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
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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 me the 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


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>2D PAGE</td>
<td>two dimensional polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>5aza</td>
<td>5′-azacytidine</td>
</tr>
<tr>
<td>ac</td>
<td>acetylation</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5′-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5′-triphosphate</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine mono phosphate</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>CpG</td>
<td>cytosine-phosphate-guanine</td>
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<tr>
<td>DRB</td>
<td>5,6-dichlorobenzimidazole</td>
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<td>EC cell</td>
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</tr>
<tr>
<td>EG cell</td>
<td>embryonic germ cell</td>
</tr>
<tr>
<td>ES cell</td>
<td>embryonic stem cell</td>
</tr>
<tr>
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</tr>
<tr>
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<td>gap 2</td>
</tr>
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</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GV</td>
<td>germinal vescicle</td>
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<tr>
<td>H</td>
<td>histone</td>
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</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>HDACi</td>
<td>histone deacetylase inhibitor</td>
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<tr>
<td>HCP</td>
<td>high CpG content promoter</td>
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<tr>
<td>HMG</td>
<td>high mobility group</td>
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<td>HMT</td>
<td>histone methyltransferase</td>
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<td>ICM</td>
<td>inner cell mass</td>
</tr>
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<td>ICP</td>
<td>intermediate CpG content promoter</td>
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<tr>
<td>Igf2</td>
<td>insulin-like growth-factor II</td>
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<td>iMS cell</td>
<td>induced multipotent stem cell</td>
</tr>
<tr>
<td>iPS cell</td>
<td>induced pluripotent stem cell</td>
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<td>LCP</td>
<td>low CpG content promoter</td>
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<tr>
<td>M</td>
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<td>metaphase II</td>
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<tr>
<td>me</td>
<td>methylated</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase</td>
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<td>mouse embryonic fibroblast</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MRL</td>
<td>Murphy Roths Large</td>
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<td>MSC</td>
<td>mesenchymal stromal cell</td>
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<tr>
<td>PcG</td>
<td>polycomb group</td>
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<td>polymerase chain reaction</td>
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<td>proximal enhancer</td>
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<td>primordial germ cell</td>
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<tr>
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<td>polymerase II</td>
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</tr>
<tr>
<td>POU</td>
<td>Pit-Oct-Unc</td>
</tr>
<tr>
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<td>polycomb repressive complex</td>
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<td>quantitative</td>
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<td>ribonuclease</td>
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<tr>
<td>RT</td>
<td>reverse transcriptase</td>
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<tr>
<td>SAHA</td>
<td>suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>SAM</td>
<td>s-adenosylmethionine</td>
</tr>
<tr>
<td>SCNT</td>
<td>somatic cell nuclear transfer</td>
</tr>
<tr>
<td>SLO</td>
<td>Streptolysin O</td>
</tr>
<tr>
<td>SSEA</td>
<td>stage-specific embryonal antigen</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TE</td>
<td>trophectoderm</td>
</tr>
<tr>
<td>TGF-E</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TRA</td>
<td>tumor rejection antigen</td>
</tr>
<tr>
<td>Trx</td>
<td>trithorax group</td>
</tr>
<tr>
<td>TSA</td>
<td>trichostatin A</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start site</td>
</tr>
<tr>
<td>VPA</td>
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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.
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 differentiation 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. 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).

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 \textit{OCT4} promoter, thus participating in the regulation of \textit{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.

\subsection*{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 characterized 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>
<thead>
<tr>
<th>Promoter class(^a)</th>
<th>Promoter activity</th>
<th>Methylation status</th>
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<tr>
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<td>Active Inactive</td>
<td>Unmethylated</td>
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<tr>
<td></td>
<td></td>
<td>No or weakly methylated</td>
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<tr>
<td>ICP</td>
<td>Active Inactive</td>
<td>Unmethylated</td>
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<td></td>
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<td>Methylated</td>
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<tr>
<td>LCP</td>
<td>Active Inactive</td>
<td>Unmethylated or methylated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unmethylated or methylated</td>
</tr>
</tbody>
</table>

\(^a\)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 \textit{OCT4} and \textit{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.

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**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 (Immuo-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 occurred 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 p21CIP1 and abolishes cell proliferation. Overexpression of c-Myc, however, is there to suppress expression of p21CIP1. 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).](image)

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-β 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

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

* 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. Initiate 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:


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-PolIII-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.
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:


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-treated 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 Wolffé, 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 target cell permeabilization 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). Furthermore, 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. Häkelien, unpublished observations), arguing that intracellular molecular crowding and/or maintenance of and isotonic osmotic 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 \textit{REX1} (\textit{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$_2$O). 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 survival 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$_2$O. 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 contain 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, which 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 pluripotency 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 complex 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 \textit{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 \textit{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 pluripotency-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 \textit{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 \textit{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 differentiation-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- to 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, whereas 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 earlier 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 concomitant 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).
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 fibroblasts. 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, virion assembly, viral DNA replication and alteration of the cell cycle (Sullivan and Pipas, 2002). Incidentally, 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 determine 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.
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DOI: 10.1091/mbc.E05–06–0572

The original publication is available at http://www.molbiolcell.org

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

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

This article was published online ahead of print in MBC in Press (http://www.molbiolcell.org) on September 29, 2005.

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 Pou1 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 (ECGs) 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...
promote cell cycle reentry and down-regulation of myogenic markers in differentiated myotubes (McCann 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 as-saying, expression of T-cell-specific genes and surface receptors, and fibroblasts (Håkelien et al., 2004), and a pneumoniae extract was recently shown to induce differentiation of ESCs into a pneumoniae phenotype (Qin et al., 2005). 293T cells were also shown to express pluripluripotency markers such OCT4 and GAPI, and down-regulate a kidney marker after coculture with extract of Xenopus eggs (Hansis et al., 2004). Despite these observations, evidence for induction of epige-netic reprogramming events in large numbers of cells by extracts is lacking. 

Teratocarcinomas are a particular type of germ cell tu-mors that contain undifferentiated stem cells and differentiated derivatives that can include endoderm, mesoderm, and ectoderm germ layers (Chambers and Smith, 2004). Undifferen-tiated 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 intro-duced into a blastocyst (Bleiloch et al., 2004). Undifferen-tiated 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 carci-noma (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 undifferen-tiated somatic cells can elicit de differentiation in a somatic cell line. Based on morphological and immunolabeling ob-servations, gene expression profiling, DNA methylation as-says, and functional assessments, we show that 293T and NIH3T3 cells can be programmed by extracts of undifferen-tiated NCCIT cells or mouse ES cells to acquire characteris-tics 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 ES cells were isolated from inner cell masses of strain avl129 blastocysts and plated on mouse fibroblast-laminated feeder layers in ESC medium (DMEM, 15% FCS, 0.1 mM β-mercaptoethanol, non-essential amino acids, and 1% penicillin/streptomycin) supplemented with 1000 U/ml of 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 RPMI 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 HEPS, pH 8.2, 50 mM NaCl, 5 mM MgCl2, 1 mM diethiothreitol, and protease inhibitors), sedimented at 400 × g, resuspended in 1 volume of cold 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 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 course material. The supernatant was aliquoted, frozen in liquid nitrogen, and stored for up to 9 mo at −80°C. Lysate of 95,583 ± 10,866 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 ± 0.4 (measured at 1 ml/ml of extract). ESC extracts (25–30 µg of protein) were similarly prepared from LIF-adapted ESC cultures. 293T, Jurkat, and NIH3T3 extracts were also pre pared as described above. If necessary, extracts were diluted with H2O before use to adjust osmolality to ≈300 mOsm.

SLO-mediated Permeabilization and Cell Extract Treatment

293T and 3T3 cells were washed in cold PBS and in cold Ca2+ and Mg2+–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 HBBS, tubes were placed in a H2O bath at 37°C for 2 min, and 2.3 µl of SLO (Sigma-Aldrich) (100 µg/ml stock diluted 1:10 in cold HBBS) was added to a final SLO concentration of 230 ng/ml. Samples were incubated horizontally in a H2O bath for 50 min at 37°C with occasional agitation and set on ice. Samples were diluted with 200 µl of cold HBBS, and cells were sedimented at 120 × g for 5 min at 4°C. Permeabilization was assessed by monitoring uptake of a 70,000-M, 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, Ger- many). The tube containing cells was incubated horizontally for 1 h at 37°C in a H2O bath with occasional agitation. To reseal plasma membranes, the extract was diluted with complete RPMI 1640 medium containing 2 mM CaCl2 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 (H 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).

PCRdDNA. First-strand cDNA was prepared as described previously (H et al., 2004) using a SMART PCRdDNA 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. PCRdDNA was purified with QIAquick columns (QIAGEN) and ethanol precipitation and dissolved in H2O.

Biotin-labeling of cRNA. Biotin-labeled cRNA was prepared from PCRdDNA using a T7 RNA polymerase MEGAscript T7 kit (Ambion, Austin, TX), purified with an RNeasy mini kit and fragmented as described (H 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 (H et al., 2004). Chips were washed, stained at 35°C for 15 min with a phycoerythrin-streptavidin 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 levels of a 5-µmol/L dehyde-dehyde 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′-GTGCTATGGGAACTGGAG-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 purified with a 12% polyacrylamide 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 system using IQ SYBR Green (Bio-Rad) and a Probelibrary probes (Exiqon, Vedbaek, 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-isoomylyalcohol extraction or by using the GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich). Bisulfite conversion was performed using the MethylEasy DNA bisulfite 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 and primer sequences for the PCR of LMNA, B, and OCT4 are given in Table S2.

