Thesis for the Master's degree in Molecular Biosciences
Main field of study in Molecular Biology.

Sanna K. Timoskainen

Treatment of epithelial 293T cells with thymocyte extract to induce RAG gene expression

60 study points

Department of Molecular Biosciences
Faculty of mathematics and natural sciences
UNIVERSITY OF OSLO 04/2006

Acknowledgements

Experimental work for this thesis was performed at the University of Oslo, Institute of Basic Medical Sciences, Department of Biochemistry. There are many people to acknowledge for making this year the most instructive and challenging year of my 'scientific' life, which has just begun. First of all I would like give my appreciations to my supervisor, Prof. Philippe Collas, who has given me this opportunity, helped and supported me throughout the whole year. Your enthusiasm and ideas are enormous inspiration for all of us. Thank you also for the amount of work and time you have put into this to help me to achieve this. I would also like to thank Prof. Jan Øivind Moskaug, who has been supporting me in my work and answering to all my questions throughout the whole year. I truly appreciate your advice and help. I also thank my supervisor at the Department of Molecular Bioscience, Prof. Odd S. Gabrielsen. I wish to thank Dr. Rønnaug Solberg, at the Department of Surgical Research, for providing us with fresh pig thymi. Special thanks to Christel and Kristine for all help and support that I have been given at all times. Moreover, I want to thank everybody else in the lab, Agate, Anita, Anne, Anne Mari, Andrew, Gaute, Helga, Inger Louise, John Arne, Lidija, Marie, Sumitra and Thomas. One always feels oneself welcome and is always met with a smile. Thanks to Line, Nezir and rest of my dearest friends that have always been there for me - caring, believing and supporting. I am also grateful for my family who has always believed in me and supported me in every possible way. I could not have managed this without you.

Table of contents

Abbreviations, gene names and cell lines

Abbreviations

ATP	Adenosinetriphosphate
CD3	T cell surface glygoprotein cd3
CD4	T cell surface glygoprotein cd4
CD8	T cell surface glygoprotein cd8
CD25	Interleukin-2 receptor α (IL-2Rα)
CD44	Phagocytic glycoprotein-1 (pgp-1)
CD117	Stem cell factor receptor (c-kit)
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT enhanser binding protein
ChIP	Chromatin immunoprecipitation
c-kit	CD117
CLB	Cell lysis buffer
Ct	Threshold cycle
CTP	Cytidine triphosphate
DNA	Deoxyribonucleic acid
DP	Double positive
Egr	Early growth response element
GATA-3	GATA binding protein
GTP	Guanosine triphosphate
HBSS	Hank's Balanced Salt Solution
H3K9	Lysine 9 of Histone 3
H3K4	Lysine 4 of Histone 4
MHC	Major histocompatibility complex
mRNA	Messanger ribonucleic acid
NBD	Nonamer binding domain
Pax-5	Paired box gene 5
PBS	Phosphate buffered saline
QRT-PCR	Real time quantitative RT-PCR
RAG1	Recombination activating gene 1
RAG2	Recombination activating gene 2
RNA	Ribonucleic acid
RSS	Recombination signal sequence
RT-PCR	Reverse transcriptase polymerase chain reaction
sca-1	Stem cell antigen 1
SLO	Streptolysin O
TCR	T cell receptor
Thy-1	Thymus cell antigen 1
TE	Tris-EDTA
TN	Triple negative
UTP	Uridine triphosphate

Cells and cell lines

ASC	Human adipose stem cells
EC	Human Embryonal carcinoma
INS-1E	Rat insulinoma cell line INS-IE
Jurkat	Human T lymphocyte cell line
293T	Human kidney epithelial cell line

Gene names

Brg1	Brahma-related gene 1
GAPDH	Glyceraldehyde-3-phosphatedehydrogenase
IL2	Interleukin 2
Oct-4	Octamer-4, a POU family transcription factor
RAG1	Recombination activating gene 1
RAG2	Recombination activating gene 2

Summary

This thesis reports an in vitro reprogramming approach with pig thymocyte extracts to induce expression of two lymphoid-specific recombination activating enzyme genes, *RAG1* and *RAG2*, in human kidney epithelial 293T cells. *RAG2* was upregulated in extract-treated cells 8 days after extract treatment, suggesting that some functional changes may have taken place in 293T cells exposed to thymocyte extract. *RAG2* upregulation was detected in high (22mg/ml) protein concentration thymocyte extract-treated cells. However, induced expression of *RAG2* was transient and decreased to basic level within 23 days. This transient expression might be explained by the nature of *RAG* expression in vivo, or it might indicate, most likely, incomplete reprogramming. Furthermore, upregulation of *RAG2* was not accompanied by *RAG1* expression or changes in morphology and growth pattern of 293T cells. Thus, we conclude that although *RAG2* upregulation is observed in extract-treated cells, further work is required to establish a proof-of-concept that RAG enzyme-initiated T cell receptor gene rearrangement can be manipulated experimentally in non-lymphocytic cells.

Introduction

Cell differentiation is a regulated process by which cells become committed to perform specific functions while loosing ability to perform others. Differentiation is accompanied by changes in chromatin structure, nuclear organization and gene expression. At least 200 distinct cell types have been identified in the human body. These cells collaborate with one another to form various tissues. Most tissues, such as bone marrow, skin, liver, brain, blood and adipose tissue, contain not only differentiated, specialized, cells, but also more pluripotent cells with stem cell properties. These so-called somatic stem cells are believed to be responsible for tissue homeostasis. Somatic stem cells divide to produce two daughter cells, of which the one will differentiate into a specific cell type, while the other daughter cell retains the stem cell function and contributes to maintaining the stem cell population. Somatic stem cells possess some degree of pluripotency and are able to give rise to several cell types. For example, bone marrow stromal stem cells can give raise to bone, fat, cartilage and fibrous connective tissue (Pittenger et al., 1999). Bone marrow contains another class of stem cells, the hematopoietic stem cells, which form all blood cell types in the body (Gunsilius et al., 2001; Weissman, 2000). Hematopoietic stem cells also give rise to specific types of progenitor cells, called thymocytes. These non-T cell lineage committed progenitors migrate from the bone marrow to the thymus where they differentiate into mature T lymphocytes (see below).

Evidence from nature and from experimental studies proposes that cell fate is not locked, but can in some instances be altered. This thesis explores the possibility of whether a non-T-cell type (such as an epithelial cell) can be instructed (or reprogrammed) to take on T-cell functions. An example of long term medical benefits of reprogramming a non-T

cell into a functional lymphocyte would be to enhance the immune system of cancer patients with low level of lymphocytes as a result of chemotherapy.

The thymus and thymic function

The thymus is our second primary lymphoid organ, located in the thorax just above the heart in humans (see Figure 1.). The thymus is a two-lobe organ, consisting of epithelial cells organized into a three-dimensional meshwork. Vertebrate thymus organogenesis depends on interactions of cells from all three germ layers, but the molecular mechanisms involved are not well established (Manley, 2000). The thymus undergoes morphological changes during life, being largest and most active at puberty, and regressing while being replaced largely by adipose tissue.

The thymus provides a microenvironment essential for T cell development. Immature (not T cell line committed) progenitor cells, arise from bone marrow and migrate

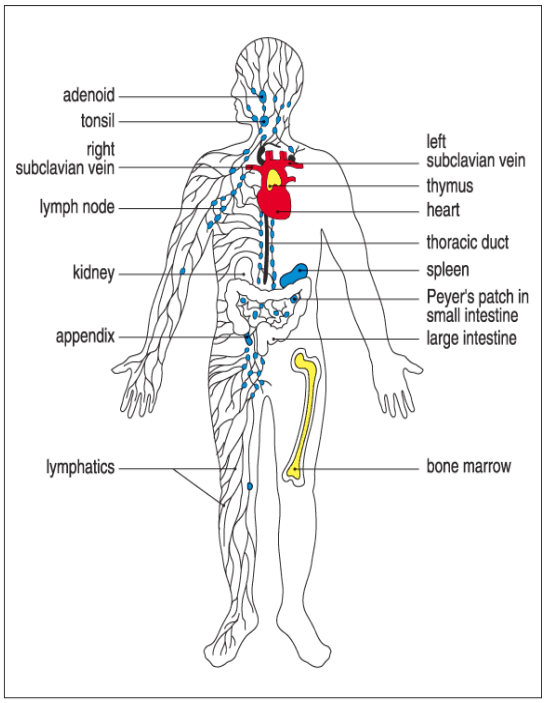

Figure 1. The thymus. Thymus is a complex epithelial organ located in upper chest cavity in humans. The thymus provides a microenvironment essential for T cell development. T cell precursors arise from bone marrow and migrate to thymus, where the combination of cellular interactions, cytokines and chemokines induce these progenitors (also called thymocytes) to undergo differentiation program leading to development of functional T cells. (Taken from http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=imm.figgrp.45)

to the thymus where they undergo highly specific maturation and selection processes to differentiate into T cells (also called T lymphocytes). While in the thymus, these progenitor cells are referred to thymocytes. During maturation of thymocytes into T cells, thymic epithelial cells secrete peptide factors (interleukins, thymosins, thymopoietin and thymulin) that influence T cell development. T cells that have successfully undergone all differentiation and selection processes are exported from the thymus and migrate to peripheral lymphoid sites.

