gene	Exp. 1	Exp. 2	293T extract	293T	NCCIT
OCT4[b]					
	p = 0.365	X			
p values	p < 0.001		X	X	X
		p < 0.001	X	X	X
			p = 0.629	X	
			p < 0.0001		X
				p < 0.0001	X
NANOG[c]					
	p = 0.198	X			
p values	p = 0.01		X	X	
	p < 0.001				X
		p = 0.01	X	X	
		p < 0.001			X
			p = 0.827	X	
			p < 0.0001		X
				p < 0.0001	X

[a] Unpaired *t* tests. Data expressed as two-tailed p values with X referring to treatment or cell type with which comparison is made.
[b] Regions 1–9 were analyzed (see Figure 3).
[c] Regions 1–3 were analyzed (see Figure 3).

Absence of methylation in the *LMNA* promoter in NCCIT cells, despite the lack of *LMNA* expression, is reminiscent of the unmethylated state of silent gene promoters poised for transcription in undifferentiated ES cells (Azuara *et al.*, 2006). Profiles of 5′-3′ CpG methylation in the *OCT4*, *NANOG*, and *LMNA* regulatory regions in 293T and NCCIT cells are represented graphically in Figure 3C as the average methylation state of a given CpG on the basis of 10 sequences per amplicon.

CpG methylation in the *OCT4* and *NANOG* promoters were next examined in two independent reprogramming experiments, 4 wk after exposure to EC extract (experiments 1 and 2) or to 293T extract (Figure 3, B and D). *OCT4* was partially demethylated in both experiments to reach methylation levels of 73 and 66%, compared with 91% in 293T extract-treated controls ($p < 0.001$; chi-square test) and 89% in untreated 293T cells ($p < 0.001$; chi-square test; see Table 1 for *t* test analyses). Nevertheless, demethylation did not occur consistently throughout the *OCT4* regulatory locus. The most susceptible areas were *OCT4* regions 2 and 3 in the DE, region 5 in the PE, and region 9 surrounding the TSS in the PP. *OCT4* regions 6 and 7, which are relatively methylated in NCCIT cells, remained unaffected by extract treatment. Furthermore, although *OCT4* methylation was slightly mosaic between 293T cells (presumably as an artifact of extended culture; Figure 3A), mosaicism was enhanced after extract treatment (Figure 3B), most likely due to a variable response of the cells to extract. We concluded that EC extract promotes partial demethylation of *OCT4*, in agreement with transcriptional activation of the gene.

The *NANOG* promoter was also demethylated within 4 wk of extract exposure in both reprogramming attempts (Figure 3, B and D; $p < 0.001$, chi-square tests; see *t* test analyses in Table 1). As little as 39% methylation was detected in the regions examined (Figure 3B, experiment 2). Again, demethylation did not occur in all cells or alleles, and it resulted in a mosaic methylation pattern (Figure 3B). Nonetheless, all regions examined were affected. In particular, CpGs no. 3, 4, and 11–14 were significantly demethylated in both experiments relative to 293T extract-treated cells ($p < 0.001$). We did not notice any changes in methylation of the *LMNA* promoter in treated or control cells relative to 293T cells (Figure 3, B and D).

We next determined how early *OCT4* demethylation took place after extract treatment, focusing on regions that showed the most pronounced demethylation in the previous experiment, namely, regions 2, 3, and 9. Demethylation occurred as early as 1 wk after treatment with EC extract in two additional experiments (experiments A and B) but not in 293T extract-treated cells (57 and 56% methylation in experiment A and B versus 89% in 293T extract-treated cells; $p < 10^{-3}$ [chi-square test] and $p < 10^{-4}$ [*t* tests]; Figure 4, A and B). Demethylation was most pronounced in DE region 2 and PP region 9, near the TSS. Demethylation was enhanced by week 2 (region 9; $p < 0.001$ [*t* test] relative to week 1; Figure 4, A and B) and correlated with transcriptional activation of *OCT4* (Figure 4D). To our surprise, however, demethylation of *NANOG* (region 1) was not detected by week 1 or 2 (Figure 4C), despite activation of the gene (Figure 4D). This suggests that demethylation may have occurred elsewhere in the *NANOG* promoter. Note that *NANOG* expression at week 1 in experiment A and B (Figure 4D) was apparently higher than that at week 4 in experiment 1 and 2 (Figure 1B), despite the relatively higher methylation level of region 1 (compare Figures 3B and 4C). However, *NANOG* expression in untreated 293T cells is barely detectable by real-time RT-PCR, and small variations in PCR efficiency and/or in background *NANOG* mRNA levels in 293T cells between experiments may translate into dramatic differences in the relative mRNA level calculated in extract-treated cells. Thus, expression levels in experiment 1 and 2 (Figure 1B) may not be compared with those of Figure 4D.

Collectively, our results indicate that EC extract is capable of inducing demethylation of *OCT4* and *NANOG* regulatory regions in exogenous genomes. Different regions across the *OCT4* promoter and enhancer are differentially demethylated, and CpGs around the TSS of *OCT4* seem to be particularly susceptible to demethylation. Demethylation in the *NANOG* promoter was more uniform than that of *OCT4*, but this might have been due to fewer numbers of CpGs examined.

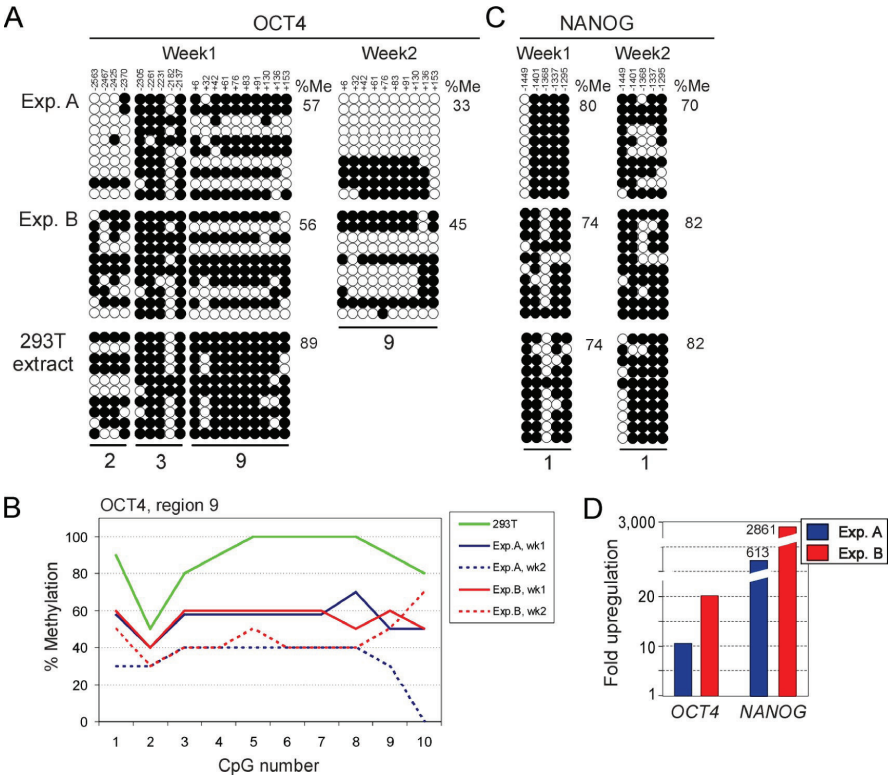


Figure 4. DNA demethylation of *OCT4* correlates with transcriptional activation in EC extract-treated cells. (A) Bisulfite sequencing analysis of *OCT4* regions 2, 3, and 9 at weeks 1 and 2 after EC extract (experiments A and B) and 293T extract treatments. (B) Percentages of methylation of each CpG in *OCT4* region 9 at weeks 1 and 2. (C) Bisulfite sequencing analysis of *NANOG* region 1 at weeks 1 and 2 in cells as described in A. (D) Real-time RT-PCR analysis of *OCT4* and *NANOG* expression in two reprogramming experiments 1 wk after EC extract treatment. Means of triplicate RT-PCRs (SE bars are negligible).