Induction of Neural Differentiation
To generate neuronal derivatives (Stewart et al., 2003), cells were seeded in complete RPMI 1640 medium at 5 × 10⁵ 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 (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 Alizarin red staining. Endothelial differentiation was performed as described previously (Planat-Bezard et al., 2004). Briefly, NCCIT extract-treated cells and controls were harvested by flask shaking and plated at 2 × 10⁵ cells per milliliter in 3 ml of methylcellulose (Methocult GF H4434; Stem Cells 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) (1:30 dilution; mAb X3B10; BABCO, Covance Research Products, Grand Rapids, MI), and a rabbit polyclonal antibody against a peptide of human B-type lamin (1:1000) (Chaudhary and Courvalin, 1993). Rabbit polyclonal antibodies against Oct4 (1:1000; Santa Cruz Biotechnology), NeuN (1:500; Chemicon International, Temecula, CA), and anti-β3 integrin antibody (1:2000; Stem Cell Technologies, Vancouver, British Columbia, Canada) were used. The cells were stained with Cy3-conjugated anti-mouse and anti-rabbit antibodies and Cy5-conjugated anti-rabbit antibodies (1:1000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). Direct immunolabeling, cells (~300,000) were incubated with fluorescein isothiocyanate-conjugated mouse anti-human CD11B, anti-human CD144 antibody (1:100 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 (1:5000), 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 (20 μg/ml) bound to protein A/G-Sepharose beads. After 1 h incubation in extract at 4°C, bead complexes were removed by sedimentation at 4000 × 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 × g supernatant at 25 μg/ml protein) from indicated cell types were spotted on a strip of 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 Alk-Phos Direct detection solution (GE Healthcare, Piscataway, NJ). Light emission on film was quantitated 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 an 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 (Häkelien 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 (Hutcheson and Worman, 2004), from 293T cells. mAbs against lamina 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 (Hutcheson and Worman, 2004) remained unaltered (Figure 2, A–C).

Induction of OCT4 (POLSF1) 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
(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 PDGFrA, 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 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.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>293T extract</th>
<th>NCCIT extract</th>
<th>NCCIT cells</th>
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<tbody>
<tr>
<td>AC133</td>
<td>2^△</td>
<td>260^△</td>
<td>271^△</td>
</tr>
<tr>
<td>APL</td>
<td>2^△</td>
<td>35^△</td>
<td>26^△</td>
</tr>
<tr>
<td>CD9</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>DNMT3B</td>
<td>1</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>DNMT3L</td>
<td>3^△</td>
<td>36</td>
<td>23</td>
</tr>
<tr>
<td>NANO2</td>
<td>1</td>
<td>2513</td>
<td>1541^△</td>
</tr>
<tr>
<td>OCT3</td>
<td>2^△</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>POU5F1</td>
<td>1</td>
<td>600</td>
<td>410</td>
</tr>
<tr>
<td>REX1</td>
<td>1</td>
<td>1994</td>
<td>1985^△</td>
</tr>
<tr>
<td>SOX2</td>
<td>1</td>
<td>18</td>
<td>27</td>
</tr>
<tr>
<td>STELLA</td>
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<td>88</td>
<td>48</td>
</tr>
<tr>
<td>UTF1</td>
<td>2^△</td>
<td>121</td>
<td>133</td>
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<tr>
<td>LMNA</td>
<td>2^▼</td>
<td>370</td>
<td>15^▼</td>
</tr>
<tr>
<td>LMNB1</td>
<td>1</td>
<td>1.5^△</td>
<td>1</td>
</tr>
</tbody>
</table>

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-GenR 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-GenR 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-GenR cells, but not NCCIT cells, contained the SV40 large T anti-
gen 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-GenR 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 (GenR) 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-GenR 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.
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), cell signaling (10 and 8%), and cytoskeletal organization (6 and 3%), metabolism (4 and 10%), protein synthesis and processing (6 and 4%), 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
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.

**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.

**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

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<th>Name</th>
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* Relative to 293T cells.

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expression profile analyses of mouse ESCs (Fortunel putative marker of "stemness" shared between three gene markers up-regulated were the CD44 stem cell antigen, plas- TERT erase (of Oct4-dependent functions in reprogrammed cells. Telom- relative to transcript levels in 293T cells exposed to 293T extract. Quantitative RT-PCR analysis of expression of indicated Figure 6. 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, placent- al 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 EGCs 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 expres- 

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 isofoms 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-regu- lated 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, TSC2, BRCA1, BRCA2, CDKN1A (p16), CDKN1B (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 re- mained unchanged in extract-treated cells. Because it is un- likely 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 phe- notype obtained is independent of NCCIT lesions or that genetic mutations that gave rise to the NCCIT tumor phe- notype 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 ac- quired 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 dispersed aggregation cultures (Figure 7A). Suspensions of all cell types formed disorga- nized 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). After 2 d in the presence of mitotic inhibitors, cells released from NCCIT extract-treated 293T cells. Immunolabeling and real-time RT-PCR analyses con- firmed 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 immuno- fluorescence 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 inter- mediate filament nestin (NES), a marker of neuronal precur- sor cells (Cattaneo and McKay, 1990), was induced at the transcrip- 

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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 Alizarin red-stained mineralized nodules were detected in NCCIT extract-treated 293T cells compared with any other cell type (Figure 8B, arrows; p < 10^{-5}; 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-
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 PolIII inhibitor 5,6-dichloro-1-β-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 was not detected in 3T3 cells incubated in their own extract or in intact (non-permeabilized) 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
(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′-[3-thio]triphosphate or adenosyl-5′-imidodiphosphate, or GTP removal or substitution with guanosine 5′-[3-thio]triphosphate or guanosine 5′-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 first Oct4 elevation

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).
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.
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 stimulation. 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 PolIII-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...


Upregulated genes
- 33%
- 19%
- 3%
- 10%
- 1%
- 4%
- 8%
- 2%
- 8%
- 4%
- 3%
- 1%

Downregulated genes
- 33%
- 19%
- 3%
- 10%
- 3%
- 3%
- 19%

Legend:
- Unknown
- Transcription
- Cell cycle
- Cytoskeleton
- Metabolism
- Hematopoietic
- Prot. synthesis / processing
- Heat shock
- Extracellular matrix
- Signaling
- Chromatin
- Secreted
- Secreted
- Apoptosis
- Other
**Supplementary Table S1.** Real-time RT-PCR primers used in this study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer (F) 5’→3’</th>
<th>Reverse primer (R) 5’→3’</th>
<th>SYBR® Green or Probe no. (ProbeLibrary)</th>
<th>Product size (bp)</th>
</tr>
</thead>
</table>
| AC133     | F: GGGGAGAAACAATAATAGGATATTGTGA  
   R: CGATGCCACTTTTCTCAGGAT | Probe 86 | | 75 |
| ACTB      | F: CCAACCGCGAGAAGAGATGA  
   R: TTGTCAACCGAGGATCAAAGCA | Probe 64 | | 97 |
| Actb      | F: TGGACGAGGAGCAGGAGAG  
   R: CGCTCAGGAGAGGAGATG | Not real time PCR | | 75 |
| APL       | F: CCTACCAAGCTCATGCATAACATC  
   R: TGAGCTTTTCTCGTACTCTCATA | SYBR® Green | | 114 |
| APOA2     | F: AGGACCGAGAGAAGGACCTT  
   R: CCAATATCGAGGAGGCTGTC | Probe 68 | | 84 |
| CD9       | F: TCGGATTTAECTTCATCTTCTTG  
   R: GTGCAATCGAGCCCATAGC | Probe 56 | | 71 |
| CD31      | F: CCACCTGAGTAGTACAGCTG  
   R: TGGCTTTTCTCTTGATCGAT | Probe 12 | | 72 |
| CD144     | F: GCAGTCCAACCGGAGCGAGAA  
   R: CATCTTCCAACCGAGGAGAAG | Probe 30 | | 65 |
| DNMT3B    | F: CTGCTTTGCAGCAACACG  
   R: CAGCACCCTCCAGCAGC | Probe 74 | | 60 |
| DNMT3L    | F: CTCTCAAGCTCGGTTTCACC  
   R: GTACAGGAAGGAGGCACTCA | SYBR® Green | | 189 |
| DSPG3     | F: CAGGAGCCTGAAATCACGAGG  
   R: CCAGAAGACAGGTTGGAAAGTCT | Probe 57 | | 66 |
| GAPDH     | F: TCGGAGTAGCAACGGAGTTTGGT  
   R: TTGCCATGGGTTGGAATCTA | SYBR® Green | | 148 |
| LMNA      | F: CTGGTAGTGAGCAGGCGAG  
   R: TGCGGTAGCTGCGAGTGA | SYBR® Green | | 240 |
| Lmna      | F: AGCAAGTTGGTGGAGAGGAGTT  
   R: ACAAGCTCCCCCCCTCTTCTTG | Not real time PCR | | 64 |
| LMNB1     | F: AGGGCAGGAGAGAGGTTGAAG  
   R: GCGGAATGAGAGATGCTAACACT | | | 163 |
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence Details</th>
<th>Tm</th>
</tr>
</thead>
</table>
| *Lnmnb1* (mouse) | Forward: GACCACCATACCCGAGGAGG  
 | | Reverse: AATGGGCACAGCTTTATTCCA  
 | | Not real time PCR                                                                 | 118  |
| *MBP*   | Forward: GGGCAGCCTTTTCAAATAATGACAG  
 | | Reverse: CCATGGGTGATCCAGATCTCG  
 | | Probe 33                                                                 | 62   |
| *NANOG* | Forward: CAAAGGCAAACACCCCACCTT  
 | | Reverse: TCTGCTGAGGTAGGTATCTCG  
 | | SYBR® Green                                                                       | 158  |
| *NTR1*  | Forward: GATCAGCAAATTAGGAAATGACAG  
 | | Reverse: GGCTATCCAGGTATCTCG   
 | | Probe 53                                                                 | 78   |
| *NTS*   | Forward: AGCTTCTGGAGTCTGGTCTC  
 | | Reverse: GTGCAAGAAATCCTGCTTCAATGC  
 | | Probe 35                                                                 | 66   |
| *OTX2*  | Forward: GGTACCCAGACATCTTGCATGC  
 | | Reverse: CTTAGCTCTTCGATCTTTAAACCATAAC  
 | | Probe 10                                                                 | 95   |
| *POU5F1* | Forward: AAGCGATCAAGCAGCCGACTAT  
 | | Reverse: GGAAAAGGGACCGAGGATACA  
 | | SYBR® Green                                                                       | 127  |
| *Pou5f1* (mouse) | Forward: GTTGGAGAAAGGTGGAACCAA  
 | | Reverse: CTCCTTCTGAGGGCTTTC  
 | | SYBR® Green                                                                       | 61   |
| *REX1*  | Forward: CAGAACAgAAAGGGCTTCAC  
 | | Reverse: TCTGATGGACTGTCTTTCAAGCAA  
 | | Probe 62                                                                 | 73   |
| *SOX2*  | Forward: GCGCCCTGCAGTACACTCTC  
 | | Reverse: GCTGGCCTCGAATCCTGGAC  
 | | SYBR® Green                                                                       | 140  |
| *STELLA* | Forward: GACCAACAAACAAAGGGAGACCTAAG  
 | | Reverse: AGAAGGATCCATCAATAGACA  
 | | SYBR® Green                                                                       | 95   |
| *Tubb* (mouse) | Forward: CAATGTATACTACAATATGAAGCAACTGG  
 | | Reverse: CCAGACCTGACTGAAGATCCATT  
 | | Not real time PCR                                                                 | 96   |
| *UTF1*  | Forward: ACCAGCTGTACCTTGAAAC  
 | | Reverse: TTGAACGTACCCAGAAGCAG  
 | | SYBR® Green                                                                       | 230  |
| *VWF*   | Forward: AGTGCAGACCCAACCTTCACC  
 | | Reverse: GTGGGGACACTCTTTTTGAC  
 | | 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