T cell development

As thymocytes differentiate into T cells in the thymus, they start to synthesize T cell receptor (TCR) proteins and insert them into the plasma membrane. The T cell receptor is a heterodimeric (αβ or γδ) membrane bound receptor that helps initiate immune responses by recognizing fractions of antigens presented by major histocompatibility complex (MHC) proteins on the surface of antigen-presenting cells. During T cell development, TCR α, β, γ and δ genes are assembled from germ line V, D and J gene segments by a site-specific recombination reaction (called the V(D)J recombination) to compose functional TCRαβ or TCRγδ loci (Gellert, 1992; Tonegawa, 1983).

T-cell progenitors arise from bone marrow and migrate into the thymus. Although the multipotential nature of these early intrathymic progenitors is not well defined, it is generally accepted that the first stage of lineage commitment occurs prior to or immediately after thymic colonization. The earliest intrathymic progenitors cells exhibit a $CD4^{lo}$, $CD44^{+}$, Thy-1^{-},sca-1^{+}-, c-kit^{+} phenotype (Wu et al., 1991). These cells are not committed to the T cell lineage and retain the ability to differentiate into natural killer cells, dentritic cells, T cells or B cells (Ardavin et al., 1993; Matsuzaki et al., 1993; Moore and Zlotnik, 1995; Wu et al., 1996). The $CD4^{lo}$ cells are referred as triple negative cells

(TN), as they lack expression of CD3, CD4 and CD8 antigens ($CD3^-$, $CD4^-$, $CD8^-$). TN cells can further be subdivided according to expression of other cell surface markers (e.g., CD44, CD25, CD117). The TCR genes in $CD4^{lo}$ TN cells are shown to be in a germ line configuration (Wu et al., 1991), indicating that no rearrangement of TCR genes have taken place.

At the TN stage, cells begin to increase in size and rearrange their β, γ and δ-chains, leading to irreversible T cell lineage commitment (Bhandoola and Sambandam, 2006; Gill et al., 2003). The expressed TCRβ-chain combines with pre-TCRα-chain (encoded by non-rearranged alpha loci) and with a collection of proteins (the CD3/ζ complex) on the cell (Groettrup et al., 1993; Saint-Ruf et al., 1994). Thymocytes that have undergone productive TCR β-chain rearrangements are selected for further maturation (by β-selection) (Wiest et al., 1999). Cells emerging from β-selection start to express the cell surface markers CD4 and CD8, leading to double positive (DP) stage cells ($CD4^+$, $CD8^+$), and to rearrangement of the TCRα chain loci to produce the second component of the mature TCRαβ, the α-chain. Transition to the DP stage is promoted by the Egr family of transcriptional factors (Carleton et al., 2002) and is followed by a burst in proliferation. It is estimated that 90% of the lymphoid cells in the thymus of a young individual consist of immature DP TCRαβ-expressing cells.

After the transition to the DP stage, thymocytes undergo highly specific selection processes whereby cells that cannot produce a functional TCR are removed. Cells that are unable to recognize self-peptides die by neglect, while cells that recognize self-peptide-MHC are chosen for maturation by positive selection. Furthermore, cells that respond with too high affinity to self-peptides are deleted by negative selection. Thus, these selection processes ensure that mature T-lymphocytes are able to recognize and react against a wide variety of foreign molecules, although they are still not self-reactive. During thymic T-cell

development, it is estimated that ~95% of thymocytes are lost by these selection processes (Scollay et al., 1980). Cells that have successfully undergone thymic maturation to single positive ($CD4^+$ or $CD8^+$) cells are exported from thymus at a constant rate of 1-2% of total thymocytes per day. The exported cells mainly home into lymph nodes, the spleen and Peyer's patches (Scollay et al., 1980).

RAG1 and RAG2

The V(D)J recombination (where VDJ stands for variable, diversity and joining, respectively) is a site-specific recombination where the antigen receptors of T and B cells are created from V, D and J gene segments (Gellert, 1992; Tonegawa, 1983). The lymphoid-specific recombination activating genes *RAG1* and *RAG2* code for proteins essential for TCR synthesis by enabling V(D)J recombination (Oettinger et al., 1990). *RAG1* and *RAG2* are conserved between variety of species that carry out V(D)J recombination (Oettinger et al., 1990; Schatz et al., 1989). Furthermore, Rag1- or Rag2-deficient mice have shown not to develop mature lymphocytes and are immune-deficient, illustrating the importance of the RAG genes for lymphocyte development and establishment of a functional immune system (Mombaerts et al., 1992; Shinkai et al., 1992).

RAG1 and RAG2 mediate V(D)J recombination

RAG1 and RAG2 are the core components of the DNA recombinase that mediates the V(D)J recombination of TCR genes in T (and B) lymphocytes. Purified RAG1 and RAG2 alone are able to mediate recombination (McBlane et al., 1995). RAG1 and RAG2 direct DNA cleavage by recognizing recombination signal sequences (RSS) that flank the V, D and J gene segments of the TCR locus, creating double strand breaks at the border of the

RSS and coding flank (see Figure 2). This RAG-mediated cleavage occurs in a synaptic complex of two RSSs (Hiom and Gellert, 1997; van Gent et al., 1995). An RSS is composed of a conserved heptamer and nonamer separated by a spacer of either 12 or 23 base pairs. An efficient recombination occurs only between a 12-bp RSS and a 23-bp RSS (Tonegawa, 1983). Furthermore, RAG1 and RAG2 complexes are suggested to have the ability to recognize and open the hairpin coding ends that is important for the generation of junctional diversity during V(D)J recombination (Besmer et al., 1998; Shockett and Schatz, 1999).

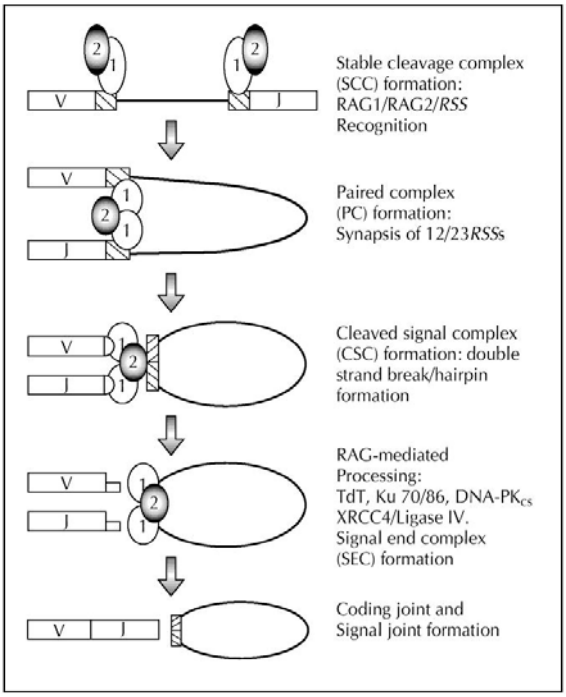

Figure 2. Schematic representation of the V(D)J recombination process. The white and grey circles represent the RAG1 and RAG2 proteins, and hatched boxes identify the Recombination Signal Sequences (RSS). RAG1 recognizes the RSS after which RAG2 is recruited and RAG protein complex is formed. The RAG1/RAG2 complex directs the DNA cleavage at the border of the RSS and the coding flank, leading to formation of blunt signal ends and hairpin coding ends. (Taken from Notarangelo et al., 2001)

RAG1 and RAG2 expression and regulation

In developing thymocytes, *Rag-1* and *Rag-2* are expressed in two waves which correlate with the rearrangement of TCR α and β chains (Wilson et al., 1994). First, *RAG1* and *RAG2* expression is induced at the triple negative (TN) stage, leading to TCR β-chain rearrangement. Expressed TCR-β chains combine to pre-TCR with CD3/ζ proteins, which leads to downregulation of the *RAG1* and *RAG2* genes. Induction of cell surface marker CD4 and CD8 expression leads to the transition of cells to the DP stage, where *RAG1* and

RAG2 expression is reinduced and TCR α-chain is rearranged. This *RAG1* and *RAG2* expression is not terminated until positive selection takes place. Although little is known about the regulation of *RAG1* and *RAG2* gene expression, *RAG*s can be downregulated both through specific stimuli (antigen presented by MHC molecules) (Brandle et al., 1992), and by non-specific stimuli, such as TCR cross-linking and treatment by phorbol esters (Turka et al., 1991). Regulation of chromatin structure and additional control elements outside promoter region has been shown to be responsible for tissue- and stage-specific expression of *RAG*s (Kitagawa et al., 1996; Kurioka et al., 1996; Wei et al., 2002).

Natural cell plasticity

Differentiated cells have long been thought to be committed to their fate, which is determined by their developmental history. However, there is natural and experimental evidence to indicate that cell type switching through both dedifferentiation and transdifferentiation processes can take place. Dedifferentiation refers to a process where differentiated cells progress towards a less differentiated state. Dedifferentiation is characterized by a loss of differentiation markers and a subsequent gain of proliferative capacity (Soll and Waddell, 1975; Takeuchi and Sakai, 1971). Transdifferentiation refers to the conversion of one differentiated cell type into another differentiated cell type (Tosh and Slack, 2002; Slack and Tosh, 2001).

Natural dedifferentiation

Classical evidence of dedifferentiation occurring in nature comes from urodele amphibians. In response to injury or damage, an adult newt can replace its limbs, jaws, lens and large sections of heart (Brockes and Kumar, 2002; Tosh and Slack, 2002). Furthermore, the outcome seems to depend on the plasticity (change in cell behaviour or

cell type in response to environmental cues) of differentiated cells that remain after tissue removal. After amputation of the limb, for example, differentiated cells localized to the zone of injury or amputation surface dedifferentiate and become blastemal cells (progenitor cells of the regenerate). These cells divide to form a blastema (a mesenchymal growth zone) and give rise to a new limb by a process of proliferation, differentiation and morphogenesis (reviewed in Brockes and Kumar, 2002; Tsonis, 2000).