EC Extract Modifies Histone Lysine Methylation and Acetylation on OCT4 and NANOG

In addition to DNA methylation, posttranslational modifications of the amino-terminal tails of core histones, notably histone H3, contribute to the regulation of gene expression. We determined whether treatment of 293T cells with EC extract modified lysines (K) 4, 9, and 27 on histone H3. To accommodate relatively small cell numbers available for ChIP analysis in this study, and to optimize detection of small differences in lysine methylation and acetylation, we recently modified a conventional ChIP protocol (Spencer *et al*., 2003). Q^{2}ChIP minimizes sample loss with a cross-linking step in suspension, it preserves acetylated epitopes by inhibiting histone deacetylase activity early in the process, and it enhances ChIP specificity by eliminating background through a tube-shift step after washes of the ChIP material (Dahl and Collas, 2007). Four weeks after EC extract (experiments 1 and 2; see above) or 293T extract treatment, chromatin was prepared for ChIP analysis of changes in three marks of transcriptionally active chromatin (H3K9ac, H3K4m2, and H3K4m3) and in three repressive marks (H3K9m2, H3K9m3, and H3K27m3) on *OCT4*, *NANOG* and on the constitutively active *GAPDH* promoter. Regions examined are shown in Figure 2, and the data are illustrated in Figure 5.

We first examined histone modifications in 293T and NCCIT cells. As expected from expression in NCCIT cells, the *OCT4* promoter and enhancer contained acetylated H3K9 and barely detectable di- and trimethylated H3K9 or trimethylated H3K27 (Figure 5A). In contrast, 293T cells harbored H3K9m2, H3K9m3, and H3K27m3 but low levels of acetylated H3K9. Di- and trimethylated H3K4 was detected in both cell types, in agreement with expression, or potential for expression, of the gene (Figure 5A). All histone modifications occurred similarly throughout the *OCT4* proximal promoter (*OCT4*-E:PP amplicon), the proximal enhancer (*OCT4*-D:PE and *OCT4*-C:PE amplicons), and the distal enhancer (*OCT4*-B:DE and *OCT4*-A:DE amplicons). The *NANOG* promoter displayed high levels of H3K9ac, H3K4m2, and H3K4m3 together with low levels of H3K9 and H3K27 methylation in NCCIT cells (Figure 5B), again consistent with expression of the gene. In contrast, heterochromatin marks (H3K9m2, H3K9m3, and H3K27m3) were abundant in 293T cells (Figure 5B). Last, no significant differences were detected for any histone H3 modification on the *GAPDH* promoter (Figure 5C). In agreement with its constitutive expression, *GAPDH* exhibited acetylated H3K9, di- and trimethylated H3K4, and background levels of methylation on H3K9 or H3K27.

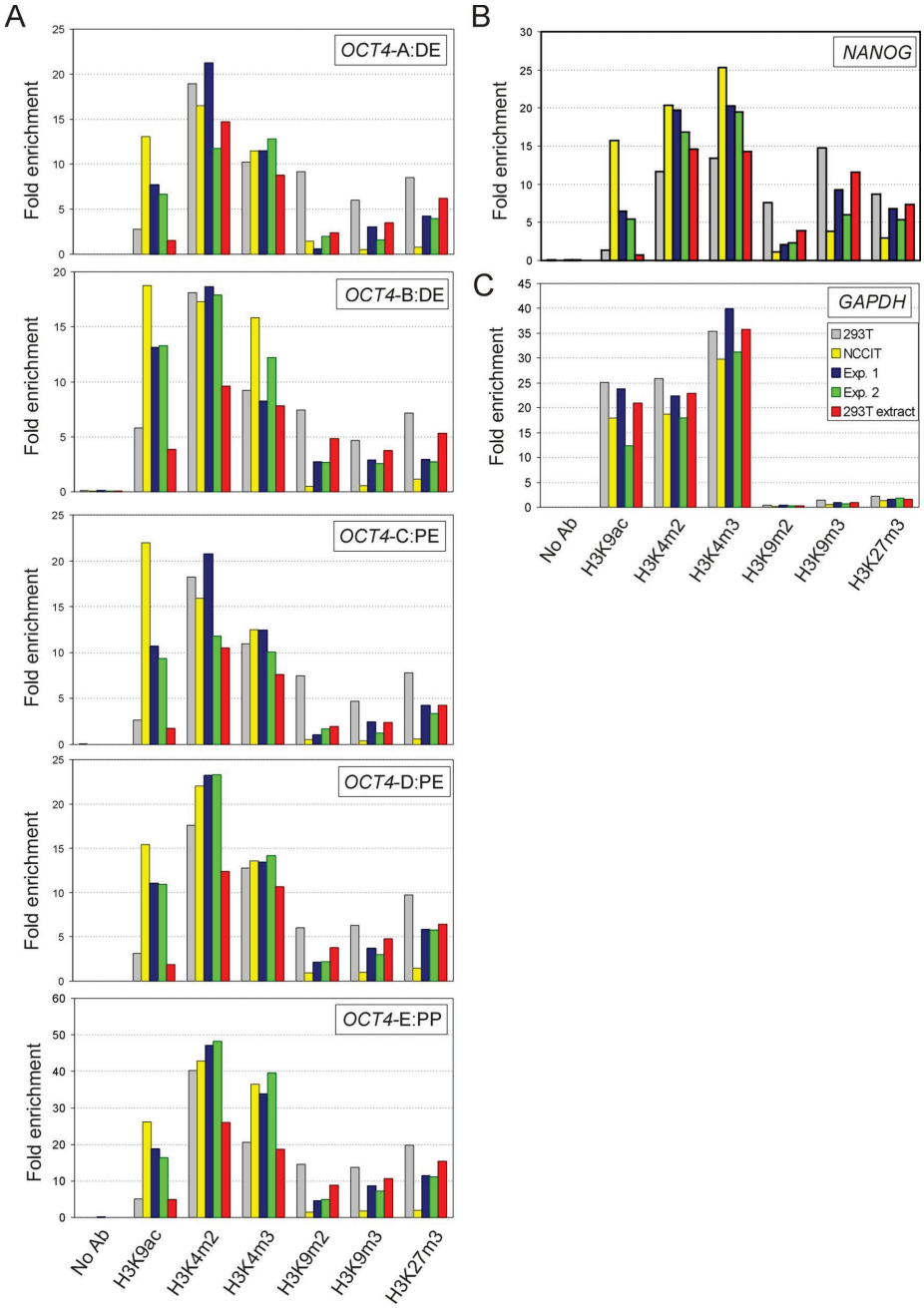


Figure 5. EC extract treatment elicits changes in histone H3 methylation and acetylation on *OCT4* and *NANOG*. 293T cells were treated with EC extract in two separate experiments (experiments 1 and 2) or exposed to 293T extract. Cells were cultured for 4 wk and analyzed by Q^2ChIP for indicated histone modifications. Untreated 293T and NCCIT cells were also examined. Data are presented as -fold enrichment of precipitated DNA associated with a given histone modification relative to a 100-fold dilution of input chromatin. (A) Five genomic regions on *OCT4* (*OCT4*-A to *OCT4*-E) were analyzed (see Figure 2, ChIP primers). (B) *NANOG* promoter. (C) *GAPDH* promoter. Each data point is from a triplicate real-time PCR (error bars are negligible and are not shown).