<table>
<thead>
<tr>
<th>Genebank Accession No.</th>
<th>Description</th>
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<tr>
<td>293T extract-treated cells Upregulated genes (n=112)</td>
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<tr>
<td>NM_005345.3</td>
<td>Heat shock 70kD protein 1A (HSPA1A)</td>
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<tr>
<td>NM_004039.1</td>
<td>Annexin A2 (ANXA2)</td>
</tr>
<tr>
<td>BC002666.1</td>
<td>Guanylate binding protein 1, interferon-inducible</td>
</tr>
<tr>
<td>AW117368 a</td>
<td>KIAA0942 protein</td>
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<tr>
<td>NM_012200.2</td>
<td>Beta-1,3-glucuronyltransferase 3 (glucuronosyltransferase 1) (B3GAT3)</td>
</tr>
<tr>
<td>NM_000227.1</td>
<td>Laminin, alpha 3</td>
</tr>
<tr>
<td>BE791251</td>
<td>Claudin 3</td>
</tr>
<tr>
<td>NM_001218.2</td>
<td>Carbolic anhydrase XII (CA12)</td>
</tr>
<tr>
<td>NM_023037.1 a</td>
<td>Putative gene product (13CDNA73)</td>
</tr>
<tr>
<td>AM580546.1</td>
<td>Neuroplin-2 soluble isoform 9 (NRP2)</td>
</tr>
<tr>
<td>AA502912 a</td>
<td>KIAA0906 protein</td>
</tr>
<tr>
<td>AK026815.1 a</td>
<td>KIAA1102 protein</td>
</tr>
<tr>
<td>AW043713 a</td>
<td>KIAA1077 protein</td>
</tr>
<tr>
<td>AL045513 a</td>
<td>KIAA0180 protein</td>
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<tr>
<td>AI282485</td>
<td>HLA-B associated transcript-1</td>
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<tr>
<td>BE901081 a</td>
<td>H2A histone family, member X</td>
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<tr>
<td>AL023584 a</td>
<td>Contains the HIVEP2 (Schnurri-2)</td>
</tr>
<tr>
<td>AK026529.1</td>
<td>Highly similar to transducin (beta)-like 2</td>
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<tr>
<td>AL569804 a</td>
<td>KIAA1095 protein</td>
</tr>
<tr>
<td>BF968960</td>
<td>Glucose phosphate isomerase</td>
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<td>AL401612 a</td>
<td>DKFZP434M154 protein</td>
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<tr>
<td>R68573 a</td>
<td>Mitochondrial ribosomal protein S12</td>
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<tr>
<td>T90013</td>
<td>C1orf20 gene, partial sequence</td>
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<tr>
<td>AU117487</td>
<td>cAMP responsive element binding protein-like 1</td>
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<td>NM_019009.1</td>
<td>TOLLIP protein (LOC54472)</td>
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<td>AW189430 a</td>
<td>KIAA0244 protein</td>
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<td>NM_024952.1 a</td>
<td>Hypothetical protein FLJ20950</td>
</tr>
<tr>
<td>NM_016234.2</td>
<td>Long-chain fatty acid coenzyme A ligase 5 (FACL5)</td>
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<tr>
<td>NM_016046.1 a</td>
<td>Homolog of yeast exosomal core protein CSL4 (CSL4)</td>
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<tr>
<td>NM_024118.1 a</td>
<td>Hypothetical protein MGC4692</td>
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<tr>
<td>NM_017842.1 a</td>
<td>Hypothetical protein FLJ20489</td>
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<td>NM_020672.1</td>
<td>S100-type calcium binding protein A14</td>
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<td>AW304174 a</td>
<td>Chitobiase, di-N-acetyl</td>
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<td>NM_014322.1</td>
<td>Opsin 3 (encephalopsin) (OPN3)</td>
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<tr>
<td>NM_023923.1</td>
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<td>Hypothetical protein PRO2714</td>
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<td>NM_018190.1 a</td>
<td>Hypothetical protein FLJ10715</td>
</tr>
<tr>
<td>NM_018219.1</td>
<td>Hypothetical protein FLJ10786</td>
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<tr>
<td>NM_014421.1</td>
<td>Dickkopf (Xenopus laevis) homolog 2 (DKK2)</td>
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<tr>
<td>NM_005021.1</td>
<td>Ectonucleotide pyrophosphatase phosphodiesterase 3 (ENPP3)</td>
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<tr>
<td>NM_007167.1</td>
<td>Zinc finger protein 258 (ZNF258)</td>
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<td>NM_022741.1 a</td>
<td>Hypothetical protein FLJ11850</td>
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<tr>
<td>NM_024708.1 a</td>
<td>Hypothetical protein FLJ2255</td>
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<td>NM_012282.1</td>
<td>Potassium voltage-gated channel, Isk-related family, 1-like (KCNE1L)</td>
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<tr>
<td>NM_016644.1</td>
<td>Mesenchymal stem cell protein DSC54</td>
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<td>NM_017805.1 a</td>
<td>Hypothetical protein FLJ20401</td>
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<tr>
<td>NM_024923.1</td>
<td>Hypothetical protein FLJ22389</td>
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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 FLJ11798
NM_014147.1 a HSPC047 protein (HSPC047)
NM_013348.1 Potassium inwardly-rectifying channel, subfamily J, 14 (KCNJ14)
NM_024720.1 Hypothetical protein FLJ23510
NM_013348.1 Potassium inwardly-rectifying channel, subfamily J, 14 (KCNJ14)
NM_005721 a Weakly similar to human tyrosine kinase receptor Tie-1 precursor
NM_018547.1 v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (SRC)
NM_013348.1 Potassium inwardly-rectifying channel, subfamily J, 14 (KCNJ14)
NM_013348.1 Potassium inwardly-rectifying channel, subfamily J, 14 (KCNJ14)
NM_013453.1 Sperm protein associated with nucleus, X chromosome, member A1 (SPANXA1)
NM_014137.1 a PRO0650 protein
NM_030974.1 a Hypothetical protein DKFZp434N1923
NM_024374.1 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_01279.1 a Cell death-inducing DFFA-like effector a (CIDEA)
NM_005092.1 Tumor necrosis factor (ligand) 18 (TNFSF18)
NM_011275.1 Testis expressed sequence 12 (TEX12)
NM_013453.1 Sperm protein associated with nucleus, X chromosome, member A1 (SPANXA1)
AY009410.1 WNT6 precursor (WNT6)
AA456973 Activated RNA polymerase II transcription cofactor 4
NM_00393.1 Collagen, type V, alpha 2 (COL5A2)
AL540260 a Human PAC clone RP3-515N1 from 22q11.2-q22
AL571721 a Weakly similar to human tyrosine kinase receptor Tie-1 precursor
AI138993 Uncharacterized hematopoietic stem cells protein MDS026
AI089655 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 Dyrk1b (disintegrin and metalloproteinase domain-containing protein 5)
AI279819 a DKFZp564G1763
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 a CLONE=IMAGE:1524202
BF732879 a FLJ14307
AI478455 Empty spiracles (Drosophila) homolog 2
CA1933199 Neuroexophilin 4
AW006750 a Hypothetical protein FLJ20059
AI199589.1 Hypothetical protein DKFZp544E1723
AK026747.1 FLJ23094 hypothetical protein
AC003982.1 Sirtuin (silent mating type information regulation 2, S. cerevisiae, homolog) 4
AK026947.1 3-phosphoinositide dependent protein kinase-1
AA129909.4 Moderately similar to ALU7_HUMAN ALU SUBFAMILY SQ
AW301397 a CLONE=IMAGE:2766893
AW541043 Olfactory receptor, family 7, subfamily E, member 47 pseudogene