Limb regeneration ability is limited in mammals. In contrast to urodele amphibian organ regeneration, mammalian regeneration generally utilizes pre-existing stem cells that differentiate to specific cell types (Carlson, 2005). However, there are sporadic instances of regeneration in mammals that show similarities to regeneration processes observed in amphibians. An example is ear hole closure in healer MRL mice (in contrast to non-healer BG mice), where similarities in mammalian and amphibian regeneration are seen at the histological and gene expression levels (Clark et al., 1998; Heber-Katz, 1999). Moreover, as regeneration involves replacement and restoration of adult tissue mass with normal architecture and function, the healer MRL mouse displays a capacity to attain full closure of ear punches with normal tissue architecture. No scar tissue formation is observed, as would be expected in the case of wound repair (Heber-Katz, 1999). Thus, ear hole closure is a form of regeneration (Clark et al., 1998; Heber-Katz, 1999).

Naturally occurring transdifferentiation

Evidence of naturally occurring transdifferentiation can be observed in insects, amphibians and in many vertebrates (Slack and Tosh, 2001). In mammals, a well-documented example of transdifferentiation is the appearance of hepatocytes in the pancreas of rats in response to treatments such as a copper deficient diet (Rao et al., 1988; Rao et al., 1989; Reddy et al., 1991). Copper deficiency has been shown to cause an irreversible depletion of over

80% of the acinar cells in the pancreas, combined with differentiation of hepatocytes from pancreatic ductular cells and interstitial cells (Rao et al., 1988; Rao et al., 1989; Reddy et al., 1991). Furthermore, pancreatic hepatocytes express several liver-specific genes, such as *Albumin* and *Urate oxidase* (Rao et al., 1988; Rao et al., 1989; Reddy et al., 1991). Another example of transdifferentiation is lens regeneration in amphibians after surgical removal (reviewed in Tosh and Slack, 2002; Tsonis, 2000). After lens removal, pigmented cells of the iris become depigmented and proliferate to form a new lens indistinguishable from the original (Tosh and Slack, 2002; Tsonis, 2000).

Experimental manipulations of cell fate

Evidence of cell plasticity in nature has led to experimental efforts to induce dedifferentiation and transdifferentiation of somatic cells in the laboratory. Changing the fate of a differentiated cell involves a reprogramming of the transcriptional program of that cell. Several lines of evidence indicate that cell fate can, at least to some extent, be manipulated – or reprogrammed. These reprogramming approaches include transplantation of a somatic nucleus into an enucleated egg, cell hybridization, and treatment with cytoplasmic and nuclear extracts prepared from a chosen "target" cell type.

Manipulation of cell function by nuclear transplantation

Perhaps the clearest evidence of nuclear reprogramming emanates from nuclear transplantation of somatic nuclei in to unfertilized oocytes and the birth of resulting cloned offspring. Injection of somatic nucleus of an adult *Xenopus laevis* into a *Xenopus* egg showed to yield normal hatched tadpoles (Gurdon, 1962). Similarly, *Xenopus laevis* somatic nuclei transplanted into a newt oocyte are also reprogrammed, as shown by the activation of genes normally inactive in somatic cells but active in early embryos (De

Robertis and Gurdon, 1977). Furthermore, permeabilized mammalian thymocyte nuclei can be reprogrammed after injection into *Xenopus* oocytes: injection was followed by induction of a pluripotency marker (*oct-4*) and down regulation of a differentiation marker (*thy-1*), demonstrating the transcriptional specificity of reprogramming (Byrne et al., 2003). DNA demethylation of the *oct-4* promoter was shown to be necessary for induction of *Oct-4* expression in these nuclei (Simonsson and Gurdon, 2004).

Several mammalian species have been cloned successfully from an adult somatic donor cell nuclei transplanted into unfertilized oocytes (Cibelli et al., 1998; Polejaeva et al., 2000; Wakayama et al., 1998; Wilmut et al., 1997). Nevertheless, mammalian cloning remains highly inefficient to date, as most reconstructed embryos fail to develop beyond implantation (Cibelli et al., 2002). This argues that complete functional reprogramming of somatic nuclear function, even through nuclear transplantation, remains a challenging issue.

Manipulation of nuclear function by cell fusion

Cell fusion experiments have provided evidence to indicate the reprogramming a somatic genome could be possible by placing a somatic nucleus into a distinct cellular environment (Blau and Blakely, 1999). In the late 1960s, cells were fused to create intra- and inter-species hybrids with separate nuclei; as such, these hybrids qualified as heterokaryons (Harris and Watkins, 1965). Fusion of human differentiated cells with chick erythrocytes created heterokaryons in which the chick nuclei was observed to swell and resume transcription to code for human proteins (Ringertz et al., 1971). Additionally, human muscle genes have been shown to be turned on in human skin cells (Blau et al., 1983) and hepatocytes (Miller et al., 1988) fused to myocytes. Therefore, studies involving somatic cell hybrids have provided evidence that cell fusion can alter cell fate. Importantly, the

creation of non-dividing heterokaryons showed that cell fate could be changed in absence of DNA replication or cell division (Blau and Blakely, 1999). These observations indicate that the ability to alter cell fate requires interaction of trans-acting molecules brought together by the cell fusion process. Interestingly, fusions of a differentiated cell with various stem cell types have further shown that epigenetic reprogramming, including DNA demethylation and histone modifications, can take place in the somatic nuclei (Kimura et al., 2004; Tada et al., 1997; Tada et al., 2001).

Functional and medical evidence for mammalian cell fusion in vivo has emerged from transplantation studies. Bone marrow cells transplanted into lethally irradiated *Mdx* mice (a mouse model of Duchenne muscular dystrophy) was shown to fuse with skeletal muscle myofibers (Gussoni et al., 2002). Furthermore a mouse with mutations in the *Fumarylacetoacetate hydrolase* gene that cause liver failure was observed to regain liver function and form regenerative normal liver nodules after transplantation of bone marrow cells, indicating fusion of donor bone marrow cells with host hepatocytes (Vassilopoulos et al., 2003).

Reprogramming with cell-free extracts

Nuclear transplantation and cell fusion approaches have been valuable in establishing proof of concept that differentiated cell nuclei could be reprogrammed. However, these systems are not readily amenable to manipulations for altering reprogramming conditions and identifying factors involved in nuclear reprogramming. Indications that nuclear and cytoplasmic factors from one cell type are capable of reprogramming the gene expression pattern and epigenetic status of the genome of another cell type led to cell extract-based reprogramming approaches (see Figure 3). Nuclear and cytoplasmic extracts prepared from

a “target” cell type are believed to contain regulatory factors essential to establish the transcriptional program specific for the “target” cell.

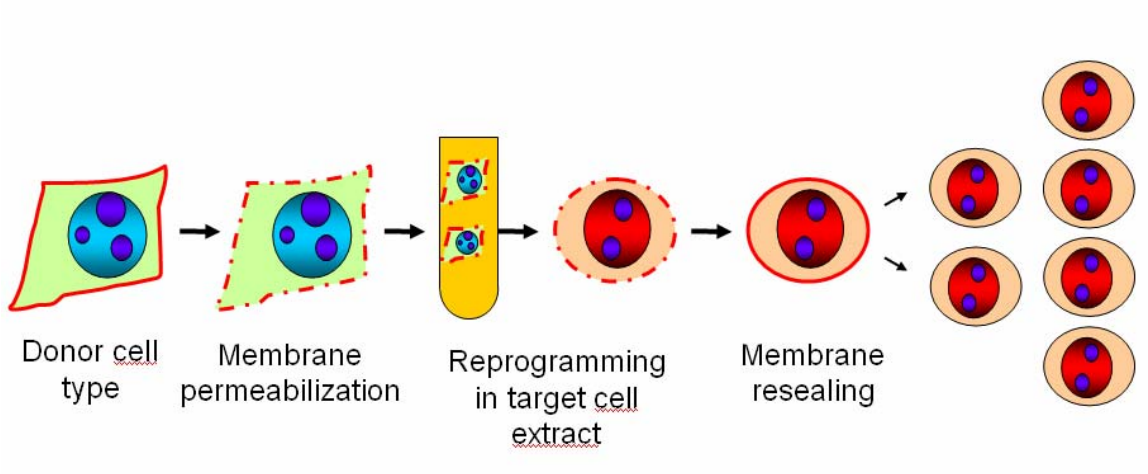

Figure 3. Schematic illustration of reprogramming system. Permeabilized donor cells are incubated in extract of target cell type and cell membranes are resealed with $CaCl_2$ containing complete medium. Extract-treated cells are cultured further and investigated for changes in transcriptional program. (Modified from Collas and Håkelien, 2003).

Extract from egg, embryos and various somatic cells have been developed to reproduce some aspects of nuclear function in vitro, and to manipulate and identify factors essential for nuclear reprogramming. The dynamics of the nuclear envelope and chromosomes at mitosis or at fertilization has been reproduced in extracts of *Xenopus* eggs (Lohka and Maller, 1985; Wilson and Newport, 1988), sea urchin eggs (Cameron and Poccia, 1994; Collas and Poccia, 1995), *Drosophila* embryos (Ulitzur et al., 1997), clam oocytes (Longo et al., 1994) and mammalian mitotic cells (Burke and Gerace, 1986; Collas et al., 1999). Extracts can also be used to perform nuclear functions such as DNA replication and transcription (Harish et al., 2001; Martins et al., 2003; Wang et al., 1995). Moreover, *Xenopus* egg extracts have been used to identify some of the molecules involved in adenosine trisphosphate (ATP)-dependent chromatin remodelling (Kikyo et al., 2000).