Treatment with EC extract promoted acetylation and demethylation of H3K9 (m2 and m3) together with a reduction of H3K27m3 on *OCT4* (Figure 5A, blue and green columns). Demethylation of H3K9m2 was consistently more pronounced than that of H3K9m3 or H3K27m3, suggesting that a trimethylated state is more stable than a dimethylated state. As expected from our observations in 293T and NCCIT cells, no changes in H3K4 methylation were detected. Furthermore, 293T extract treatment maintained low H3K9ac and elevated H3K4m2 and H3K4m3 levels (Figure 5A). Demethylation of H3K9m2 and H3K9m3 occurred, however, albeit to a lesser extent than in EC extract-treated cells, particularly in the *OCT4*-E:PP, *OCT4*-D:PE, and *OCT4*-B:DE regions. *OCT4*-C:PE and *OCT4*-A:DE regions were demethylated to the same extent. In addition, 293T extract-treated cells maintained elevated H3K27m3 particularly in the DE (*OCT4*-A:DE, *OCT4*-B:DE) but showed similar H3K27m3 patterns as in EC extract-treated cells in the PE and PP (Figure 5A).

The *NANOG* promoter also underwent H3K9 acetylation in EC extract-treated cells, together with moderate increases in di- and trimethylated H3K4, demethylation of H3K9m2, moderate demethylation of H3K9m3, and weak demethylation of H3K27m3 (Figure 5B). Moreover, histone modification profiles in 293T extract-treated cells were similar to those of 293T cells, except for some demethylation of H3K9m2 (Figure 5B). Last, no changes took place on the *GAPDH* promoter (Figure 5C), indicating that changes on *OCT4* and *NANOG* were specific.

In summary, histone modification changes detected on *OCT4* and *NANOG* regulatory regions after EC extract treatment are indicative of a remodeling of chromatin on these promoters to acquire an epigenetic state characteristic of pluripotent cells. Acetylation and demethylation of H3K9 occur in an EC extract-specific manner and are indicative of transcriptional activation of these genes.

Retinoic Acid Causes Remethylation of the OCT4 Promoter in Reprogrammed Cells

To ascertain the specificity of *OCT4* demethylation elicited by EC extract, we determined whether the promoter was responsive to induction of differentiation with RA. First, stimulation of NCCIT cells with 10 μM RA for 3 wk strongly down-regulated *OCT4* and *NANOG* expression and activated nestin (*NES*) transcription, an early marker of neuronal differentiation (Figure 6A). *OCT4* repression correlated with heavy DNA methylation in the PP (region 9) and DE (regions 2 and 3), establishing the responsiveness of these regions to RA in NCCIT cells (Figure 6B; compare with Figure 3A, NCCIT).

Second, stimulation of reprogrammed cells with RA starting 3 wk after extract treatment (experiments A and B) down-regulated *OCT4* expression (Figure 6C). Remarkably, remethylation of *OCT4* occurred in both batches of reprogrammed cells (Figure 6, D and E; see Table 2 for *t* test analyses). Note that only region 9 was examined here, because it was previously shown to be very responsive to EC extract treatment. *OCT4* remethylation in reprogrammed cells occurred to the same extent as in RA-treated NCCIT cells (Figure 6E and Table 2). In contrast, reprogrammed cells kept in culture for 6 wk without RA maintained a relatively hypomethylated profile, in agreement with elevated *OCT4* mRNA levels (Figure 6, C–E). Finally, as anticipated, *OCT4* remained highly methylated in 293T extract-treated cells exposed to RA (Figure 6E). We concluded that demethylation of *OCT4* elicited by EC extract treatment is a functionally significant epigenetic response, because it can be reverted by induction of differentiation.

DISCUSSION

This report demonstrates the epigenetic reprogramming of *OCT4* and *NANOG* as a result of transient treatment of 293T epithelial cells with extract of EC cells. We previously reported an nonquantitative assessment of demethylation of eight CpGs in EC extract-treated cells within *OCT4* region 5 in the PE (Taranger *et al*., 2005). We now show mosaic CpG demethylation throughout the *OCT4* regulatory region and in the *NANOG* promoter. Targeted *OCT4* and *NANOG* demethylation is specific for EC extract, and it does not occur in 293T extract-treated cells. *OCT4* demethylation is physiologically relevant, because it is associated with activation of the gene, whereas RA-mediated differentiation elicits its remethylation along with transcriptional repression. DNA demethylation is accompanied by methylation and acetylation changes on lysines 4, 9, and 27 of histone H3 on the *OCT4* PP, PE, and DE as well as on the *NANOG* promoter, to create a chromatin configuration compatible with transcriptional activation.

Reprogramming of DNA Methylation on OCT4 and NANOG Regulatory Regions

EC extract-induced demethylation produces mosaic methylation profiles on *OCT4* and *NANOG* upstream regulatory sequences. On the basis of previous immunological observations of Oct4 protein expression (Taranger *et al*., 2005), not all cells are expected to be reprogrammed to the same extent. Our results are reminiscent of partial demethylation of *Oct4* and *Nanog* in mouse fibroblasts constitutively overexpressing Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). Apparent partial reprogramming in our system may be due the examination of a heterogeneous cell population, or assuming that enzyme(s) causing demethylation originate from the EC extract, to a restricted enzyme access to target sequences. This may be alleviated by incubation of purified nuclei or deproteinized chromatin, which in *Xenopus* eggs accelerates demethylation (Simonsson and Gurdon, 2004), rather than cells. Interestingly, reprogramming of *OCT4* methylation is as efficient in somatic–ES cell hybrids (Cowan *et al*., 2005) as by nuclear transplantation (Simonsson and Gurdon, 2004), two situations where nuclei are directly exposed to the putative reprogramming factors.

Reprogramming of *OCT4* DNA methylation by extract treatment is targeted to specific, nonrandom areas, which may be more sensitive to demethylation. The most significant DNA demethylation detected occurs in regions 2 and 3 in the DE and in region 9 in the PP. Regions 2 and 3 encompass putative elements for transcription factors, including COUP-T, MZF1, GATA-2, HNF4, and three Sp1 elements. Region 9, surrounding the TSS, covers several MZF1, ADR1 (whose promoter binding is promoted by loss of histone deacetylation; Verdone *et al*., 2002), HSF, GATA-1, GATA-2, and Sp1 elements (www.cbrc.jp/htbin/nph-tfsearch). The *NANOG* promoter region demethylated by extract treatment is rich in putative HSF; ADR1, CdxA, AP-1/4, IRF-1, Cap, and c-Rel elements. Whether all these elements are involved in transcription activation and whether methylation modulates their binding to DNA remain uncertain. Nonetheless, DNA demethylation, together with hyperacetylation and hypomethylation of H3K9 (see below), contribute to loosening chromatin structure and thereby to the binding of transcription factors.

Demethylation in the *OCT4* PP (region 9) took place within 1 wk of extract treatment and was slightly enhanced by week 2. By week 1, extract-treated cells have undergone three rounds of replication (cells are quiescent for the first