**Downregulated genes (n=36)**

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<tr>
<th>Accession</th>
<th>Description</th>
</tr>
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<tr>
<td>L13852</td>
<td>Ubiquitin-activating enzyme E1 related protein</td>
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<tr>
<td>S72904 b</td>
<td>AKP1 antigen=MAb KI recognized</td>
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<tr>
<td>AB011174 b</td>
<td>KIAA0602 protein</td>
</tr>
<tr>
<td>L06147 b</td>
<td>Human (clone SY11) golgin-95 mRNA</td>
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<tr>
<td>D87470 b</td>
<td>KIAA0280 protein</td>
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<tr>
<td>U46024 b</td>
<td>Myotubularin (MTM1)</td>
</tr>
<tr>
<td>AB029343</td>
<td>HCR (alpha-helix coiled-coil rod homologue)</td>
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</table>
X74496 b Prolyl oligopeptidase
AJ984221 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 Myelinated 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 b 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_020739.1 1,2-alpha-mannosidase IC (HMIC)
NM_014650.1 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-associated 1 (HHLA1)
NM_025042.1 b Hypothetical protein FLJ22367
AI948472 b Paired box gene 8
AK021672.1 b Hypothetical protein FLJ12820
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)
AA77285 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_02420.2 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 superfamly member (G6E)

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

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

Gene not detected in 293T extract-treated cells. Total numbers of NCCIT genes up- or
downregulated in Jurkat extract-treated cells are 70 (58 annotated a + 12 annotated c) and 28 (annotated b),
respectively.
**Supplementary Table S3.** NCCIT genes consistently up- or downregulated over eight weeks after treatment with NCCIT extract

<table>
<thead>
<tr>
<th>Upregulated genes (n=686)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster Incl. AB006533: Homo sapiens RecQ5 mRNA for DNA helicase, complete cds</td>
</tr>
<tr>
<td>Cluster Incl. M24899: Human triiodothyronine (t3) mRNA, complete cds</td>
</tr>
<tr>
<td>Cluster Incl. AB015331: Homo sapiens HRHFB2017 mRNA, partial cds</td>
</tr>
<tr>
<td>Cluster Incl. M96789: Homo sapiens connexin 37 (GJA4) mRNA, complete cds</td>
</tr>
<tr>
<td>Cluster Incl. A1003763: ou91e02.x1 Homo sapiens cDNA, 3' end</td>
</tr>
<tr>
<td>Cluster Incl. AL047020: DKFZp586N1517_s1 Homo sapiens cDNA, 3' end</td>
</tr>
<tr>
<td>Cluster Incl. AA402435: zt60g10.r1 Homo sapiens cDNA, 5' end</td>
</tr>
<tr>
<td>Cluster Incl. W72694: zt68f10.s1 Homo sapiens cDNA, 3' end</td>
</tr>
<tr>
<td>gb:BC001643.1 /DEF=Human sapiens, tumor necrosis factor, alpha-induced protein 1 (endothelial)</td>
</tr>
<tr>
<td>gb:AF198997.1 /DEF=Human sapiens PRO2760 mRNA, complete cds.</td>
</tr>
<tr>
<td>gb:NM_022844.1 /DEF=Human sapiens myosin, heavy polypeptide 11, smooth muscle (MYH11), transcript variant SM2, mRNA.</td>
</tr>
<tr>
<td>gb:NM_000552.2 /DEF=Human sapiens von Willebrand factor (VWF), mRNA.</td>
</tr>
<tr>
<td>gb:NM_005562.1 /DEF=Human sapiens laminin, gamma 2, mRNA.</td>
</tr>
<tr>
<td>Consensus includes gb:BC000023.1 /DEF=Human sapiens, ribosomal protein S19, clone MGC:1630, mRNA, complete cds.</td>
</tr>
<tr>
<td>Consensus includes gb:AI467916 AXL receptor tyrosine kinase</td>
</tr>
<tr>
<td>gb:NM_006129.2 /DEF=Human sapiens bone morphogenetic protein 1 (BMP1), transcript variant BMP1-3, mRNA.</td>
</tr>
<tr>
<td>gb:NM_014672.1 /DEF=Human sapiens KIAA0391 gene product (KIAA0391), mRNA.</td>
</tr>
<tr>
<td>Consensus includes gb:AW117498 forkhead box O1A (rhadomyosarcoma)</td>
</tr>
<tr>
<td>Consensus includes gb:W72082 complement component C1q receptor</td>
</tr>
<tr>
<td>gb:NM_002342.1 /DEF=Human sapiens lymphotixin beta receptor (TNFR superfamily, member 3 (LTBR), mRNA.</td>
</tr>
<tr>
<td>gb:AF097493.1 /DEF=Human sapiens glutaminase kidney isoform mRNA, complete cds.</td>
</tr>
<tr>
<td>Consensus includes gb:AB007869.1 /DEF=Human sapiens KIAA0409 mRNA, partial cds.</td>
</tr>
<tr>
<td>Consensus includes gb:AW139152 Notch (Drosophila) homolog 3</td>
</tr>
<tr>
<td>gb:NM_000702.1 /DEF=Human sapiens ATPase, Na+K+ transporting, alpha 2 (+) polypeptide (ATP1A2), mRNA.</td>
</tr>
<tr>
<td>gb:NM_014782.1 /DEF=Human sapiens KIAA0512 gene product (KIAA0512), mRNA.</td>
</tr>
<tr>
<td>Consensus includes gb:T79216 KIAA1046 protein</td>
</tr>
<tr>
<td>gb:NM_012306.1 /DEF=Human sapiens lifeguard (KIAA0950), mRNA.</td>
</tr>
<tr>
<td>gb:NM_002444.1 /DEF=Human sapiens CD163 antigen (CD163), mRNA.</td>
</tr>
<tr>
<td>gb:NM_002996.1 /DEF=Human sapiens small inducible cytokine subfamily D (Cys-X3-Cys), member 1 (SCYD1), mRNA.</td>
</tr>
<tr>
<td>gb:NM_014961.1 /DEF=Human sapiens KIAA0871 protein (KIAA0871), mRNA.</td>
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<tr>
<td>gb:NM_020991.2 /DEF=Human sapiens chorionic somatomammotropin hormone 2 (CSH2), transcript variant 1, mRNA.</td>
</tr>
<tr>
<td>gb:NM_005781.2 /DEF=Human sapiens activated p21cdc42Hs kinase (ACK1), mRNA.</td>
</tr>
<tr>
<td>gb:NM_001998.1 /DEF=Human myeloperoxidase mRNA, complete cds.</td>
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<tr>
<td>gb:NM_006888.1 /DEF=Human sapiens N-myc (and STAT) interactor (NMI), mRNA.</td>
</tr>
<tr>
<td>gb:NM_000570.1 /DEF=Human sapiens N-myc (and STAT) interactor (NMI), mRNA.</td>
</tr>
<tr>
<td>gb:NM_000314.1 /DEF=Human sapiens phosphatase and tensin homolog (mutated in multiple advanced cancers 1) (PTEN), mRNA</td>
</tr>
<tr>
<td>gb:NM_014963.1 /DEF=Human sapiens KIAA0963 protein (KIAA0963), mRNA.</td>
</tr>
<tr>
<td>Consensus includes gb:AI655714 KIAA1052 protein</td>
</tr>
<tr>
<td>Consensus includes gb:AA772285 vitamin D (1,25- dihydroxyvitamin D3) receptor</td>
</tr>
<tr>
<td>gb:NM_006017.1 /DEF=Human sapiens prominin (mouse)-like 1 (PROML1), mRNA.</td>
</tr>
<tr>
<td>Consensus includes gb:BF303580 G-2 and S-phase expressed 1 /FL=gb:AF223408.1 gb:NM_016426.1</td>
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<tr>
<td>Consensus includes gb:N46430 zinc finger protein 202 /FL=gb:NM_003455.1 gb:AF027218.1 gb:AF027219.1</td>
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<tr>
<td>gb:BC000737.1 /DEF=Human sapiens, regulator of G-protein signalling 4, clone MGC:2124, mRNA, complete cds.</td>
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</table>
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:NM_000610.1 /DEF=Homo sapiens CD44 antigen (homing function and Indian blood group system) (CD44), mRNA.

gb:NM_014808.1 /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_006368.1 /DEF=Homo sapiens vironectin (VTN), mRNA.

Consensus includes gb:U38321.1 /DEF=Homo sapiens clone rasi-11 matrix metalloproteinase RASI-1 mRNA, complete cds.

gb:NM_004966.1 /DEF=Homo sapiens hepatocyte nuclear factor 3, alpha (HNF3A), mRNA.

gb:NM_004809.1 /DEF=Homo sapiens stomachin-like 1 (STOML1), mRNA.

gb:NM_000351.1 /DEF=Homo sapiens aldolase B, fructose-bisphosphate (ALDOB), mRNA.

gb:NM_001302.1 /DEF=Homo sapiens coagulation factor V (proaccelerin, labile factor) (F5), mRNA.

Consensus includes gb:NM_002667.1 /DEF=Homo sapiens phospholamban (PLN), mRNA.

gb:NM_002832.1 /DEF=Homo sapiens glycophorin A2 (GPA2), mRNA.

gb:NM_002594.1 /DEF=Homo sapiens protein tyrosine phosphatase, receptor type, N (PTPRN), mRNA.

gb:NM_014001.1 /DEF=Homo sapiens GPI-anchored metastasis-associated protein homolog (C4.4A), mRNA.

Consensus includes 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.

Consensus includes gb:NM_002211.1 /DEF=Homo sapiens mature T-cell proliferation 1 (MTCP1), mRNA.

Consensus includes gb:NM_000238.1 /DEF=Homo sapiens potassium voltage-gated channel, subfamily H (eag-related), member 2 (KCNH2), mRNA.

Consensus includes gb:NM_000259.1 /DEF=Homo sapiens leukemia inhibitory factor (cholinergic differentiation factor) (LIF), mRNA.

Consensus includes gb:NM_001200.1 /DEF=Homo sapiens bone morphogenetic protein 2 (BMP2), mRNA.