Cellular extracts have also been used to reprogram gene expression of somatic cells and nuclei. An illustration of dedifferentiation comes from the use of extracts of

regenerating newt limbs (McGann et al., 2001). After exposure to extract prepared from regenerating newt limbs, terminally differentiated murine myocytes were shown to re-enter the cell cycle and to downregulate muscle markers (McGann et al., 2001). Furthermore extracts from *Xenopus* eggs and early embryo have shown to promote upregulation of two pluripotency markers (*OCT4* and *germ cell alkaline phosphatase*) in 293T cells and leukocytes (Hansis et al., 2004). Moreover, the 293T cells showed downregulation of natriuretic peptide receptor and kidney markers (Hansis et al., 2004).

Our laboratory has also provided evidence that extracts of somatic cells of one type can at least partly reprogram the nucleus of another somatic cell type. Kidney epithelial 293T cells permeabilized with Streptolysin O (SLO) were exposed to extract of Jurkat T cells or primary human T cells (Håkelien et al., 2002; Håkelien et al., 2005; Landsverk et al., 2002). Extract-treated 293T cells were shown to acquire T cell functions, such as synthesis of IL2 following stimulation of the TCR-CD3 signalling pathway (Håkelien et al., 2002; Håkelien et al., 2005). Extract-treated cells also induced expression of T cell-specific genes, such as induction of *IL2* gene expression, and expression of the T cell-specific cell surface receptors CD3 and CD4 (Håkelien et al., 2002). Downregulation of genes encoding fibroblast growth factors, adhesion molecules and cytoskeletal proteins was also observed (Håkelien et al., 2002; Håkelien et al., 2005). Furthermore, extract-treated cells showed some evidence of chromatin remodelling, including histone acetylation and uptake of transcriptional regulators of the *IL2* gene, demonstrating the reprogramming capacity of somatic cell extracts (Håkelien et al., 2002; Landsverk et al., 2002). Moreover, extracts of cardiomyocytes or insulinoma cells have been shown to elicit expression of cardiomyocyte or beta-cell markers in human adipose stem cells (ASCs) (Gaustad et al., 2004) and fibroblasts (Håkelien et al., 2004). Permeabilized ASCs exposed to rat cardiomyocyte extracts prepared from perfused rat hearts induced expression of

several cardiomyocyte markers, including desmin, cardiac troponin I and sarcomeric α-actinin (Gaustad et al., 2004). Furthermore formation of binucleated and striated cell, as well as upregulation of expression of the cell differentiation marker, nuclear lamin A/C, provided additional evidence of an ongoing differentiation process induced by extract treatment (Gaustad et al., 2004). Moreover, the exposure of permeabilized primary rat fetal fibroblast to an extract of INS-1E rat insulinoma cells promoted morphological changes, activation of β-cell specific genes and insulin synthesis in the rat fetal fibroblasts (Håkelien et al., 2004). More recently, studies have been extended to show that undifferentiated human embryonal carcinoma (EC) cells could be used to reprogram 293T cells to take on properties of undifferentiated stem-like cells (Taranger et al., 2005). 293T cells exposed to EC extract indicated both changes in morphology and upregulation of several genes normally expressed in embryos or in EC cells (Taranger et al., 2005). Collectively, these studies indicate that cell extracts contain factors essential to elicit at least some form of trans- or dedifferentiation in somatic cell of another type.

This thesis examines whether the lymphocyte-specific *RAG1* and *RAG2* genes could be induced in human epithelial 293T cells by treatment with an extract of primary porcine thymocytes. The results show some indication of transient upregulation of *RAG2* by this approach.

Materials and Methods

Cells

Human kidney epithelial 293T cells (American Type Culture collection, Manassas, VA) were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal calf serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 1 mM sodium pyruvate (Invitrogen, Gibco-BRL, Paisley, UK), non-essential amino acids (Sigma-Aldrich, St. Louis, MO) and antibiotics (1% penicillin-streptomycin) (Invitrogen, Gibco-BRL, Paisley, UK) (complete RPMI-1640). When approximately 80% confluent, cells were harvested by tapping the flask and sub-cultured after a 1:8 or 1:10 dilution. 293T cells treated with cellular extracts (see below) were seeded at 10,000 to 40,000 cells per well of a 24-well plate (Corning, Corning, NY) and cultured in 700 µl of complete RPMI-1640. Extract-treated cells were harvested by transferring cell cultures into 24- or 6-well cell plates (Corning, Corning, NY) and further passaged by 1:3 to 1:8 dilutions. No trypsinization was required for passaging 293T or extract-treated cells.

Isolation of thymocytes from pig thymus

Pig thymocytes were obtained by isolation from freshly collected pig thymi. Thymi (from 2-36 hour old piglets) were collected at the Department of Surgical Research at the University of Oslo in ice-cold phosphate buffered saline (PBS) in 50 ml plastic tubes. A cell sieve (CD1; Sigma-Aldrich, St. Louis, MO) was placed in a 60 mm Petri dish and approximately 10 ml of cold complete RPMI-1640 was added to the sieve. The thymus was cut in smaller pieces which were placed in the sieve strainer. A cell suspension was prepared by pushing the tissue through the mesh with a glass pestle. The cell suspension was transferred into a 50 ml tube, centrifuged at 300 g for 10 min at 4^{o}C and washed 4

times by suspension and sedimentation in cold PBS. Erythrocytes were lysed by incubating the cell suspension in 25 ml of ATC buffer (2.06 g/l Tris Base, pH 7.2, and 7.49 g/l NH_4Cl). Remaining cells were washed twice in cold PBS. Connective tissue was observed as a white aggregate and removed with a pipette. Cells were counted and centrifuged at 300 g for 10 min at 4^{o}C. PBS was removed and cell pellets were quick-frozen in liquid nitrogen and stored at -80^{o}C until use.

Cell extracts

To prepare a whole cell extract from 293T cells, cells were harvested, washed twice in ice-cold PBS and once in cell lysis buffer (20 mM Hepes, pH 8.2, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM phenylmethylsulfonylfluoride), centrifuged at 300 g, resuspended in one volume of cell lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO; 10 µl 100x stock solution/ml cell lysis buffer) and incubated on ice for 45 min. Two hundred µl cell aliquots were sonicated using a Labsonic-M pulse 3-mm diameter probe sonicator (Model Labsonic M, B. Braun Biotech International; Melsungen, Germany; 1.5 min, 35% power, 0.4 sec intervals) until all cells and nuclei were lysed, as judged by phase contrast microscopy. Lysates were sedimented at 15,000 g for 15 min at 4 ^{o}C in a fixed angle rotor. The supernatant (extract) was transferred to a new 1.5 ml tube and aliquoted into 100 µl in 200 µl tubes, quick-frozen and stored at -80^{o}C. Protein concentrations of extracts ranged from 24.6 to 31.98 mg/ml (Bradford assay; BioRad, Hercules, CA), pH ranged from 6 to7 and osmolarity was 375-387 mOsm (measurements from 3 extract batches).

Thymocyte extracts were prepared from frozen thymocytes isolated as described above. Thymocytes were thawed by warming the tube by hand, pellet size was estimated and cells were resuspended in one volume of ice-cold cell lysis buffer with protease

inhibitor cocktail. The extract was prepared as described for 293T cells. Protein concentrations of extracts ranged from 6.25 mg/ml to 22.5 mg/ml, pH ranged from 6 to 7 and osmolarity was 368-395 mOsm (measurements from 3 extract batches).

Treatment of 293T cells with thymocyte extract

293T cells were harvested and washed twice in cold PBS and once in cold Hank's Balanced Salt Solution (HBSS, Ca^{2+}/Mg^{2+}-free; Sigma-Aldrich, St. Louis, MO). One aliquot of 100,000 cells/100 µl HBSS was prepared for each reprogramming reaction and samples were preheated at 37^{o}C for 2 min.

Membrane permeabilization with Streptolysin O (SLO)

An SLO working solution was prepared by thawing one aliquot of SLO stock (Sigma-Aldrich, St. Louis, MO; at 100 µg/ml), and diluting it 1:10 in HBSS on ice. One µl of ice-cold SLO working solution was then added to each tube containing 100,000 cells. This produces an SLO concentration of 100 ng/ml. Samples were incubated horizontally for 30 min in a 37^{o}C water bath and placed on ice. SLO was diluted by adding 100 µl ice-cold HBSS and samples were centrifuged at 120 g for 10 min at 4 ^{o}C in a swing-out bucket rotor.

Extract treatment

Following membrane permeabilization, cells were resuspended in 100 µl of thymocyte extract of high or low protein concentration, supplemented with an ATP-regenerating system (2.5 mM ATP, 62.5 µg/ml creatine kinase and 25 mM creatine phosphate; Sigma-Aldrich, St. Louis, MO), 125 mM guanosine triphosphate (GTP; Sigma-Aldrich, St. Louis, MO) and with a nucleoside trisphosphate mix (1 mM each of ATP, GTP, CTP, UTP;

Roche; Basel, Switzerland) in 1.5 ml tubes. Cells were incubated horizontally for 50 min in a 37^{o}C water bath, with occasional agitation.