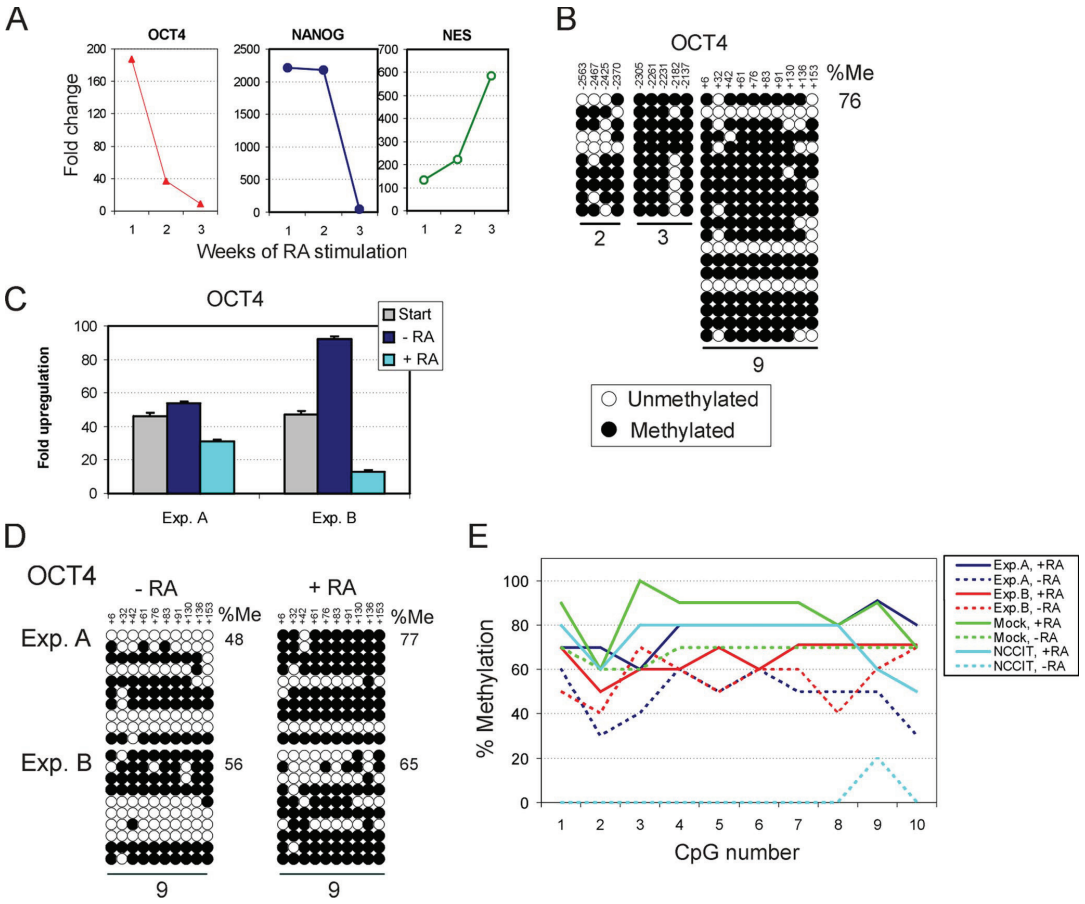


Figure 6. Retinoic acid induces remethylation of the *OCT4* promoter in EC extract-treated cells. (A) Real-time RT-PCR analysis of *OCT4*, *NANOG*, and *NES* expression in RA-stimulated NCCIT cells (mean of triplicate PCRs; error bars negligible). (B) DNA methylation of *OCT4* regions 2, 3, and 9 in NCCIT cells after 3 wk of RA stimulation. (C) Real-time RT-PCR analysis of *OCT4* expression in EC extract-treated cells (experiments A and B) 3 wk after exposure to extract (Start) and after one additional week of culture with 0 (−RA) or 10 μM RA (+RA). (D) Methylation of *OCT4* region 9 in EC extract-treated cells exposed for 3 wk to RA, starting 3 wk after extract exposure as described in C. (E) Percentage of methylation of individual CpGs in indicated cell types. For *t* test analyses, see Table 2.

48 h after extract treatment), so whether reprogramming-associated demethylation is a replication-dependent process or an active replication-independent process remains unknown. This also holds true for cell fusion or transduction reprogramming strategies, because clonal selection of the reprogrammed cells occurs before analysis (Cowan *et al.*, 2005; Takahashi and Yamanaka, 2006). However, reprogramming of *Oct4* methylation in *Xenopus* oocytes occurs in the absence of replication, transcription, or protein synthesis (Simonsson and Gurdon, 2004), and as such, it may involve active demethylating activity. Interestingly, the Aid/Apobec 1 members of the family of 5-methylcytosine deaminases are expressed in a cluster containing *Nanog* and other pluripotency genes in oocytes, embryonic germ cells, and ES cells (Morgan *et al.*, 2004), three cell types known to be able to reprogram somatic genomes (Tada *et al.*, 1997, 2001; Wilmut *et al.*, 2002; Gurdon *et al.*, 2003). Activity of Aid/Apobec deaminases results in C→T transitions in methylated DNA, or to demethylation in connection with repair of the T:G mismatch (Morgan *et al.*, 2004). As such, Aid/Apobec deaminases may play a role in epigenetic reprogramming.

Retinoic acid stimulation of the reprogrammed cells promotes *OCT4* remethylation and transcriptional down-regulation. Down-regulation of *OCT4* is expected to correlate with the establishment of a repressive chromatin structure as demonstrated previously in NCCIT cells (Dahl and Collas, 2007), and with the dissociation of transcription regulators from the PP (Minucci *et al.*, 1996) to ensure long-term silencing. Our data indicate that in reprogrammed cells, the *OCT4* locus behaves epigenetically as in NCCIT cells, in which *OCT4* is fully methylated in regions 2, 3 (DE), and 9 (PP) after RA stimulation (Figure 6; also see Deb-Rinker *et al.*, 2005). So, the (partially) demethylated *OCT4* promoter in reprogrammed cells retains the ability to undergo further ad hoc epigenetic modifications upon differentiation.

Table 2. *t* test analysis of the numbers of methylated CpGs in the *OCT4* proximal promoter (region 9) between extract-treated cells, 293T cells, and NCCIT cells exposed to 0 or 10 μM retinoic acid

Comparison of nos. of methylated CpGs between cell types (p values)[a]							
Exp. A		Exp. B		293T extract		293T	NCCIT
+RA	−RA	+RA	−RA	+RA	−RA	+RA	+RA
$p < 0.0001$	X						
$p > 0.1$				X		X	X
		$p = 0.04$	X				
		$p < 0.05$		X		X	
		$p = 0.147$					X
				$p < 0.01$	X		X
				$p = 0.621$		X	
	$p < 0.0001$				X		
			$p = 0.004$		X		
						$p = 0.15$	X

[a] Unpaired *t* tests. Data expressed as two-tailed p values with X referring to treatment or cell type with which comparison is made. Cells not exposed to RA (−RA) were analyzed 6 wk after EC extract (exp. A and exp. B) or 293T extract treatment. Cells treated with RA were exposed to 10 μM RA for 3 wk starting 3 wk after extract treatment. 293T and NCCIT cells were also exposed to 10 μM RA.

Remodeling Chromatin through Posttranslational Modifications of Histone H3 on OCT4 and NANOG

The most prominent histone modification specifically elicited by EC extract on the *OCT4* PP, PE, and DE and on the *NANOG* promoter is acetylation of H3K9. This takes place in the context of minimal hypermethylation of already di- and trimethylated H3K4, which mark genes either transcriptionally active (H3K4m3) or competent for transcription (H3K4m2) (Santos-Rosa *et al*., 2002). These changes are consistent with chromatin remodeling on the *Oct4* promoter in mouse thymocyte–ES cell hybrids, except for the marked trimethylation of (initially unmethylated) H3K4 detected in the thymocyte nuclei (Kimura *et al*., 2004). Transcriptional activation of *Oct4* in mouse fibroblasts treated with ES cell extract requires ATP hydrolysis, most likely for nuclear import of transcription factors (Håkelien *et al*., 2002; Landsverk *et al*., 2002) and for the activity of chromatin remodeling complexes (Kingston and Narlikar, 1999; Aalfs *et al*., 2001). *Oct4* activation by ES cell extract or *Xenopus* egg extract also requires the Brg1 subunit of the SWI/SNF complex (Hansis *et al*., 2004; Taranger *et al*., 2005). Promoter-specific targeting of SWI/SNF may involve H3K4 (hyper)methylation on *OCT4* and *NANOG* and prime the loci for further transcription-permissive remodeling (H3K4 methylation per se is not sufficient to allow transcription as H3K4 is methylated on both *OCT4* and *NANOG* in 293T cells). This additional remodeling presumably occurs by recruitment of histone acetyl transferases, whose activity results in the marked acetylation of H3K9 on *OCT4* and *NANOG*. Therefore, a key reprogramming event is relaxation of chromatin to create a conformation compatible with transcriptional activation.