Consensus includes gb:NM_002584.1 /DEF=Homo sapiens parvalbumin (PVALB), mRNA. /FEA=mRNA

Consensus includes gb:NM_002053.1 /DEF=Homo sapiens gap junction protein, beta 3, 31kD (connexin 31) (GJB3), mRNA.

Consensus includes gb:NM_005401.1 /DEF=Homo sapiens gap junction protein, beta 1, 31kD (connexin 37) (GJA1), mRNA.

Consensus includes gb:NM_000130.2 /DEF=Homo sapiens coagulation factor V (proaccelerin, labile factor) (F5), mRNA.

Consensus includes gb:NM_001302.1 /DEF=Homo sapiens coagulation factor V (proaccelerin, labile factor) (F5), mRNA.

Consensus includes gb:NM_002594.1 /DEF=Homo sapiens protein tyrosine phosphatase, receptor type, N (PTPRN), mRNA.

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Consensus includes gb:NM_002216.1 /DEF=Homo sapiens inter-alpha (globulin) inhibitor, H2 polypeptide (ITIH2), mRNA.

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Consensus includes gb:NM_000238.1 /DEF=Homo sapiens potassium voltage-gated channel, subfamily H (eag-related), member 2 (KCNH2), mRNA.

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Consensus includes gb:NM_002584.1 /DEF=Homo sapiens parvalbumin (PVALB), mRNA. /FEA=mRNA

Consensus includes gb:NM_002053.1 /DEF=Homo sapiens gap junction protein, beta 3, 31kD (connexin 31) (GJB3), mRNA.

Consensus includes gb:NM_005401.1 /DEF=Homo sapiens gap junction protein, beta 1, 31kD (connexin 37) (GJA1), mRNA.

Consensus includes gb:NM_000130.2 /DEF=Homo sapiens coagulation factor V (proaccelerin, labile factor) (F5), mRNA.

Consensus includes gb:NM_002594.1 /DEF=Homo sapiens protein tyrosine phosphatase, receptor type, N (PTPRN), mRNA.

Consensus includes gb:NM_005213.1 /DEF=Homo sapiens cystatin A (stefin A) (CSTA), mRNA.

Consensus includes gb:NM_002216.1 /DEF=Homo sapiens inter-alpha (globulin) inhibitor, H2 polypeptide (ITIH2), mRNA.

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Consensus includes gb:NM_000238.1 /DEF=Homo sapiens potassium voltage-gated channel, subfamily H (eag-related), member 2 (KCNH2), mRNA.

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Consensus includes gb:NM_002053.1 /DEF=Homo sapiens gap junction protein, beta 3, 31kD (connexin 31) (GJB3), mRNA.

Consensus includes gb:NM_005401.1 /DEF=Homo sapiens gap junction protein, beta 1, 31kD (connexin 37) (GJA1), mRNA.

Consensus includes gb:NM_000130.2 /DEF=Homo sapiens coagulation factor V (proaccelerin, labile factor) (F5), 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 subtilisin/kexin type 5 (PCSK5), mRNA.
gb:NM_020980.2 /DEF=Homo sapiens aquaporin 9 (AQP9), mRNA.

gb:NM_006035.1 /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

Consensus includes gb:NM_006200.1 /DEF=Homo sapiens proprotein convertase subtilisin/kexin type 5 (PCSK5), 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.

Consensus includes gb:NM_006200.1 /DEF=Homo sapiens proprotein convertase subtilisin/kexin type 5 (PCSK5), mRNA.

Consensus includes gb:NM_005658.1 /DEF=Homo sapiens TNF receptor-associated factor 1 (TRAF1), mRNA.

Consensus includes gb:NM_002336.1 /DEF=Homo sapiens low density lipoprotein receptor-related protein 6 (LRP6), mRNA.

Consensus includes gb:NM_005658.1 /DEF=Homo sapiens TNF receptor-associated factor 1 (TRAF1), mRNA.

Consensus includes gb:NM_002336.1 /DEF=Homo sapiens low density lipoprotein receptor-related protein 6 (LRP6), mRNA.

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gb:NM_001541.1 /DEF=Homo sapiens heat shock 27kD protein 2 (HSPB2), mRNA.

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gb:NM_005012.1 /DEF=Homo sapiens receptor tyrosine kinase-like orphan receptor 1 (ROR1), mRNA.

gb:NM_002220.1 /DEF=Homo sapiens inositol 1,4,5-trisphosphate 3-kinase A (ITPKA), mRNA.

gb:NM_005658.1 /DEF=Homo sapiens TNF receptor-associated factor 1 (TRAF1), mRNA.

Consensus includes gb:NM_005658.1 /DEF=Homo sapiens TNF receptor-associated factor 1 (TRAF1), mRNA.

Consensus includes gb:NM_005658.1 /DEF=Homo sapiens TNF receptor-associated factor 1 (TRAF1), mRNA.

Consensus includes gb:NM_005658.1 /DEF=Homo sapiens TNF receptor-associated factor 1 (TRAF1), mRNA.

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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_003444.1 /DEF=Homo sapiens macrophage lectin 2 (calcium dependent) (HML2), mRNA.
gb:NM_006299.1 /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_001794.1 /DEF=Homo sapiens cadherin 4, type 1, R-cadherin (retinal) (CDH4), mRNA.

gb:NM_0015370.1 /DEF=Homo sapiens hypothetical protein (HS747E2A), mRNA.

gb:NM_000197.1 /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_003079.1 /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:NM_005666.1 /DEF=Homo sapiens H factor (complement)-like 3 (HFL3), mRNA.

gb:NM_005361.1 /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_000197.1 /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_004186.1 /DEF=Homo sapiens sema domain, immunoglobulin domain 3F (SEMA3F), mRNA.

gb:NM_006678.1 Homo sapiens CMRF35 leukocyte immunoglobulin-like receptor (CMRF35), mRNA.

gb:NM_001899.1 /DEF=Homo sapiens cystatin S (CST4), 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_003833.2 /DEF=Homo sapiens matrin 4 (MATN4), transcript variant 1, mRNA.

gb:NM_000335.1 /DEF=Homo sapiens sodium channel, voltage-gated, type V, alpha polypeptide (SCN5A), mRNA.

gb:NM_006640.1 /DEF=Homo sapiens MLL septin-like fusion (MSF), mRNA.

gb:NM_004256.1 /DEF=Homo sapiens organ cationic transporter-like 3 (ORCTL3), mRNA.

gb:NM_001794.1 /DEF=Homo sapiens cadherin 4, type 1, R-cadherin (retinal) (CDH4), mRNA.

gb:NM_002363.1 /DEF=Homo sapiens melanoma antigen, family B, 1 (MAGEB1), mRNA.

gb:NM_001531.1 /DEF=Homo sapiens major histocompatibility complex, class I-like sequence (HLALS), mRNA.

gb:NM_00451.1 /DEF=Homo sapiens short stature homeobox (SHOX), transcript variant SHOXa, 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_0025068.1 /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), 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_002188.1 /DEF=Homo sapiens interleukin 13 (IL13), mRNA.
gb:NM_006664.1 /DEF=Homo sapiens small inducible cytokine subfamily A (Cys-Cys), member 27 (SCYA27), 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, eAMP-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:AI953123 CLONE=IMAGE:2464769 /UG=Hs.57548 ESTs
Consensus includes gb:AL033377 Contains an exon similar to parts of BMP and Tolloyd 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: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. /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 homogenitase 1,2-dioxygenase (homogenitase 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:AB092023 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:R99037 acetyl-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:AF070571.1 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:AU275469 /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:AU066701.1 /DEF=Homo sapiens mRNA for putative serinethreonine protein kinase, partial.
Consensus includes gb:AK023845.1 weakly similar to PROBABLE UBQUTIN CARBOXYLY-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 Monoxygenase 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:SB33390.1 /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 IIla, 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 IMAGE:1963178, mRNA sequence.
Consensus includes gb:AU150691 Homo sapiens cDNA FLJ10577 fis, clone NT2RP003367
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:AI147194 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 phospholipase 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:AU148005 Homo sapiens cDNA FLJ12105 fis, clone HEMBB1002699
Consensus includes gb:AK026040.1 /DEF=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:AK022450.1 highly 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 DKFZp434I10523
Consensus includes gb:AU159276 Homo sapiens cDNA FLJ13867 fis, clone THYR01001262
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 DKFZp434C2012
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: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 prostataglandin receptor pseudogene
Consensus includes gb:AC003079 Human BAC clone GS1-303P24 from 7q21-22
Consensus includes gb:L37198.1 /DEF=Homo sapiens (clone B33E13) 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:AK000388.1 /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:AL346187 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=CS0Dj008Y09 (5 prime)
Consensus includes gb:BF484596 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:A0685172 Highly similar to KPCM_HUMAN PROTEIN KINASE C, MU TYPE H.sapiens
Consensus includes gb:AA479678 Moderately similar to ALU13_HUMAN ALU SUBFAMILY SX
gb:NM_003498.1 /DEF=Homo sapiens stannin (SNN), mRNA.
Consensus includes gb:BC001080.1 hypothetical protein MGCC2749
gb:NM_015894.1 /DEF=Homo sapiens SGC10-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 cell (E.coli) homolog 1 (ELAC1), mRNA.
gb:NM_024526.1 /DEF=Homo sapiens hypothetical protein FLJ21522 (FLJ21522), mRNA.

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gb:NM_024712.1 /DEF=Homo sapiens hypothetical protein FLJ13824 (FLJ13824), mRNA.
gb:NM_020659.1 /DEF=Homo sapiens tawney (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 (WDFC1), mRNA.
gb:NM_013246.1 /DEF=Homo sapiens cardiotoxin-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_025400.1 /DEF=Homo sapiens likely ortholog of mouse polydom (POLYDOM), mRNA.
gb:NM_024515.1 /DEF=Homo sapiens hypothetical protein MGC4645 (MGC4645), mRNA.
gb:NM_012311.1 /DEF=Homo sapiens calcequestrin 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_024777.1 /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 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-apoptosis (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 (KCNNB3L), 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.
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 FLI20839 (FLI20839), 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 FLJ21232 (FLJ21232), 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_014100.1 /DEF=Homo sapiens PRO1770 protein (PRO1770), mRNA.
gb:NM_023038.1 /DEF=Homo sapiens 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 (LOCS1501), 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 hypothetical protein FLJ23407.