Plasma membrane resealing

Plasma membranes were resealed following extract treatment by incubation in $CaCl_2$-containing medium. Complete RPMI-1640 containing 2 mM $CaCl_2$ (Sigma, St. Louis, MO) was prepared, preheated to 37^{o}C and 100 µl was added in each tube containing extract-treated cells. Cells were transferred into a 24-well culture plate, 500 µl of complete RPMI-1640 with 2 mM $CaCl_2$ were added to each well and cells were incubated for 2 h at 37^{o}C. Cell cultures were then observed by phase contrast microscopy to monitor adhesion to the bottom of the well. Floating cells and $CaCl_2$-containing medium were removed and replaced with complete RPMI-1640 for further culture. Control 293T cells treated with 293T cell extracts were treated and cultured as above.

Cell pellet preparation

Cell pellets were prepared for transcription analysis from 0.5–1x10^{6} 293T cells exposed to thymocyte extract of high or low protein concentration at indicated time periods after extract treatment (see Results). Cells were harvested and counted. Appropriate amount of cells were transferred to 1.5 ml or 15 ml tube and centrifuged at 1,000 g for 10 min at room temperature. Cell pellets were washed once in PBS and centrifuged at 1,000 g for 10 min. The supernatant was removed and the cell pellets were quick frozen in liquid nitrogen and stored at -80^{o}C.

RNA isolation

Total RNA was isolated from cell pellets using a Qiagen RNeasy mini Kit (Qiagen, Hilden, Germany) as described by the manufacturer. In short, buffer RLT containing 1% (v/v) 2-mercaptoethanol was added to the cell pellet and cells were homogenized through a 21-gauge needle. One volume of 70% ethanol was added and the lysate was homogenized by pipetting. The lysate was removed to an RNeasy column and centrifuged at 9,000 g for 15 sec. The flow through was discarded and the collection tube was replaced. Buffer RW1 was added to the sample and centrifuged at 9,000 g for 15 sec. The RNeasy column was placed into a new collection tube, buffer RPE was added and the sample was centrifuged as above. This step was repeated once. The RNeasy column was replaced into a new collection tube and centrifuged at 18,000 g for 1 min. The RNeasy column was placed into a 1.5 ml tube and 30-50 µl of RNase-free water were added. The sample was centrifuged at 9,000 g for 1 min.

DNase treatment of RNA

The RNA preparation was treated with DNase to remove any contaminating genomic DNA. We used two protocols for this purpose, 1) DNase treatment in a column (RNase-free DNase set; Qiagen, Hilden, Germany) and 2) RQ1 DNase treatment (Promega, Madison, WI). The first protocol was done during the RNA isolation in the RNeasy column. RNA isolation was as described above, until buffer RW1 was added. The sample was centrifuged at 9,000 g for 15 sec and 10 µl of DNase I solution (prepared by devolving solid DNase (1500 Kunitz units) in 550 µl RNase-free water) mixed with 70 µl Buffer RDD was added to the silica-gel. The sample was incubated for 15 min, buffer RW1 was added and the sample was centrifuged at 9,000 g for 15 sec. The column was placed into a new collection tube, buffer RPE was added and the sample was centrifuged at 9,000 g for 2 min. The column was replaced into a new collection tube and centrifuged at 18,000 g for 1

min. Next, the column was placed into a 1.5 ml tube, 50 µl of RNase free water was added and the sample was centrifuged at 9,000 g for 1 min. RNasin (Promega, Madison, WI; RNasin® Plus RNase Inhibitor; 2 µl/50 µl RNA) was added to the flow-through. The resulting RNA was used as template for cDNA synthesis.

In the RQ1 DNase treatment, 6 µl RQ1 10x buffer, 2 µl RQ1 DNase and 2 µl of RNasin (Promega, Madison, WI; RNasin® Plus RNase Inhibitor) were added per 50 µl RNA preparation and the mixture was incubated for 20 min in a 37 ^{o}C water bath. After incubation, RNA was cleaned up from the DNase reaction by adjusting the sample to 100 µl with RNase-free water. Next, buffer RLT containing 1% (v/v) of 2-mercaptoethanol was added and all reagents were mixed well. 96% ethanol was added to the column and the sample was removed to the RNeasy column in a 2 ml collection tube and centrifuged at 9,000 g for 15 sec. The column was placed into a new collection tube, buffer RPE was added and the sample was centrifuged as above. The flow through was discarded, buffer RPE added and the sample was centrifuged at 9,000 g for 2 min. The RNeasy column was placed into a new collection tube and centrifuged at 18,000 g for 1 min. The column was placed into a 1.5 ml tube, 50 µl of RNase-free water was added and the sample was centrifuged at 9,000g for 1 min. RNasin (Promega, Madison, WI; RNasin® Plus RNase Inhibitor; 2 µl/50 µl RNA) was added to the flow-through. The resulting RNA was used as template for cDNA synthesis.

cDNA synthesis

cDNA was prepared from 1 µg RNA using the iScript™ cDNA synthesis Kit (BioRad, Hercules, CA). Four µl of 5x iScript reaction mix, 1 µl iScript reverse transcriptase, 0-15 µl nuclease–free water and 1 µg RNA were mixed in a total volume of 20 µl. cDNA synthesis without reverse transcriptase was performed to control for the presence of

genomic DNA for each RNA preparation. cDNA synthesis conditions were 25^{o}C for 5 min, 42^{o}C for 30 min and 85^{o}C for 5 min. cDNA samples were stored at -20^{o}C.

Primer design

Nucleotide sequences for pig (*Sus scrofa*) *RAG1* and *RAG2* were obtained from the NCBI database using Nucleotide search. Primers specific for pig *RAG1* and *RAG2* were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and Net primer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) programs. The Primer 3 program picks out primers from a given DNA sequence and proposes the best primer alternatives. The Net primer program analyses primer sequence for dimerization, hairpin formation and %CG. Nucleotide sequences for human (*Homo sapiens*) *RAG1* and *RAG2* were obtained from the NCBI database using Nucleotide search. Human primers for *RAG1* and *RAG2* were designed using the oligo6-primer design program (htpp://oligo.net). Primer sequences used in this study are shown in Table 1 and Figures 1A and 1B.

Homology determination

The homology between human and pig *RAG1* and *RAG2* coding sequences and between human and pig nucleotide sequences amplified by human and pig RAG1 and RAG2 primers were determined by two sequence BLAST (Tatusova and Madden, 1999). Sequence alignment was generated by this tool using the BLAST engine.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR reactions were carried out in triplicates using IQ TM SYBRGreen Superscript (BioRad, Hercules, CA) on a BioRad MyCycler Thermocycler. Amplification was carried out in a total volume of 25 µl in an Icycler IQ™ PCR 96 well

plate (BioRad, Hercules, CA). Reaction mixture was composed of 12.5 µl IQ TM SYBR Green supermix (BioRad, Hercules, CA), 1 µl of a primer set (0.4 mM of each primer), 1 µl of cDNA mixed with 4 µl H_2O or Tris-EDTA (TE, pH 7.6) and 6.5 µl H_2O. RT-PCR conditions were 95^{o}C for 3 min, and 40 cycles of 95^{o}C 30 sec, 60^{o}C 30 sec and 72^{o}C 30 sec. Melting point analysis started at 65^{o}C, with an increase of 0.5^{o}C every 10 sec up to 95^{o}C. PCR products were stored at -20^{o}C. The MyiQ Single-Color Real Time PCR Detection software was utilized for data analysis.

The relative expression levels of human *RAG1* and *RAG2* in extract treated cells compared to untreated 293T cells were calculated from mean threshold cycle (Ct) values and PCR effiency of each primer (Pfaffl, 2001). PCR efficiency of each primer pair used was determined by the LinREG program (Ramakers et al., 2003). The human housekeeping gene GAPDH was used as a reference gene for normalization of data. RT-PCR primers for human and pig RAG1 and RAG2 and human GAPDH are shown in Table 1.

Analysis of human *RAG1* and *RAG2* expression

In order to investigate *RAG1* and *RAG2* expression in extract-treated 293T cells, we determined the relative expression of *RAG1* and *RAG2* in untreated 293T cells compared to pig thymocytes. cDNA from 293T cells and thymocytes was analyzed in triplicates by QRT-PCR using human RAG1 and RAG2 primers. Ratios between human *RAG1* and *RAG2* expression in 293T cells compared to thymocytes were calculated from assumed PCR efficiency of 80% (expressed as 1.8 in calculations) raised to the power of difference between a mean threshold value (Ct) of one triplicate and a mean threshold value (Ct) of another triplicate ($1.8^{(\text{mean Ct of triplicate 1} - \text{mean Ct of triplicate 2})}$).

Agarose gel electrophoresis

1.5% agarose gel electrophoresis with ethidium bromide staining was used to visualize RT-PCR products. Products were electrophoresed for ~ 20 min at 100 V. Gels were visualized using a GEL DOC 2000 system (BioRad, Hercules, CA). Product sizes were determined using 123 bp (Invitrogen, Carlsbad, CA) and 100 bp (Promega, Madison, WI) ladders.

Framework and objectives of the study

In vitro reprogramming of gene expression may be useful for investigation of processes regulating gene expression and for, potentially, producing replacement cells for the treatment of a wide variety of diseases (Collas and Håkelien, 2003). The research group of Philippe Collas has shown that somatic cell extract can be used to reprogram gene expression in differentiated nuclei of permeabilized human epithelial 293T cells. **The objective of this study was to test the hypothesis that 293T cells could be induced to express lymphoid cell-specific *RAG1* and *RAG2* genes by treatment with extract of primary pig thymocytes.** Because the large numbers of cells are required to prepare reprogramming extract, isolation of thymocytes from pig thymus is therefore a well suitable method to obtain cells for reprogramming experiment, offering an unlimited access to materials.