Trimethylation of H3K27 is a facultative heterochromatin mark that promotes the recruitment of Polycomb group proteins for gene silencing (Cao *et al*., 2002; Czermin *et al*., 2002; Orlando, 2003). Interestingly, in ES cells, H3K27m3 can also mark transcriptionally silent, albeit acetylated, promoters for activation upon differentiation (Azuara *et al*., 2006). We detected some H3K27m3 demethylation on *OCT4* in EC extract-treated cells but essentially none on NANOG. Because both promoters are acetylated on H3K9, this again illustrates the heterogeneity of the cell populations examined and suggests that the loci have been partially reprogrammed. To support this view, these modifications were not as prominent as in NCCIT cells, in which both genes are transcribed at a higher level than in reprogrammed cells. Notably, H3K27m3 seems to be fully demethylated in thymocyte–ES cell hybrids, suggesting a more extensive chromatin remodeling in this system (Kimura *et al*., 2004). Globally, however, the changes reported as a result of EC extract treatment reflect a remodeling of chromatin on *OCT4* and *NANOG* indicative of a transition from a potentially active to an active promoter.

Our results indicate that the primary epigenetic determinants of *OCT4* and *NANOG* reprogramming by EC cell extract are DNA demethylation, and acetylation and demethylation of H3K9. Demethylation of H3K9m2 clearly occurs; however, trimethylated histone marks tend to remain more stable: demethylation of H3K9m3 or H3K27m3 was less pronounced than that of H3K9m2. Interestingly, modulation of repressive histone modifications such as H3K9 trimethylation is a feature of fertilized embryos, which is also not faithfully reproduced by somatic cell nuclear transfer (Santos *et al*., 2003). Furthermore, nuclear transplantation into *Xenopus* oocytes has shown that, indeed, repressive complexes do not readily disassemble (Kikyo and Wolffe, 2000). It is clear, therefore, that demethylation of trimethylated repressive histone marks remains a limiting factor in nuclear reprogramming, irrespective of the approach. Identification of the molecular mechanism driving histone demethylation (Shi *et al*., 2004; Armstrong *et al*., 2006; Schneider and Shilatifard, 2006) is likely to constitute a significant step toward improving nuclear reprogramming efficiency.

ACKNOWLEDGMENTS

We thank Lidja Stijac for assistance with bisulfite sequencing. This work was supported by the Research Council of Norway (FUGE, STORFORSK, YFF, and STAMCELLE programs).

REFERENCES

Aalfs, J. D., Narlikar, G. J., and Kingston, R. E. (2001). Functional differences between the human ATP-dependent nucleosome remodeling proteins BRG1 and SNF2H. J. Biol. Chem. *276*, 34270–34278.

Antequera, F. (2003). Structure, function and evolution of CpG island promoters. Cell Mol. Life Sci. *60*, 1647–1658.

Armstrong, L., Lako, M., Dean, W., and Stojkovic, M. (2006). Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. Stem Cells *24*, 805–814.

Azuara, V. *et al*. (2006). Chromatin signatures of pluripotent cell lines. Nat. Cell Biol. *8*, 532–538.

Blelloch, R., Wang, Z., Meissner, A., Pollard, S., Smith, A., and Jaenisch, R. (2006). Reprogramming efficiency following somatic cell nuclear transfer is influenced by the differentiation and methylation state of the donor nucleus. Stem Cells *24*, 2007–2013.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039–1043.

Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce de Leon, F., and Robl, J. M. (1998). Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. Nat. Biotechnol. *16*, 642–646.

Cowan, C. A., Atienza, J., Melton, D. A., and Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science *309*, 1369–1373.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell *111*, 185–196.

Dahl, J. A., and Collas, P. (2007). Q^2ChIP, a quick and quantitative chromatin immunoprecipitation assay unravels epigenetic dynamics of developmentally regulated genes in human carcinoma cells. Stem Cells Feb. 1 [Epub ahead of print].

Deb-Rinker, P., Ly, D., Jezierski, A., Sikorska, M., and Walker, P. R. (2005). Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. J. Biol. Chem. *280*, 6257–6260.

Feldman, N., Gerson, A., Fang, J., Li, E., Zhang, Y., Shinkai, Y., Cedar, H., and Bergman, Y. (2006). G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. Nat. Cell Biol. *8*, 188–194.

Gurdon, J. B., and Byrne, J. A. (2003). The first half-century of nuclear transplantation. Proc. Natl. Acad. Sci. USA *100*, 8048–8052.

Gurdon, J. B., Byrne, J. A., and Simonsson, S. (2003). Nuclear reprogramming and stem cell creation. Proc. Natl. Acad. Sci. USA *100* (suppl 1), 11819–11822.

Håkelien, A. M., Landsverk, H. B., Robl, J. M., Skålhegg, B. S., and Collas, P. (2002). Reprogramming fibroblasts to express T-cell functions using cell extracts. Nat. Biotechnol. *20*, 460–466.

Hansis, C., Barreto, G., Maltry, N., and Niehrs, C. (2004). Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. Curr. Biol. *14*, 1475–1480.

Kikyo, N., and Wolffe, A. P. (2000). Reprogramming nuclei: insights from cloning, nuclear transfer and heterokaryons. J. Cell Sci. *113*, 11–20.

Kimura, H., Tada, M., Nakatsuji, N., and Tada, T. (2004). Histone code modifications on pluripotential nuclei of reprogrammed somatic cells. Mol. Cell. Biol. *24*, 5710–5720.

Kingston, R. E., and Narlikar, G. J. (1999). ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. Genes Dev. *13*, 2339–2352.

Lachner, M., and Jenuwein, T. (2002). The many faces of histone lysine methylation. Curr. Opin. Cell Biol. *14*, 286–298.

Landsverk, H. B., Håkelien, A. M., Küntziger, T., Robl, J. M., Skålhegg, B. S., and Collas, P. (2002). Reprogrammed gene expression in a somatic cell-free extract. EMBO Rep. *3*, 384–389.

Minucci, S., Botquin, V., Yeom, Y. I., Dey, A., Sylvester, I., Zand, D. J., Ohbo, K., Ozato, K., and Scholer, H. R. (1996). Retinoic acid-mediated down-regulation of Oct3/4 coincides with the loss of promoter occupancy in vivo. EMBO J. *15*, 888–899.

Morgan, H. D., Dean, W., Coker, H. A., Reik, W., and Petersen-Mahrt, S. K. (2004). Activation-induced cytidine deaminase deaminates 5-methylcytosine in DNA and is expressed in pluripotent tissues: implications for epigenetic reprogramming. J. Biol. Chem. *279*, 52353–52360.

Munsie, M. J., Michalska, A. E., O'Brien, C. M., Trounson, A. O., Pera, M. F., and Mountford, P. S. (2000). Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. Curr. Biol. *10*, 989–992.

Orlando, V. (2003). Polycomb, epigenomes, and control of cell identity. Cell *112*, 599–606.

Pells, S., Di Domenico, A. I., Callagher, E. J., and McWhir, J. (2002). Multipotentiality of neuronal cells after spontaneous fusion with embryonic stem cells and nuclear reprogramming in vitro. Cloning Stem Cells *4*, 331–338.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, e45

Santos, F., Zakhartchenko, V., Stojkovic, M., Peters, A., Jenuwein, T., Wolf, E., Reik, W., and Dean, W. (2003). Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. Curr. Biol. *13*, 1116–1121.

Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emre, N. C., Schreiber, S. L., Mellor, J., and Kouzarides, T. (2002). Active genes are tri-methylated at K4 of histone H3. Nature *419*, 407–411.