Consensus includes gb:BC000794.1 /DEF=Homo sapiens, pre-mRNA splicing factor similar to S. cerevisiae Prp18 mRNA.

Consensus includes gb:BC000122.1 /DEF=Homo sapiens, Similar to nuclear localization signals binding protein 1, clone MGC:3104, mRNA.

Consensus includes gb:AL4046979 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:AI439556 upregulated by 1,25-dihydroxyvitamin D-3
Consensus includes gb:NM_021079.1 /DEF=Homo sapiens Upregulated by 1,25-dihydroxyvitamin D-3 (VDUP1), mRNA.

Consensus includes gb:M97935.1 /DEF=Homo sapiens transcription factor ISGF-3 mRNA, complete cds.

Downregulated genes (n=161)

Cluster Incl. M99436:Human transducin-like enhancer protein (TLE2) mRNA, complete cds
Cluster Incl. AA209463:zq48h11.s1 Homo sapiens cDNA, 3 end /clone=IMAGE-648357

Consensus includes gb:A1439556 upregulated by 1,25-dihydroxyvitamin D-3

Cluster Incl. AA209463:zq48h11.s1 Homo sapiens cDNA, 3 end /clone=IMAGE-648357

Consensus includes gb:A1439556 upregulated by 1,25-dihydroxyvitamin D-3 (VDUP1), mRNA.

Consensus includes 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:zq48h11.s1 Homo sapiens cDNA, 3 end /clone=IMAGE-648357

Consensus includes gb:A1439556 upregulated by 1,25-dihydroxyvitamin D-3

Consensus includes gb:A1439556 upregulated by 1,25-dihydroxyvitamin D-3 (VDUP1), mRNA.

Consensus includes 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:zq48h11.s1 Homo sapiens cDNA, 3 end /clone=IMAGE-648357
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 polyt(c)-binding protein 4, clone MGC:2386, mRNA, complete cds.
gb:BC004443.1 /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
Consensus includes 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:AF283890.1 /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:U80918.1 /DEF=Homo sapiens transcription factor (NF-ATcC) mRNA, complete cds.
gb:A067524.1 /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:L20942.1 /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:AL363319 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:AI676426 KIAA1547 protein
Consensus includes gb:AL583340 KIAA0602 protein
Consensus includes gb:A1992251CLONE=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
Consensus includes 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: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 chromosome 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:NM_016031.1 /DEF=Homo sapiens elongation of very long chain fatty acids-like 1 (ELOVL1), mRNA.
Consensus includes gb:NM_025082.1 /DEF=Homo sapiens hypothetical protein FLJ13111 (FLJ13111), mRNA.
Consensus includes gb:NM_022917.1 /DEF=Homo sapiens hypothetical protein FLJ121959 (FLJ121959), mRNA.
Consensus includes gb:NM_018231.1 /DEF=Homo sapiens hypothetical protein FLJ10815 (FLJ10815), mRNA.
Consensus includes gb:NM_024585.1 /DEF=Homo sapiens hypothetical protein FLJ22160 (FLJ22160), mRNA.
Consensus includes gb:NM_013385.2 /DEF=Homo sapiens pleckstrin homology, Sec7 and coiledcoil domains 4 (PSCD4), mRNA.
Consensus includes gb:NM_017586.1 /DEF=Homo sapiens chromosome 9 open reading frame 7 (C9orf7), mRNA.
Consensus includes gb:NM_017511.1 /DEF=Homo sapiens glioma tumor suppressor candidate region gene 1 (GLTSCR1), mRNA.
Consensus includes gb:NM_020228.1 /DEF=Homo sapiens PR domain containing 10 (PRDM10), mRNA.
Consensus includes gb:NM_014353.1 /DEF=Homo sapiens RAB26, member RAS oncogene family (RAB26), mRNA.
Consensus includes gb:NM_020664.1 /DEF=Homo sapiens 2,4-dienoyl CoA reductase 2, peroxisomal (DECR2), mRNA.
Consensus includes gb:NM_017741.1 /DEF=Homo sapiens hypothetical protein FLJ20280 (FLJ20280), mRNA.
Consensus includes gb:NM_00908.1 /DEF=Homo sapiens natriuretic peptide receptor C guanylate cyclase C (NPR3), mRNA.
Consensus includes gb:NM_017726.1 /DEF=Homo sapiens hypothetical protein FLJ20251 (FLJ20251), mRNA.
Consensus includes gb:NM_018063.1 /DEF=Homo sapiens hypothetical protein FLJ10339 (FLJ10339), mRNA.
Consensus includes gb:NM_007059.1 /DEF=Homo sapiens kaptin (actin-binding protein) (KPTN), mRNA.
Consensus includes gb:NM_030915.1 /DEF=Homo sapiens hypothetical protein DKFZp564D0372 (DKFZP564D0372), mRNA.
Consensus includes gb:NM_031301.1 /DEF=Homo sapiens hypothetical protein DKFZp564D0372 (DKFZP564D0372), mRNA.
Consensus includes gb:NM_018482.1 /DEF=Homo sapiens KIAA1249 protein (KIAA1249), mRNA.
Consensus includes gb:NM_023076.1 /DEF=Homo sapiens hypothetical protein FLJ23360 (FLJ23360), mRNA.
Consensus includes gb:NM_022467.1 /DEF=Homo sapiens N-acetylglalactosamine-4-O-sulfotransferase (GALNAC-4-ST1), mRNA.
Consensus includes gb:HC002382.1 /DEF=Homo sapiens, COX15 (yeast) homolog, cytochrome c oxidase assembly protein, mRNA.
Consensus includes gb:U15642.1 /DEF=Human transcription factor E2F-5 mRNA, complete cds.
Consensus includes gb:AF22167.1 /DEF=Homo sapiens DRC3 mRNA, complete cds.
Consensus includes gb:AV741657 leucine zipper protein 1
Consensus includes gb:AK024269.1 weakly similar to TROPOMYSIN 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:AV700403 /FEA=EST /DB_XREF=gi:10302374 /DB_XREF=est:AV700403 /CLONE=GBKDA01
Consensus includes gb:AW974816 Weakly similar to ALU1_HUMAN ALU SUBFAMILY J

\textsuperscript{a} Genes represented in Figure 6G, left graph, week 8, gray bar.

\textsuperscript{b} Genes represented in Figure 6G, right graph, week 8, gray bar.
**Supplementary Table S4.** Changes in expression level of markers of dedifferentiation and multilineage differentiation potential in 293T cells treated with Jurkat extract

<table>
<thead>
<tr>
<th>Name</th>
<th>Jurkat cells (fold upregulation)</th>
<th>Jurkat extract-treated cells (fold upregulation over 8 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Somatic cell markers</strong></td>
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<td></td>
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<tr>
<td>LMNA</td>
<td>0.9</td>
<td>0.9 – 2.1</td>
</tr>
<tr>
<td>LMNB1</td>
<td>0.7</td>
<td>1.2 – 2.2</td>
</tr>
<tr>
<td>LMNB2</td>
<td>1.4</td>
<td>0.5 – 1.3</td>
</tr>
<tr>
<td>NPR3</td>
<td>1.4</td>
<td>1.4 – 3.4</td>
</tr>
<tr>
<td><strong>Embryonic, germ cell and stem cell markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCT4 and Oct4-responsive genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>POU5F1</td>
<td>0.2</td>
<td>0.05 – 0.2</td>
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<tr>
<td>SOX2</td>
<td>0.25</td>
<td>0.8 – 1.2</td>
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<tr>
<td>UTF1</td>
<td>0.1</td>
<td>0.05 – 0.1</td>
</tr>
<tr>
<td>REX1</td>
<td>0.4</td>
<td>0.6 – 2.3</td>
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<tr>
<td>FOXD3</td>
<td>0.75</td>
<td>0.9 – 1.9</td>
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<tr>
<td>Telomerase and telomerase-associated factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TERT</td>
<td>0.9</td>
<td>0.6 – 1.8</td>
</tr>
<tr>
<td>TERF1</td>
<td>0.3</td>
<td>0.6 – 5.2</td>
</tr>
<tr>
<td>TERF2</td>
<td>1.3</td>
<td>1.6 – 3.1</td>
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<td>Others</td>
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<td></td>
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<td>POU3F1</td>
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<td>0.7 – 1.8</td>
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<td>ALP1</td>
<td>1.4</td>
<td>0.7 – 1.5</td>
</tr>
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<td>ALP1</td>
<td>1.3</td>
<td>0.8 – 2.2</td>
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<td>CD44</td>
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<td>0.7 – 1.1</td>
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<tr>
<td>LIF</td>
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<td>0.2 – 1.5</td>
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<td>FZD9</td>
<td>0.4</td>
<td>0.8 – 1.9</td>
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<td>TEF</td>
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<tr>
<td>SCGF</td>
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<td>GCNF</td>
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<td>0.6 – 1.0</td>
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<td>SPINK2</td>
<td>3.0</td>
<td>0.9 – 2.7</td>
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<td>DKK2</td>
<td>0.8</td>
<td>5.5 – 9.3</td>
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<tr>
<td>INTA6</td>
<td>1.9</td>
<td>0.4 – 1.2</td>
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<tr>
<td><strong>Markers of potential lineage-specific differentiation</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Osteogenic lineage</strong></td>
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<tr>
<td>BMP1</td>
<td>0.2</td>
<td>0.5 – 1.9</td>
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<tr>
<td>BMP2</td>
<td>0.5</td>
<td>0.5 – 2.5</td>
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<td>OGN</td>
<td>1.5</td>
<td>1.4 – 2.7</td>
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<tr>
<td>CTSK</td>
<td>1.1</td>
<td>1.2 – 1.6</td>
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<tr>
<td>TNFRSF11B</td>
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<td>0.3 – 0.5</td>
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<td><strong>Endothelial lineage</strong></td>
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<tr>
<td>VWF</td>
<td>3.5</td>
<td>0.4 – 2.7</td>
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<tr>
<td>NOS3</td>
<td>1.8</td>
<td>0.7 – 1.3</td>
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<tr>
<td><strong>Myogenic lineage</strong></td>
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<tr>
<td>MYF5</td>
<td>0.9</td>
<td>0.2 – 1.0</td>
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<tr>
<td>TMP1</td>
<td>2.2</td>
<td>0.9 – 3.9</td>
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<td>MYH11</td>
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<td><strong>Neurogenic lineage</strong></td>
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<tr>
<td>NTS</td>
<td>3.0</td>
<td>1.9 – 2.8</td>
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<tr>
<td>NRG1</td>
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<td>0.8 – 1.1</td>
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<tr>
<td>MBP</td>
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<td>0.8 – 1.3</td>
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<td>MOBP</td>
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<td>0.3 – 1.6</td>
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<tr>
<td>NCAM1</td>
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<td>0.5 – 1.0</td>
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<td>CD56</td>
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<td>0.5 – 1.5</td>
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<td><strong>Adipogenic lineage</strong></td>
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22
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<th>Gene</th>
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<th>Range</th>
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<td>APOA2</td>
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<td>0.5 – 1.1</td>
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<td>APOD</td>
<td>0.3</td>
<td>0.6 – 1.0</td>
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<td>APOE</td>
<td>2.1</td>
<td>0.3 – 1.0</td>
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<td>APOC1</td>
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<td>0.8 – 1.9</td>
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<tr>
<td>PPARG2</td>
<td>0.2</td>
<td>0.3 – 0.6</td>
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<td>FAD1</td>
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<td>0.2 – 2.3</td>
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<td>Chondrogenic lineage</td>
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<td>COL4A3</td>
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<td>COL5A2</td>
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<td>0.5 – 1.9</td>
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<td>FN1</td>
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*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