The specific aims of the study were to:

1. Design quantitative RT-PCR primers for human and pig *RAG1* and *RAG2* transcripts.

2. Isolate thymocytes from pig thymuses and prepare reprogramming extracts from these thymocytes.

3. Examine changes in 293T cell morphology over time after exposure to thymocyte extract.

4. Investigate whether extract of pig thymocytes can induce expression of *RAG1* and *RAG2* in 293T cells.

Results

Specificity of human and porcine RAG1 and RAG2 RT-PCR primers

This work relies on the use of porcine thymocyte extracts to reprogram human epithelial 293T cells in order to induce *RAG1* and *RAG2* expression. To this end, we needed a set of primers specific for human and pig *RAG1* and *RAG2* transcripts. *RAG1* and *RAG2* sequences were found to be highly homologous between pigs and humans, with a homology of 88% for *RAG1* and 91% for *RAG2*. Coding sequences for human and pig *RAG1* and *RAG2* genes are shown in Figures 4A and 4B. To distinguish between human and pig *RAG1* and *RAG2* transcripts, primers for both human and pig RAG1 and RAG2 were designed using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), the Net primer program (www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) and the Oligo6 program (http://oligo.net). Positions of primers designed for this work are shown in Figures 4A and 4B. Human and pig primers were designed within a common exon, since exon spanning primers for human and pig *RAG1* and *RAG2* sequences gave a too high %CG value to be accepted for primer design (a high %CG correlates with low primer specificity). Homologies between human and pig sequences amplified by human RAG1 and RAG2 primers were 93% and 87%, respectively, and those between human and pig sequences amplified by pig RAG1 and RAG2 primers were 84% and 90%, respectively.

Next, we tested the species-specificity of human and pig primers by RT-PCR analysis and visualized PCR products by 1.5% agarose gel electrophoresis and ethidium bromide staining. Human RAG1 and RAG2 primers amplified PCR products from 293T cell cDNA of expected sizes, namely 186 and 147 bp, respectively (Fig. 4C, left panel, lanes 1, 2); however, pig primers did not amplify any product from 293T cell cDNA (Fig.

A Pig versus Human RAG 1 nucleotide sequences **Fig.4**


B Human versus Pig RAG2 nucleotide sequences
H ATGTCTCTGCAGATGGTAACAGTCAGTAATAACATAGCCTTAATTCAGCCAGGCTTCTCACTGATGAATTTTGATGGACAAGTTTTCTTCTTTGGACAAAAAGGCTGGCCCAAAAGATCCTGCCCCACTGGAGTTTTCCATCT
P ATGTCACTACAGATGATAACAGTTGGTAATAACATGGCCTTAATTCAGCCAGGCTTCTCATTGATGAATTTTGATGGGCAAATCTTCTTCTTTGGCCAAAAAGGCTGGCCCAAGAGGTCCTGCCCCACTGGAGTTTTTCATTT
H GGATGTAAAGCATAACCATGTCAAACTGAAGCCTACAATTTTCTCTAAGGATTCCTGCTACCTCCCTCCTCTTCGCTACCCAGCCACTTGCACATTCAAAGGCAGCTTGGAGTCTGAAAAGCATCAATACATCATCCATGGAG
P TGATGTAAAGCATAACCATCTCAAACTGAAGCCTGCACTTTTCTCTAAGGATTCCTGCTACCTTCCTCCTCTCCGCTACCCAGCCACTTGCACATTCAAAAGCAGCTTAGAGTCTGAAAAACATCAGTACATCATCCATGGAG
H GGAAAACACCAAACAATGAGGTTTCAGATAAGATTTATGTCATGTCTATTGTTTGCAAGAACAACAAAAAGGTTACTTTTCGCTGCACAGAGAAAGACTTGGTAGGAGATGTTCCTGAAGCCAGATATGGTCATTCCATTAAT
P GGAAAACACCAAATAATGAGCTTTCGGATAAGATTTATGTCATGTCTGTGGTTTGCAAGAACAACAAAAAAGTTACTTTTCGCTGCAGAGAGAAAGACTTGGTAGGAGATGTTCCTGAAGGCAGATATGGTCATTCCATTGAT
H GTGGTGTACAGCCGAGGGAAAAGTATGGGTGCTCTCTTTGGAGGACGCTCATACATGCCTTCTACCCACAGAACCACAGAAAAATGGAATAGTGTAGCTGACTGCCTGCCCTGTGTTTTCCTGGTGGATTTTGAATTTGGGTG
P GTCGTGTATAGTCGAGGGAAAAGTATGGGTGTTCTCTTTGGAGGACGGTCATACATCCCTTCTGCTCAAAGAACCACAGAAAAATGGAATAGTGTAGCTGACTGCCTGCCCCACATTTTCTTGGTAGATTTTGAATTTGGTTG
pRAG2 5595L20
H TGCTACATCATACATTCTTCCAGAACTTCAGGATGGGCTATCTTTTCATGTCTCTATTGCCAAAAATGACACCATCTATATTTTAGGAGGACATTCACTTGCCAATAATATCCGGCCTGCCAACCTGTACAGAATAAGGGTTG
P CTCTACATCATACATTCTTCCAGAACTTCAAGATGGGCTATCTTTTCATGTCTCCATTGCCAGAAATGATACCATTTATATTTTAGGAGGACACTCACTTGCCAATAACATCCGTCCTGCCAATCTATATAAAATAAGGGTTG
pRAG2 5827R19
H ATCTTCCCCTGGGTAGCCCAGCTGTGAATTGCACAGTCTTGCCAGGAGGAATCTCTGTCTCCAGTGCAATCCTGACTCAAACTAACAATGATGAATTTGTTATTGTTGGTGGCTATCAGCTTGAAAATCAAAAAAGAATGATC
P ATCTCCCCCTGGGTAGCCCAGCTGTGACTTGCACAGTCCTGCCAGGAGGAATCTCTGTCTCCAGTGCAATCCTGACTCAAACGAGCAGTGATGAATTTGTTATTGTTGGTGGCTATCAGCTTGAAAATCAAAAAAGAATGGTC
hRAG2 1133L24
H TGCAACATCATCTCTTTAGAGGACAACAAGATAGAAATTCGTGAGATGGAGACCCCAGATTGGACCCCAGACATTAAGCACAGCAAGATATGGTTTGGAAGCAACACGGGAAATGGAACTGTTTTTCTTGGCATACCAGGAGA
P TGCAACATCATCTCTTTCAAGGACAACAAGATAGGAATTCATGAGATGGAAACTCCAGATTGGACCCCAGATATTAAGCACAGCAAGATATGGTTTGGAAGCAACATGGGAAATGGAACCGTTTTCCTTGGCATACCAGGAGA
hRAG2 1255R25
H CAATAAACAAGTTGTTTCAGAAGGATTCTATTTCTATATGTTGAAATGTGCTGAAGATGATACTAATGAAGAGCAGACAACATTCACAAACAGTCAAACATCAACAGAAGATCCAGGGGATTCCACTCCCTTTGAAGACTCTG
P CAATAAACAGGCTCTTTCAGAAGCATTCTATTTCTATACATTGAAATGTACTGAAGACGATGTGAACGAAGATCAAAAAACATTCACAAATAGTCAGACATCAACAGAAGATCCAGGGGACTCCACTCCCTTTGAAGACTCAG
H AAGAATTTTGTTTCAGTGCAGAAGCAAATAGTTTTGATGGTGATGATGAATTTGACACCTATAATGAAGATGATGAAGAAGATGAGTCTGAGACAGGCTACTGGATTACATGCTGCCCTACTTGTGATGTGGATATCAACACT
P AAGAATTTTGTTTCAGTGCAGAAGCAAATAGTTTTGATGGTGATGATGAATTTGACACCTATAACGAAGATGATGAGGAAGATGAGTCTGAGACGGGCTACTGGATTACATGCTGCCCTACTTGTGATATGGATATCAACACT
H TGGGTACCATTCTATTCAACTGAGCTCAACAAACCCGCCATGATCTACTGCTCTCATGGGGATGGGCACTGGGTCCATGCTCAGTGCATGGATCTGGCAGAACGCACACTCATCCATCTGTCAGCAGGAAGCAACAAGTATTA
P TGGGTACCATTTTATTCAACTGAGCTCAACAAACCTGCCATGATCTACTGCTCTCATGGAGATGGGCATTGGGTCCATGCCCAGTGCATGGATCTGGCAGAACACACACTCATCCATCTGTCAGAAGGAAGCAGCAAGTATTA
H CTGCAATGAGCATGTGGAGATAGCAAGAGCTCTACACACTCCCCAAAGAGTCCTACCCTTAAAAAAGCCTCCAATGAAATCCCTCCGTAAAAAAGGTTCTGGAAAAATCTTGACTCCTGCCAAGAAATCCTTTCTTAGAAGGT
P CTGCAAGGAGCATGTGGAGATAGCAAGAGCACTGCAAACCCCCAAAAGAGTTTTACCCTTAAAAAAGCCTCCACTGAAATCCCTCCACAAAAAAGGTTCTGGGAAAATTATTACCCCTGCCAAGAAATCCTTTCTTAGAAGAT
H TGTTTGATTAG
P TATTCGATTAG

Fig. 4 (cont.)

4C, left panel, lanes 3, 4). Human RAG1 and RAG2 primers also amplified PCR products from pig thymocyte cDNA with expected sizes (186 and 147 bp, respectively; Fig. 4C, right panel, lanes 5, 6). Pig RAG1 and RAG2 primers also amplified products of expected size (222 bp and 251 bp, respectively) from thymocyte cDNA (Fig. 4C, right panel, lanes 7, 8). These results indicate that whereas the human primers detect both human and porcine transcripts, the pig primers are pig-specific.