Schneider, J., and Shilatifard, A. (2006). Histone demethylation by hydroxylation: chemistry in action. ACS Chem. Biol. *1*, 75–81.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A., and Shi, Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941–953.

Simonsson, S., and Gurdon, J. (2004). DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. Nat. Cell Biol. *6*, 984–990.

Spencer, V. A., Sun, J. M., Li, L., and Davie, J. R. (2003). Chromatin immunoprecipitation: a tool for studying histone acetylation and transcription factor binding. Methods *31*, 67–75.

Tada, M., Tada, T., Lefebvre, L., Barton, S. C., and Surani, M. A. (1997). Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. EMBO J. *16*, 6510–6520.

Tada, M., Takahama, Y., Abe, K., Nakastuji, N., and Tada, T. (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr. Biol. *11*, 1553–1558.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Taranger, C. K., Noer, A., Sorensen, A. L., Hakelien, A. M., Boquest, A. C., and Collas, P. (2005). Induction of dedifferentiation, genome-wide transcriptional programming, and epigenetic reprogramming by extracts of carcinoma and embryonic stem cells. Mol. Biol. Cell *16*, 5719–5735.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E., and Scott, E. W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416*, 542–545.

Verdone, L., Wu, J., van Riper. K., Kacherovsky, N., Vogelauer, M., Young, E. T., Grunstein, M., Di, M. E., and Caserta, M. (2002). Hyperacetylation of chromatin at the ADH2 promoter allows Adr1 to bind in repressed conditions. EMBO J. *21*, 1101–1111.

Wakayama, T., Tabar, V., Rodriguez, I., Perry, A. C., Studer, L., and Mombaerts, P. (2001). Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science *292*, 740–743.

Warnecke, P. M., Stirzaker, C., Song, J., Grunau, C., Melki, J. R., and Clark, S. J. (2002). Identification and resolution of artifacts in bisulfite sequencing. Methods *27*, 101–107.

Wilmut, I., Beaujean, N., De Sousa, P. A., Dinnyes, A., King, T. J., Paterson, L. A., Wells, D. N., and Young, L. E. (2002). Somatic cell nuclear transfer. Nature *419*, 583–586.

Ying, Q. L., Nichols, J., Evans, E. P., and Smith, A. G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545–548.

Supplementary Fig. 1

OCT4 genomic sequence GenBank No. AJ297527

Translation initiation start codon
Area covered by bisulfite primer pair: Forward primer; reverse primer; overlapping primer (3' end of reverse/5' end of forward
Green: Exons (mRNA sequence GenBank No.NM_002701)
t Transcription start site (TSS)
CpG examined

```
-3204 ggagaggtgg gcctcacctg ggtcccctgg cagtgctctg tgaagggtct tgacattgca
-3144 cctgtaataa taaaggtgtg tgtgaagtat ctttttatgg tgactttcta aaacccaggg
-3084 aatcatggga ccagttctga tgactcagcc tggcttccag tctcctccag gcccaacggt
-3024 ggcccccagc actggttggg gcctgggaga gctggccttg gctgaagtga agccacctac
-2964 ccttcaggca taacaggaca gtgagaagga aggaaagcct gcctcaacct cccatcagcc
-2904 ctgagcaccc cagaaggggg ccggctagga gtctaggcat gcaggaggct gacccctgac
-2844 tgggctcata tccagccaca aggcagccag ggatccaggc acccacccct tgtctgcgtc
-2784 cctctcggga atgggcctct tgcccaggcc agaaatacac cacctacagt acaaattata
-2724 atctaaaaac aagagggtgg tgttgagtgg ggaaattggg gaaggtgttt taggagccac
-2664 taggaaaatg ggcagcaggg actctctgga ctggcttggg aagagcgctt ttggggaacc
-2604 tggaggatgg caagctgaga aacactggtg tggagattcc agccaaatcc caggcctgcc
-2544 cctccccctc ctctgagagg ccgtcttctt ggcagacagc agagagatgc atgacaaagg
-2484 tgccgtgatg gttctgtcct ggggattgag atggctgggg aggggcctcc tcctgttccg
-2424 aagcatgttc ctcccacccc caccaggccc cataatctac gctgccttttg ggcattaaa
-2364 ggccgagaag tgaacacagc tgcaacccca ctgccttgta gaccttccgg cagacctgtg
-2304 gcaggtattg aaatgcacgc atacaattag gctcaaaaag tctacacaga caggagatgg
-2244 gcacacgaac agaggcaaca taagagtggg ggaaaagtct caaaagactc acggatgcca
-2184 ccaagatgaa gacagctggc cacggacac ccatcccctt agaaggcaga tagagccact
-2124 gaccccagca gacaagccca ggcagggctg agcctggagc ctgcaatgag aagccttact
-2064 taagtcgaca gaggtcagcg tgcccagtcc agacctggcc ttctggcctt cgaagctgtg
-2004 gggagccctg gcccagagcc ccctctggag cccccagact taccccaggc cctccactga
-1944 gatcaagttt tgggagcaga cagacaaaca tcatccctca cagacaggca ttccgttggc
-1884 tattctcttg caaacagaat caagcactag accagcagca tgagcctcag gatactcagg
-1824 ccaggcccag aaaaacagac cctgaagggg agcttaggc agccttcctg cacgcctcca
-1764 caaatcactc tccacctcct ctgcgtcttt ctgccagcca gccccactaa acaaagcaca
-1704 tccctcaatc tgccaggctc ggggagggac gcacgatgaa gctggacgcc tgagtccccc
-1644 agaggaagga ggaactagat acctaggtcc ctgtgggggg cccttggtgc ccgtctgagg
-1584 ctcagtcttt gaggggattg cagaggggcg ttgctggagc tccttttagc gtctctgaag
-1524 gggattctgt gtgaggggat tgggactggg gggttgggga gcaggaagca gtccccaggg
-1464 gagccatcca ggcccattca agggttgagc acttgtttag ggttagagct gccccctctg
-1404 gggaccggga ttgtccagcc aaggccattg tcctgccccc ttcccccagt ccctcccagg
-1344 cttctttgaa cctgaagtca gatatttttt ctccacaccc cccacccccc ggttttcccc
-1284 acccagggcc tagggctgga ggcctgggcc agggaggtgg gggagggaga acggggccta
-1224 ccgtggtatt agatgtctga gttttggttg agaggggagc aaggaacctg atgtgcaggt
-1164 tccatagtgg agggggccca aagcgggtgt cttatcactc tgtttcagca aaggttggga
-1104 aactgaggcc cagtcagtcc aaagtctggt cccttgaagg ggaagtaggg accaacccct
-1044 tagtctgtta gatgaggaga gtctggagtc tgattctgga agacggaggg gtggggggat
 -984 ggggggggtgg ggggatatag cacggaggcc ttgtctggca gtctactctt gaagatgggg
 -924 tgaaatttgg caggctgggc agatggtgcc aggcacccag gctgcggggt ggctggattt
 -864 ggccagtatc gggatgggaa tgcctaggat tctggatgga tcgggggaag gcataaggga
 -804 gcagctggcc attgtgctta tggctgttga tgcattgagg gatagcgcca cacacacatt
```

Supplementary Fig. 1 (cont.)

Supplementary Fig. 1 (cont.)