<table>
<thead>
<tr>
<th>Name</th>
<th>Genebank Accession No.</th>
<th>Description</th>
<th>NCCIT cells (fold upregulation)</th>
<th>NCCIT extract-treated cells (fold upregulation)</th>
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<tr>
<td></td>
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<td></td>
<td>Week 1</td>
<td>Week 2</td>
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<tr>
<td><strong>Housekeeping genes</strong></td>
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<tr>
<td>18S</td>
<td>M10098.1</td>
<td>18S ribosomal RNA</td>
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<td>1.3</td>
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<td>28S</td>
<td>M11167.1</td>
<td>28S ribosomal RNA</td>
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<td>1.0</td>
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<td>GAPDH</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>1.4</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Tubulin, alpha</td>
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<td>TUBA3</td>
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<td>Tubulin, gamma 2</td>
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<td>HPRT1</td>
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<td>Hypoxanthine phosphoribosyltransferase 1</td>
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<td>ACTB</td>
<td>X00351.1</td>
<td>Beta actin</td>
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**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

<table>
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<tr>
<th></th>
<th></th>
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<th>0.8 – 1.2</th>
<th>0.8 – 1.2</th>
<th>0.8 – 1.2</th>
<th>0.8 – 1.2</th>
<th>0.8 – 1.2</th>
</tr>
</thead>
</table>

*a Relative to 293T cells.*
Epigenetic Reprogramming of OCT4 and NANOG Regulatory Regions by Embryonal Carcinoma Cell Extract

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; Munsch et al., 2001; 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 (Hakelien et al., 2002) or of undifferentiated, pluripotent ES or EC cells (Teranger 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 (Teranger 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 (Blieloch 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

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Vol. 18, 1543–1553, May 2007
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 quantitative 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-ChIP) 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 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 (Ab8850) 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 (NCIT) were cultured in RPMI 1640 medium containing 10% fetal calf serum (complete RPMI medium) (Taranger et al., 2005). Cells were treated with extracts seeded were harvested. Cells were sonicated on ice; the lysate was sedimented at 10,000 g for 15 min, and the supernatant was aliquoted, frozen in liquid nitrogen, and stored at −80°C. Extracts were diluted with H2O 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 Ca2+- and Mg2+-free Hank’s balanced salt solution (HBBS) and resuspended in 490 μl of cold ice-cold HBBS. Tubes were placed in a H2O bath at 37°C for 2 min, and 10 μl of Streptolysin O (SLO; 100 μg/ml stock diluted 1:10 in cold HBBS; Sigma-Aldrich) was added (final concentration, 200 ng/ml). Samples were incubated horizontally in a H2O 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 HBBS, and cells were sedimented at 120 × g for 5 min at 4°C. Permeabilization was assessed by uptake of a fluorescent dextran in separate samples 24 h after rescaling and resealing the cells (Taranger et al., 2005).

Permeabilized cells (500,000) were suspended in 500 μl of NCIT. 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 H2O bath with occasional agitation. Torcode membranes, the extract was diluted with complete RPMI medium containing 2 mM CaCl2, 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 isooamylcohol extractions, followed by one extraction with chloroform isoamyl alcohol, and then the DNA was ethanol-precipitated. DNA was dissolved indifferently in H2O 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/index.html) (Supplemental Table 1). PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 1 min, 55°C/55/55°C for 1 min (temperature was primer-dependent; see Supplemental Table 1), and 72°C for 1 min. 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 the extent of methylation change (the number of CpGs deduced 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 throughout the text.

Chromatin Immunoprecipitation (Q-ChIP)

To minimize sample loss during the ChIP procedure and maximize ChIP specificity, we recently developed and validated a quick and quantitative Q-ChIP assay (Dahl and Collas, 2007) also used in this study. 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. To prepare a 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 1·105 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 × 30 s on ice to generate chromatin fragments of ~500 base pairs. The lysate and supernatant were collected, and chromatin concentration was determined by A260 from an aliquot diluted 100-fold.

Chromatin diluted (2 A260 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. The antibody 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 isooamyl alcohol, once with chloroform isooamyl 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 normalized with a gDNA input control, 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 NCIT 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 reactions in an Applied Biosystems 7500 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
formulas of (Pfaff, 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 \( \text{OCT4} \) and \( \text{NANOG} \) were strongly up-regulated from levels barely detectable by real-time RT-PCR (Figure 1B). Moderate down-regulation of \( \text{LMNA} \) 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 (\( \text{LMNB1} \)) gene was not altered by extract treatment (Figure 1B).

\( \text{OCT4} \) and \( \text{NANOG} \) 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 \( \text{OCT4} \), \( \text{NANOG} \), and \( \text{LMNA} \) promoter regions. Bisulfite sequencing analysis was carried out to establish 5′-3′ CpG methylation profiles across the \( \text{OCT4} \) 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 \( \text{OCT4} \) 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 \( \text{NANOG} \) promoter, encompassing a total of 14 CpGs within nucleotides −1503 to −163 relative to the TSS (Figure 2 and Supplemental Figure 1). The proximal \( \text{LMNA} \) promoter region examined encompassed nine CpGs within nucleotides −277 to +92 relative to the TSS (Figure 2 and Supplemental Figure 1).

The \( \text{OCT4} \) region examined was methylated in 293T cells and largely unmethylated in NCCIT cells (\( \rho < 10^{-4} \); Figure 3A; see Table 1 for statistical analysis). This methylation pattern was consistent with the pattern of expression of...
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 \( (p < 10^{-4}; \text{Figure 3A}; \text{Table 1}) \). 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 \( (p < 0.001; \text{Figure 3A}) \).
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.

### 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>293T extract</th>
<th>293T</th>
<th>NCCIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT4</td>
<td>p = 0.365</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>p values</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 0.629</td>
<td>X</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &lt; 0.0001</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>NANOG</td>
<td>p = 0.198</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>p values</td>
<td>p = 0.01</td>
<td>p = 0.01</td>
<td>p = 0.827</td>
<td>X</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.0001</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

* Unpaired t tests. Data expressed as two-tailed p values with X referring to treatment or cell type with which comparison is made.

* Regions 1–9 were analyzed (see Figure 3).

* Regions 1–3 were analyzed (see Figure 3).
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). Q²ChIP 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-EPP amplicon), the proximal enhancer (OCT4-DPE and OCT4-CPE amplicons), and the distal enhancer (OCT4-BDE and OCT4-ADE 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 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).
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 QChIP 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-DP, OCT4-DPE, and OCT4-DE regions. OCT4-CPE 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 H3K27me2 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 induces 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 to the examination of a heterogeneous cell population, or assuming that enzymatic(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, ADRI (whose promoter binding is promoted by loss of histone deacetylation; Verdon et al., 2002), HSF, GATA-1, GATA-2, and Sp1 elements (www.bcrjlp/jhbin/npf-search). The NANOG promoter region demethylated by extract treatment is rich in putative HSF; ADRI, 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
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 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 downregulation. 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.
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 hypermetylation of already di- and trimethylated H3K4, which mark genes either transcriptionally active (H3K4me3 or H3K4me2) or competent for transcription (H3K4me2) (Santos-Rosa et al., 2002). These changes are consistent with the remodeling of 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 EC cell extract requires ATP hydrolysis, most likely for nuclear import of transcription factors (Håkelien et al., 2003; Aalfs et al., 2001). OCT4 activation by ES cell extract or Xenopus egg extract also requires the BRG1 subunit of the SWI/SNF complex (Hansig 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 inactive 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 Wolfle, 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 Lidija Stijac for assistance with bisulfite sequencing. This work was supported by the Research Council of Norway (FUGE, STORFORSK, YFF, and STAMCELLE programs).