Verification of this contention was provided by analysis of melting curves. Figure 4D shows the melting curve analysis for RT-PCR products shown in Figure 4C. Since each nucleotide sequence has a characteristic melting temperature, melting curves can be used

Fig 4. (cont.)

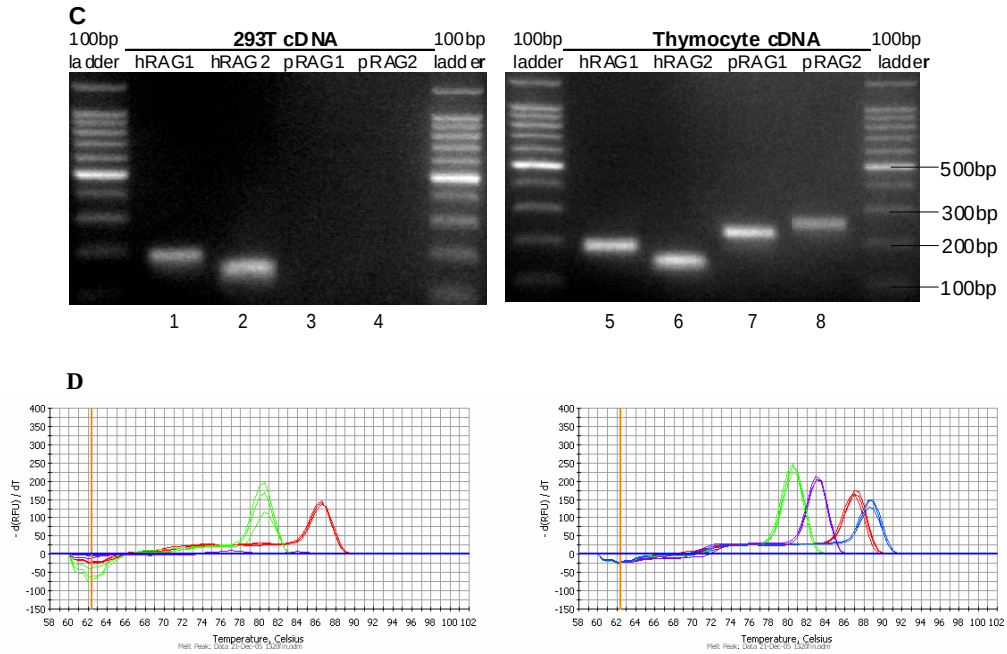

Figure 4. Specificity of human and pig RAG1 and RAG2 QRT-PCR primers. (A) Human (h, black) and pig (p, green) *RAG1* nucleotide sequences. Position of the human and pig RAG1 primers is indicated by dotted arrows (hRAG1 and pRAG1, respectively). Human and pig *RAG1* GeneBank accession numbers are NM_000448 and AB091392, respectively. (B) Human (h, black) and pig (p, green) *RAG2* nucleotide sequences. Position of the human and pig RAG2 primers is indicated by dotted arrows. Human and pig *RAG2* GeneBank accession numbers are NM_000536.1 and AB091391.1, respectively. (C) Agarose gel visualization of QRT-PCR analysis of primer specificity. Left, QRT-PCR analysis of *RAG1* and *RAG2* transcripts from 293T cells using human (h) and pig (p) primers. Right, QRT-PCR analysis of *RAG1* and *RAG2* transcripts from thymocytes using human (h) and pig (p) primers. (D) Melt curve analysis of quantitative RT-PCR products shown in (C). Left, melting curves for QRT-PCR products from 293T cell cDNA amplified with human RAG1 (red) and RAG2 (green) primers . Pig primers gave no products. Right, melting curves for QRT-PCR products from thymocyte cDNA amplified with human RAG1 (red), RAG2 (green) and pig RAG1 (blue) and RAG2 (purple).

to determine whether samples contain non-specific products (e.g., primer dimers). 293T cDNA amplified with human RAG1 and RAG2 primers gave RT-PCR products with melting temperatures of 86.6^{o}C for human RAG1 and 80.5^{o}C for human RAG2 (Fig 4D, left panel hRAG1 [red] and hRAG2 [green]. Based on these data, we used melting temperatures for human RAG1 and RAG2 as an indication of specific QRT-PCR products when 293T cDNA was amplified with the human RAG1 and RAG2 primers. Thymocyte cDNA amplified with human RAG1 and RAG2 primers gave PCR products with similar melting temperatures as with 293T cDNA (RAG1 [red], 87^{o}C; RAG2 [green]: 80.5^{o}C, Fig 4D, right panel). This indicates that human RAG1 and RAG2 primers also amplify specific products from thymocyte cDNA. Pig RAG1 and RAG2 primers with thymocyte cDNA amplified products with melting temperatures of 88.5^{o}C for RAG1 and 83.0^{o}C for RAG2 (Fig. 4D, right panel, RAG1 [blue] and RAG2 [purple]). We used the melting temperatures of pig RAG1 and pig RAG2 to specifically verify amplification of pig *RAG1* and *RAG2* transcripts. These melting curve analyses indicate that human primers amplify specific products with characteristic melting temperatures from 293T and thymocyte cDNA. Pig primers amplify only thymocyte cDNA giving PCR products with characteristic melting temperatures that reflect pig *RAG1* and *RAG2* transcripts. Furthermore, none of the QRT-PCR products were shown to contain non-specific products.

Altogether, these results indicate that the human RAG1 and RAG2 primers can amplify both human and pig transcripts, whereas the pig primers are specific for pig transcripts. Therefore, human primers were used in this study to detect any upregulation or activation of human *RAG1* and *RAG2* genes in 293T cells exposed to thymocyte extract, but only when used in addition to pig primers. Indeed, pig primers were used to determine whether the mRNA preparation of extract-treated 293T cells contained pig *RAG* mRNA of extract origin.

Relative levels of *RAG1* and *RAG2* expression in 293T cells and pig thymocytes

To investigate the induction of *RAG1* and *RAG2* expression in 293T cells exposed to thymocyte extract, we determined the relative levels of *RAG1* and *RAG2* expression in untreated 293T cells compared to thymocytes, using human RAG1 and RAG2 primers. Ratios of *RAG1* and *RAG2* expression in 293T cells relative to thymocytes were calculated from a calculated PCR efficiency of 80%. Figures 5A and 5B indicate that *RAG1* and *RAG2* were expressed 22- and 238-fold higher, respectively, in thymocytes compared to 293T cells. Melting curve analysis provided correct melting temperatures for the QRT-PCR products (Fig. 5C). Although *RAG1* and *RAG2* expression was detected in

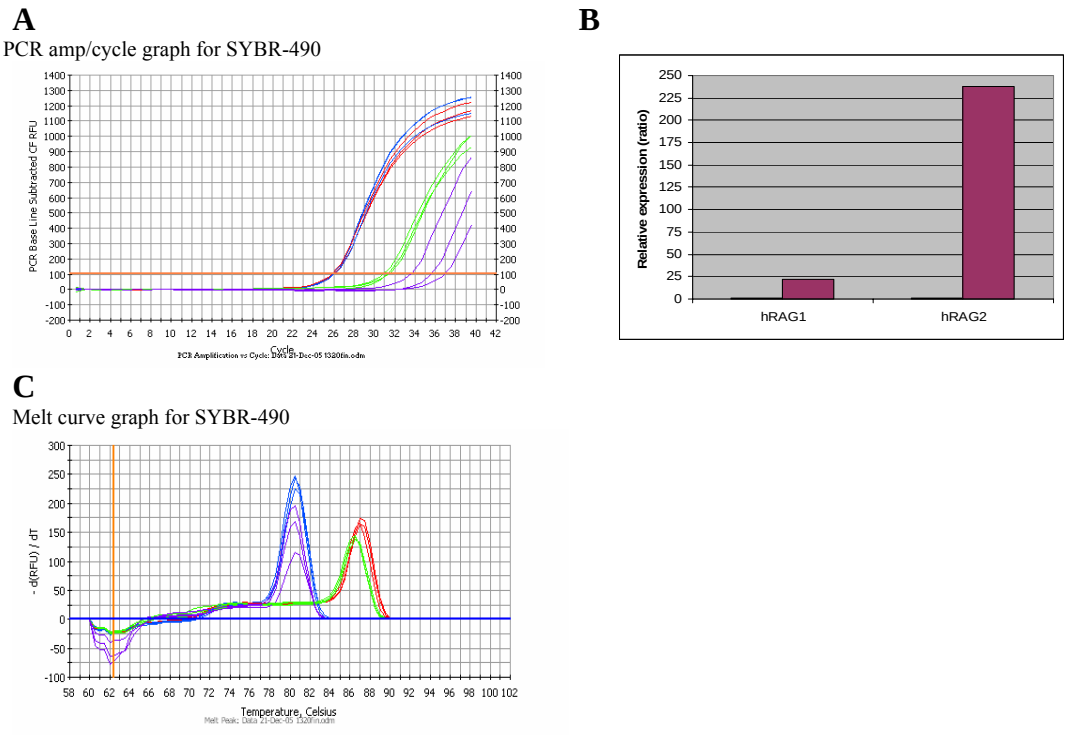

Figure 5. Relative levels of *RAG1* and *RAG2* expression in 293T cells and pig thymocytes. (A) Quantitative RT-PCR analysis of *RAG1* and *RAG2* transcripts from 293T cells (green and purple, respectively) and from pig thymocytes (red and blue, respectively), using human primers. (B) Histogram representation of relative expression levels of *RAG1* and *RAG2* in 293T cells (level 1) and in pig thymocytes (purple bars). (C) Melt curve analysis of QRT-PCR results shown in (A).