NANOG Ensembl Gene ID: ENSG00000111704
http://www.ensembl.org/Homo_sapiens/geneseqview?db=core&gene=ENSG00000111704&flank5_display=3056&flank3_display=800&exon_display=core&exon_ori=fwd&snp_display=off&line_numbering=off&submit=Update

Translation initiation start codon
Area covered by bisulfite primer pair; forward primer; reverse primer
Green: Exon (mRNA sequence GenBank No.NM_024865)
g Putative transcription start site (TSS)
CpG examined

```
-3204 tccatctcaa aaaaaaaaaa aaaaaaattg ccctcacaga gcttatattc tagtggtagt
-3144 agggggataa atcagatagg tagctagcta gctagctaga taaatagata gatagacaga
-3084 tagtatatta cctgtgtcgc ctggccaaag ggtaggttca catttggcat gtgtgtcaac
-3024 tcaagctgca cttgaccttt cctaatcatc tccaacacta cctattgccc tatccaaatc
-2964 ctatcacttg aattattcaa aaagtctccc atcttcattt tggtgggatt tggtcagctc
-2904 ctttactgca acctgtttta tcaggaaggt ctttatgacc tgtattttgt gctgacctcc
-2844 tatctcatcc tatgacttag aacaccttaa ccatctggga atgcagccca gtaggtttca
-2784 gcctcatttt acccagctcc tattgaagat ggagtcgttc tggttcacac agctctgata
-2724 gtaatctgct cactaagtgt tagttgccgt gttctctccc acaactccct cctgttgcac
-2664 ccctgccttt gagtcctgct aaacaacagt attggaaccc atcattttct ctcatgcctt
-2604 tacccaaact gttagttttc tggtattccc ttctctccac atttttcctt ctcagatcac
-2544 tgaggatcgt ccaatcttcc ctgcccatcc ctcccctacc ccaacctcca ttatttgtac
-2484 tctcttagca tccagtgcac tttttttcct tacttattac acaactttac acttacaatt
-2424 aacattataa ttgtcttttt ggtctatttg atctactcta ctaaatcttt ttttttttt
-2364 ttttgagaca aggtctcact gtgcctcctc ggctggagtg cagtggcgtg atctcggctc
-2304 gctgcaacct ccgcctctgg agttcaagtg attctcctgt ctcagcctcc ctagtagatg
-2244 ggattacagg cgtgcaccac cacgcccagc ccagttaatt tttgtatttt tagtacagac
-2184 agggtttcac catgttggtc aggctggtct tgaactcctg atctcagatg atgcacctgg
-2124 ctcggcctcg caaagtgctg ggattacagg tgtgagccac cgtgcccagc cgttagctca
-2064 ttttaacaca tccttagtcc agcctgttcc aaaaaatcta aagtcagata gcttcctaaa
-2004 cctcaacttt attccaattg ctttccttgg cgaagaatgt agtaagtcgg ccttccagcc
-1944 accagcccct tccctttggt ctttcactcc ggaggctctt accctagaca caatgggaca
-1884 gggagcgggg gatgggggaa ttcagctcag gcttttatgc aaagaccccc ttctgcaaag
-1824 aacaaagctt ctggtacctg ccctttggag agctgcgggc aagctcagcc tcggtgagtc
-1764 ttggtggcct tgacagcccc cacttaacaa actgtgctga ttaagagaga caggagggca
-1704 agtttttccc ttctttaaa gaaatcatcc tatttcctac gagacataga ctatctgcct
-1644 gaagcatgat gtactagccc cactcacgg ctccctgatg cccctatgct taatcttctc
-1584 cggaatggta gtctgagaag aaaaaagatt acgcccaatt tcatttcctt gtttcacatc
-1524 aagcaatact tttcgagtct ttgcattgtg aacaaaagtc agcttgtgtg ggagcaaagc
-1464 cagctgctct gggtgcagac ccaggagcag agtgcagagg agaatgagtc aaagagtttt
-1404 gtcttcaaaa attacataat cgggatttgc taagagttta cttttcggta tggaagactg
-1344 gaaaagagaa agaaatctta ggtttcttga atgttgggtt tgggaatagg aaggaaaatc
-1286 gaaaactgta gactttgtcc ataaatgtta gtgctggaac cccactctaa aaactttgtt
-1224 cctttggaaa acacctccct tcccccagaa acacacacac ccacacgaga tgggcacgga
-1164 gtagtcttga aagacatgac aaatcaccag acctgggaag aagctaaaga gccagaggga
-1104 aaaagccaga agtcgactac ctgggaggag ggatagacaa gaaaccaaac taaaggaaac
-1044 taaggtaggt gctgaaaaca agtaccattt tcaacattaa ctgatgcctt ggcttcatgc
 -984 tataatgcca tgttgtgttt cactataacc tcagagtgaa tgaaagagga aaatggagct
 -924 agttgaaatt tctgcctaaa ctagccagat tttgagacac taagttatct caaatcaaga
 -864 aatcacccta atgagaattt caataacctc aggaatttaa ggtgcatgca tccccccacc
 -804 ccccctttt tttttgagac gtagtcccgc tctgttgccc aggctggagt acagtggcgc
 -744 gatatcggct caccacaacc tctgcctccc aggttcaagg gattctcccg cctcagcttc
```

Supplementary Fig. 1 (cont.)

Supplementary Fig. 1 (cont.)