### REFERENCES


### 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

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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 5 wk after extract treatment. 293T and NCCIT cells were also exposed to 10 μM RA.
Epigenetic Reprogramming by Cell Extract


Supplementary Fig. 1

**OCT4 genomic sequence GenBank No. AJ297527**

- **Translation initiation start codon**
  - Dddd

- **Area covered by bisulfite primer pair:** Forward primer; reverse primer; overlapping primer (3' end of reverse/5' end of forward)
  - Area covered by bisulfite primer pair

- **Green:** Exons (mRNA sequence GenBank No.NM_002701)
  - Exons (mRNA sequence GenBank No.NM_002701)

- **CpG examined**
  - CpG examined

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Translation initiation start codon
Area covered by bisulfite primer pair; forward primer; reverse primer

Green: Exon (mRNA sequence GenBank No.NM_024865)
Putative transcription start site (TSS)
CpG examined

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Supplementary Fig. 1 (cont.)

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96 gaaaaacttac ccacctggac aatgctttct gctgagatgc ccacacccgca gactgtctct
156 cctctcttctct cctcctagga ctgctgattt cagagcaagct ctgattttcttc caccagctt
216 aaagccacac ccacccacac aagctgctcaaa aaggagagaga caggtcctca
**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)
- **Putative transcription start site (TSS)**
- **CpG examined**

```
-2606 gaatccaggg ttgcagtgag ctatgatcgt gccaccgcac tccaggtcgg gcgcacagcc
-2546 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-2486 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-2426 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-2366 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-2306 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-2246 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-2186 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-2126 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-2066 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-2006 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1946 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1886 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1826 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1766 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1706 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1646 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1586 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1526 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1466 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1406 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1346 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1286 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1226 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1166 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1106 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-1046 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-986 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-926 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-866 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-806 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-746 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-686 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
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-506 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
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-386 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-326 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-266 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-206 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-146 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-086 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
-26 aagattctca aaaaagagc gggggagtct aggacaggaa gcagctacgg cgcagctcag
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**Supplementary Table 1 (cont.)**

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<thead>
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<th>Sequence</th>
<th>Description</th>
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<td>Exon 1 (mRNA sequence GenBank No. NM_170707)</td>
<td>Putative transcription start site (TSS)</td>
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<td>Green:</td>
<td>Exon 1 (mRNA sequence GenBank No. NM_170707)</td>
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**Table 2 (cont.)**

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<td>Putative transcription start site (TSS)</td>
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<tr>
<td>Green:</td>
<td>Exon 1 (mRNA sequence GenBank No. NM_170707)</td>
</tr>
<tr>
<td>Putative transcription start site (TSS)</td>
<td>GenBank No. AL135927</td>
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**Supplementary Table 1.** Bisulfite sequencing and ChIP primers used in this study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer pair</th>
<th>Forward primer (F)</th>
<th>Reverse primer (R)</th>
<th>Seq. coverage relative to TSS</th>
<th>Annealing temp. (°C)</th>
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<tbody>
<tr>
<td><strong>Bisulfite sequencing primers</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCT4</td>
<td>Oct4-1</td>
<td>F: TTTTTAGTTTTTTTTAGGTTTA$^a$</td>
<td>R: TAAACAADAACCCTATTCC$^a$</td>
<td>-2995 to -2723</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Oct4-2</td>
<td>F: TTAGGAAATGGGTAGTAGGGATTT$^a$</td>
<td>R: TACCCAAAAAACAAATTTAATATTACACCT$^a$</td>
<td>-2609 to -2417</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Oct4-3</td>
<td>F: ATTTGTGTTTTGCTAGTTAAAGGT$^a$</td>
<td>R: CCAACTATCTCTCACTTTAATAACACCT$^a$</td>
<td>-2344 to -2126</td>
<td>58</td>
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<tr>
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<td>Oct4-4</td>
<td>F: GGATGGGAAAATTGTTAAGATGTTAGTGTTG$^a$</td>
<td>R: CCTAAACTCTCTTCCTAAAAATCTATT$^a$</td>
<td>-2136 to -1721</td>
<td>58</td>
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<tr>
<td></td>
<td>Oct4-5</td>
<td>F: AATAGATTTGGATGGAAGGGGAGTTAGG$^a$</td>
<td>R: TTCCCTCTCTCTCTCTAAAAACTCA$^a$</td>
<td>-1755 to -1574</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Oct4-6</td>
<td>F: GAAGGGGAAGTGGATGATATTAAAATT</td>
<td>R: CAACACCATAAAACACAAATAACAA$^a$</td>
<td>-1014 to -720</td>
<td>58</td>
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<tr>
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<td>Oct4-7</td>
<td>F: TAGTTGGAGTTGTAGTTAGTGTGAG $^a$</td>
<td>R: TAAACAAAACACCTCTCTCTCCTACCTC$^a$</td>
<td>-567 to -309</td>
<td>58</td>
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<td>Oct4-8</td>
<td>F: AAGTTTGTTTGGTCAGAGTTGATAT $^a$</td>
<td>R: CCACCACACTACAATACCTAACCTCTA$^a$</td>
<td>-215 to -29</td>
<td>58</td>
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<tr>
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<td>Oct4-9</td>
<td>F: GTTGAATGTTAAGGTTAGGTTG$^a$</td>
<td>R: AAACCTAAAAACTCTAAAAACTCA$^a$</td>
<td>-57 to +66</td>
<td>58</td>
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<td>NANOG</td>
<td>Nanog-1</td>
<td>F: AGAGATAGGAGGGATTAAGTTTTTTT</td>
<td>R: ACCTCCACACACAAACTTACATTAT</td>
<td>-1503 to -1254</td>
<td>58</td>
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<tr>
<td></td>
<td>Nanog-2</td>
<td>F: GAGTTAAGAGTTTTGTGGTTAAAATTAT</td>
<td>R: TCCCAAATCTATAATTTATCATATCTTTC</td>
<td>-1203 to -911</td>
<td>58</td>
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<tr>
<td></td>
<td>Nanog-3</td>
<td>F: TTAATTTATGAGTTACTATAGTGGGTTG$^a$</td>
<td>R: AAACCAAAAAACTAAAAACCAAACC</td>
<td>-334 to -163</td>
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<tr>
<td>LMNA</td>
<td>Lmna</td>
<td>F: GAAGGGTGATGTTATATGAGTTGGTAT</td>
<td>R: ACTCTTTAAAAAAACTAATCCAAATC</td>
<td>-277 to +92</td>
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<td><strong>ChIP primers</strong></td>
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<td></td>
<td></td>
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<tr>
<td>OCT4</td>
<td>Oct4-A</td>
<td>F: GAGGATGGCAAGCTGAGAAA</td>
<td>R: CTCAATCCCCAGGACAGAAC</td>
<td>-2546 to -2379</td>
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<td></td>
<td>Oct4-B</td>
<td>F: ACCCCACTGCTTGTAGACCT</td>
<td>R: CAGCCTGACCTTCGTCAGCTT</td>
<td>-2284 to -1986</td>
<td>60</td>
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<tr>
<td></td>
<td>Oct4-C</td>
<td>F: AGGCCCACTAAACAAAGCAC</td>
<td>R: GCAATCCCACCAAAAGCTGA</td>
<td>-1669 to -1508</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Oct4-D</td>
<td>F: GTTTGGGAGACGGAAGCA</td>
<td>R: GGGCAGCTCTCTACTACAACAA</td>
<td>-1435 to -1353</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Oct4-E</td>
<td>F: AGTCTGGCAGAACAAAGTGAGA</td>
<td>R: AGAAACTGAGAAGAGTGGAG</td>
<td>-262 to -94</td>
<td>60</td>
</tr>
<tr>
<td>NANO</td>
<td>Nanog</td>
<td>F: GAGGATGGCAGCTGAGAAA</td>
<td>R: CTCAATCCCCAGGACAGAAC</td>
<td>-2546 to -2379</td>
<td>60</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Gapdh</td>
<td>F: CTCGAGCAGCGGTGTCACATC</td>
<td>R: GAGGACTTTGGGAAGCGACTGAG</td>
<td>-486 to -320</td>
<td>60</td>
</tr>
</tbody>
</table>

$^a$ From Deb-Rinker et al. (2005).
**Supplementary Table 2.** Real time RT-PCR primers used in this study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer (F) 5’→3’</th>
<th>Reverse primer (R) 5’→3’</th>
<th>Annealing temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>F: TCWGAGTCAACGGATTTGGT</td>
<td>R: TTGCCATGGGTGGAATCATA</td>
<td>60</td>
</tr>
<tr>
<td><strong>LMNA</strong></td>
<td>F: CTTGTGTGTTGAGGAGCGAG</td>
<td>R: TGGCGTAGCTGCGAGGTA</td>
<td>60</td>
</tr>
<tr>
<td><strong>LMNB1</strong></td>
<td>F: AAGGCAGAAGAAGGAGTGGGAAG</td>
<td>R: GCGGAATGAGAGATGCTAACACT</td>
<td>60</td>
</tr>
<tr>
<td><strong>NANOG</strong></td>
<td>F: CAAAGGGCAAAACAACCCACTT</td>
<td>R: TCGGCTGAGGCTGAGTAT</td>
<td>60</td>
</tr>
<tr>
<td><strong>NES</strong></td>
<td>F: CACCTGTGCGAGCCCTTCCTTA</td>
<td>R: TTTCTTCCCACCTGTGTCT</td>
<td>60</td>
</tr>
<tr>
<td><strong>OCT4</strong></td>
<td>F: AAGCGATCAAGCAGCGACTAT</td>
<td>R: GGAAAGGGGACCAGGAGTACA</td>
<td>60</td>
</tr>
</tbody>
</table>
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