LMNA genomic sequence GenBank No. AL135927

Proximal promoter (Genbank No. AL 003955)
Translation initiation start codon
Area covered by bisulfite primer pair; forward primer; reverse primer
Green: Exon 1 (mRNA sequence GenBank No. NM_170707)
g Putative transcription start site (TSS)
CpG examined

```
-2606 gaattcaagg ttgcagtgag ctatgatcct gccaccgcac tccagcctgg gcgacagacc
-2546 aagattctca aaaaaagagc ggggaggtct aggacaggaa gggttaagtg tggtttagct
-2486 ttcccagcct ggaaaggaag ccaggccaag cagctgggta ggggaaaagg gggcactgag
-2426 tgccagggag gggctgaggc agtgggaaca gcatgaaccc ttccctcacg tttcactggc
-2366 ttacccctcc tgcctctgct tctggtgtgg agaagaacaa gacttttTat tgtctgggaa
-2306 gctgagggtg ggtgccacgc cctgggggag agaagccata ttctgggccc cctcatccgg
-2246 gtgcatccct agtgctcacc atgctgccca ggcaccctca ctgagatgag aactttcctt
-2186 gttcccctcc cctaataacc aaagttcctg ccttaatccc actccagtcc ctcatccacc
-2126 ccgaggccag gcttcagaac ccagaactga gggcctgtcc agccctgctt tccttgtgtt
-2066 tgagggaagc cctgatatct tggagctgta caaggtagca acataatttg atttccctgg
-2006 ttgctcatcg gaggataagc tgtgggaggc agttgggcag ggcatgcagg cagatgggac
-1946 ccaggcctca atgctgtcac ctcttagaga ggataaggtg ggacgggcat ggtggctcac
-1886 atctgtaatc ccagaacttt gggaagctga ggcaggcaga ttacctgagg tcgggagttt
-1826 gagaccagtt tggccaacat ggtgaaaccc catctctact aaaaataaaa ataaaaaaaa
-1766 aattagctgg gcttggtggc aggtgcctat aatcccagct actcaggagg ctgaggcagg
-1706 agaatctctt gaacccggga ggtggagatt gcaatgagct gagatcatgc cactgcactc
-1646 cagcctgggt gagagagcaa gacttaatct caaaaaaaca aacaaacaaa caaaaaaaac
-1586 aaacaaacag aggatgaggt ggatggtggg accaggatag gggccaggat gaggggaggg
-1526 gagtggaagg cttatcaaaa gggtccttgg tgaggcctga ggatggaggc tccagagcct
-1466 gaggtagcga accctgggga cctgagtgat ctcgttttgt gagagagcct ggcccctccc
-1406 cagagccagc tgcagaatgg acctggccag agaggaaagt agagatgagt atggtgctgg
-1346 cctttgcagc cagcaaggct gtggggtctt tgctgttcct gtcccccact accttcttgc
-1286 cccccactac cttctttctg gctgaaacca ggatagagac ccaacattgg ctgtccagcc
-1226 cccagccctg ctccccctTt caggcccctc tgggaaccac agaaatctgg gacctagtgt
-1166 cttggcaacg taatgaatgc atgcacagct ctggtatctg ttttaaatta tccattaaaa
-1106 taagtacagt tctgggggaa aaaaataagt tgactgggtg cggtaagcag tagaaaggga
-1046 ctgaaggggg aaggaaccca ggcagtgctt gggtccaaag gaaggggaca ggagatggaa
 -986 ggggcagtgc ctggctccta ttcttggctt tctttagggg acttctttag gggactgtgg
 -926 cttgttgctt gggtctaaaa acgaatgctt ggctttgaag agagatagat tggggcaaaa
 -866 gaaagaaaaa aagggacccc ccaaactcct tgatccctgg ccccaaactg ggggcataaa
 -806 ggaactcagg ttccagaact ttgctccccc cagggaaccc aggcattcct tctccacccc
 -746 actcctggca cactgagatg cagctctgaa tgggctgccc acgtgtggag gggggttggg
 -686 gtgactcact attactactg ggaggacagg gggagccagt ggtggaagaa gggtgagtca
 -626 cactgatggg caccagcctc agccctcccc ccactttcct ggctcccagc cctgcctacc
 -566 tgaccctctc ccttgctttg cgcccacttc cctctctttc tccccgaccc ttttgcccac
 -506 ccactctccc tccttggctc tgccctctag cccagaaggt ctgaggcaat gggggcaagc
 -446 ttggagccga cagtgctgag caggcaggag ccaagagagg ggaagcttga gcctcacgca
 -386 gttaggggtg cgctggagag ggtggggccc gactccgcca cacccaacg gtccttcccc
 -326 ctcctcacca ctccCgcccc caccccaaat ggatctggga ctgccccttt aagagtagtg
 -266 gcccctcctc ccttcagagg aggacctatt agagcctttg ccccggcgtc ggtgactcag
 -206 tgttcgcggg agcgccgcac ctacaccagc caacccagat cccgaggtcc gacagcgccc
 -146 ggcccagatc cccacgcctg ccaggagcaa gccgagagcc agccggccgg cgcactccga
  -86 ctccgagcag tctctgtcct tcgacccgag ccccgcgccc tttccgggac ccctgccccg
  -26 cgggcagcgc tgccaacctg ccggccatgg agaccccgtc ccagcggcgc gccacccgca
   34 gcggggcgca ggccagctcc actccgctgt cgcccacccg catcacccgg ctgcaggaga
   94 aggaggacct gcaggagctc aatgatcgct tggcggtcta catcgaccgt gtgcgctcgc
  154 tggaaacgga gaacgcaggg ctgcgccttc gcatcaccga gtctgaagag gtggtcagcc
  214 gcgaggtgtc cggcatcaag gccgcctacg aggccgagct cggggatgcc cgcaagaccc
  274 ttgactcagt agccaaggag cgcgcccgcc tgcagctgga gctgagcaaa gtgcgtgagg
  334 agtttaagga gctgaaagcg cggtgagttc gcccaggtgg ctgcgtgcct ggcggggagt
```

Supplementary Table 1. Bisulfite sequencing and ChIP primers used in this study.

Gene name	Primer pair	Forward primer (F) Reverse primer (R)	Seq. coverage relative to TSS	Annealing temp. (°C)
Bisulfite sequencing primers				
OCT4	Oct4-1	F: TTTTTAGTTTTTTTTAGGTTTAA[a] R: TAAACAAAAAACCCATTCCC[a]	-2995 to -2723	50
	Oct4-2	F: TTAGGAAAATGGGTAGTAGGGATTT[a] R: TACCCAAAAAACAAATAAATTATAAAACCT[a]	-2609 to -2417	58
	Oct4-3	F: ATTTGTTTTTTGGGTAGTTAAAGGT[a] R: CCAACTATCTTCATCTTAATAACATCC[a]	-2344 to -2126	58
	Oct4-4	F: GGATGTTATTAAGATGAAGATAGTTGG[a] R: CCTAAACTCCCCTTCAAAATCTATT[a]	-2136 to -1721	58
	Oct4-5	F: AATAGATTTTGAAGGGGAGTTTAGG[a] R: TTCCTCCTTCCTCTAAAAAACTCA[a]	-1755 to -1574	58
	Oct4-6	F: GAAGGGGAAGTAGGGATTAATTTT[a] R: CAACAACCATAAACACAATAACCAA[a]	-1014 to -720	58
	Oct4-7	F: TAGTTGGGATGTGTAGAGTTTGAGA[a] R: TAAACCAAAACAATCCTTCTACTCC[a]	-567 to -309	58
	Oct4-8	F: AAGTTTTTGTGGGGGATTTGTAT[a] R: CCACCCACTAACCTTAACCTCTA[a]	-215 to -29	58
	Oct4-9	F: GTTAGAGGTTAAGGTTAGTGGGTG[a] R: AAACCTTAAAAACTTAACCAAATCC[a]	-57 to +66	58
NANOG	Nanog-1	F: AGAGATAGGAGGGTAAGTTTTTTTT R: ACTCCCACACAAACTAACTTTTATTC	-1503 to -1254	58
	Nanog-2	F: GAGTTAAAGAGTTTTGTTTTTAAAAATTAT R: TCCCAAATCTAATAATTTATCATATCTTTC	-1203 to -911	58
	Nanog-3	F: TTAATTTATTGGGATTATAGGGGTG[a] R: AACAACAAAACCTAAAAACAAACC	-334 to -163	55
LMNA	Lmna	F: GAAGGGTGAGTTATATTGATGGGTAT R: ACTCTTAAAAAAACAATCCCAAATC	-277 to +92	58
ChIP primers				
OCT4	Oct4-A	F: GAGGATGGCAAGCTGAGAAA R: CTCAATCCCCAGGACAGAAC	-2546 to -2379	60
	Oct4-B	F: ACCCCACTGCCTTGTAGACCT R: CACGCTGACCTCTGTCGACTT	-2284 to -1986	60
	Oct4-C	F: AGCCCCACTAAACAAAGCAC R: GCAATCCCCTCAAAGACTGA	-1669 to -1508	60
	Oct4-D	F: GTTGGGGAGCAGGAAGCA R: GGGGCAGCTCTAACCCTAAA	-1435 to -1353	60
	Oct4-E	F: AGTCTGGGCAACAAAGTGAGA R: AGAAACTGAGGAGAAGGATG	-262 to -94	60
NANOG	Nanog	F: GTTCTGTTGCTCGGTTTTCT R: TCCCGTCTACCAGTCTCACC	-173 to -79	60
GAPDH	Gapdh	F: CTGAGCAGACCGGTGTCACATC R: GAGGACTTTGGGAACGACTGAG	-486 to -320	60

[a] From Deb-Rinker *et al*. (2005).

Supplementary Table 2. Real time RT-PCR primers used in this study.

Gene name	Forward primer (F) 5'→3' Reverse primer (R) 5'→3'	Annealing temp. (^{o}C)
GAPDH	F: TCGGAGTCAACGGATTTGGT	60
	R: TTGCCATGGGTGGAATCATA	
LMNA	F: CTGTGGTTGAGGACGACGAG	60
	R: TGCGGTAGCTGCGAGTGA	
LMNB1	F: AAGGCGAAGAAGAGAGGTTGAAG	60
	R: GCGGAATGAGAGATGCTAACACT	
NANOG	F: CAAAGGCAAACAACCCACTT	60
	R: TCTGCTGGAGGCTGAGGTAT	
NES	F: CACCTGTGCCAGCCTTTCTTA	60
	R: TTTCCTCCCACCCTGTGTCT	
OCT4	F: AAGCGATCAAGCAGCGACTAT	60
	R: GGAAAGGGACCGAGGAGTACA	

This article is removed.