293T cells, the differences were such that we rationalized that human RAG1 and RAG2 primers could be used to detect alterations of *RAG1* and *RAG2* expression in thymocyte extract-treated cells.

Preparation of reprogramming extracts from pig thymocytes.

We prepared whole cell extracts from 293T cells and pig thymocytes. The approach for extract preparation from pig thymocytes is illustrated in Figure 6. Particular attention was paid to completely lyse all cells by sonication, so that no remaining thymocytes would contaminate extracts (assessed by phase contrast microscopy). The procedure developed for thymocyte extract preparation is provided in the Materials and Methods.

Since human RAG1 and RAG2 primers detect both 293T and thymocyte cDNA, any upregulation of *RAG1* and *RAG2* in extract-treated cells (see below) could possibly be caused by the uptake of pig *RAG1* and *RAG2* transcripts from the extract. To examine this possibility, we determined by RT-PCR analysis whether thymocyte extracts contained

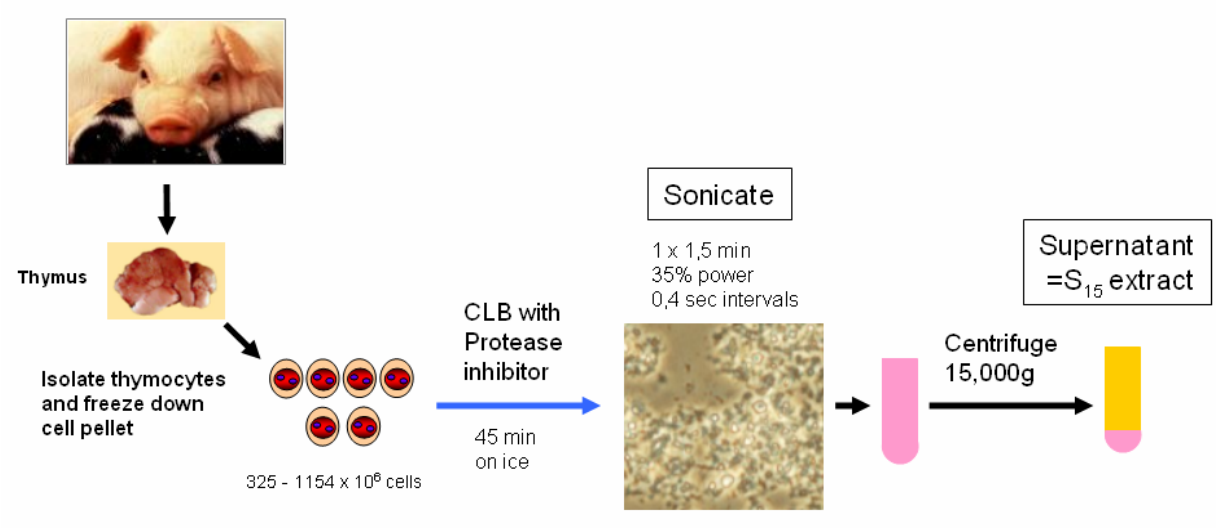

Figure 6. Schematic illustration of extract preparation from pig thymocytes. Thymus was collected and thymocytes were immediately isolated from fresh thymus. Extract was prepared from frozen thymocytes by incubation in cell lysis buffer (CLB) with protease inhibitor cocktail and further sonication. Finally, extract (supernatant) was obtained after sedimentation at 15,000 g.

RAG1 and *RAG2* transcripts. No pig transcripts were observed in thymocyte extracts (Fig. 7). Thus, any alterations in *RAG1* and *RAG2* expression which might be detected in extract-treated cells would most likely not result from *RAG1* and *RAG2* transcripts uptake from the extracts.

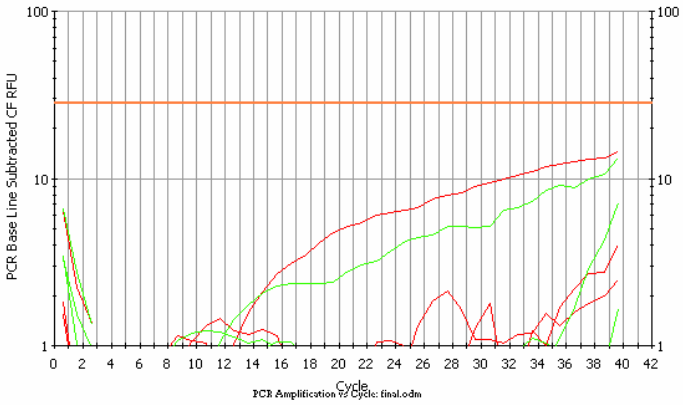

Figure 7. Quantitative RT-PCR analysis of *RAG1* and *RAG2* mRNA in thymocyte extracts. Quantitative RT-PCR analysis of pig *RAG1* (red) and pig *RAG2* (green) transcripts in thymocyte extracts (6 mg/ml and 22 mg/ml of protein). In QRT-PCR analysis, each sample was analyzed in triplicates. No *RAG1* or *RAG2* transcripts were found in thymocyte extracts.

Reprogramming of 293T cells with thymocyte extracts

In order to determine whether 293T cells could be reprogrammed to induce *RAG1* and *RAG2* expression, cells permeabilized with the pore-forming toxin Streptolysin O (SLO) were exposed for 50 min to thymocyte extract of low (6 mg/ml protein) or high (22 mg/ml protein) protein concentration. The experimental set up is illustrated in Figure 8. After extract treatment, plasma membranes were resealed and extract-treated cells were cultured for up to four weeks. Inductions of any reprogramming event were assessed by morphological changes and by QRT-PCR analyses of *RAG1* and *RAG2* expression. Untreated 293T cells and 293T cells exposed to a 293T cell extract were used as controls for permeabilization and handling effects.

As a first indication of any change occurring in 293T cells following thymocyte extract treatment, we determined whether the cells changed morphology. Figure 9 shows

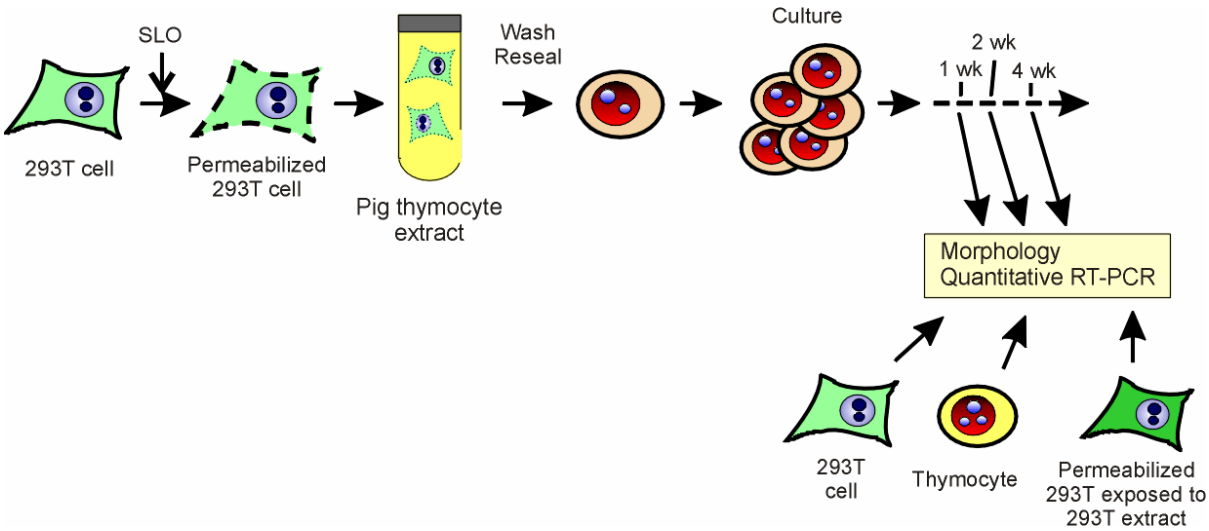

Figure 8. Experimental set up of in vitro cell reprogramming. 293T cells are permeabilized with Streptolysin O (SLO) and incubated in thymocyte extract. After incubation, cell membranes are resealed with 2 mM $CaCl_2$ after diluting the extract and cell mixture with complete RPMI-1640 medium. Over time period of 4 weeks, extract-treated cells were analysed for changes in morphology and induction of human *RAG1* and *RAG2* expression by real time RT-PCR. (Modified from Collas and Håkelien, 2003).

that, like untreated 293T cells (Fig. 9A), 293T cells treated with a control 293T cell extract or with thymocyte extract continued to grow as adherent cells with a fibroblastic morphology (Fig. 9B-D). Cell morphology was not affected by extract concentration and by duration of cultured after exposure to the extracts (Fig. 9C,D). The doubling time of the cells also remained unaltered (data not shown). We concluded that, unlike treatment with Jurkat cell extract (Håkelien et al., 2002; Håkelien et al., 2005), thymocyte extract treatment under these conditions had no obvious effect on growth pattern and morphology of 293T cells.

Expression of human *RAG1* and *RAG2* in extracts-treated cells

Despite the lack of noticeable morphological changes, we investigated whether expression of *RAG1* and *RAG2* was altered in 293T cells as a consequence of extract treatment. Expression of the housekeeping GAPDH gene was used for normalization of expression. Expression levels of *RAG1* and *RAG2* in all samples were calculated relative to those in

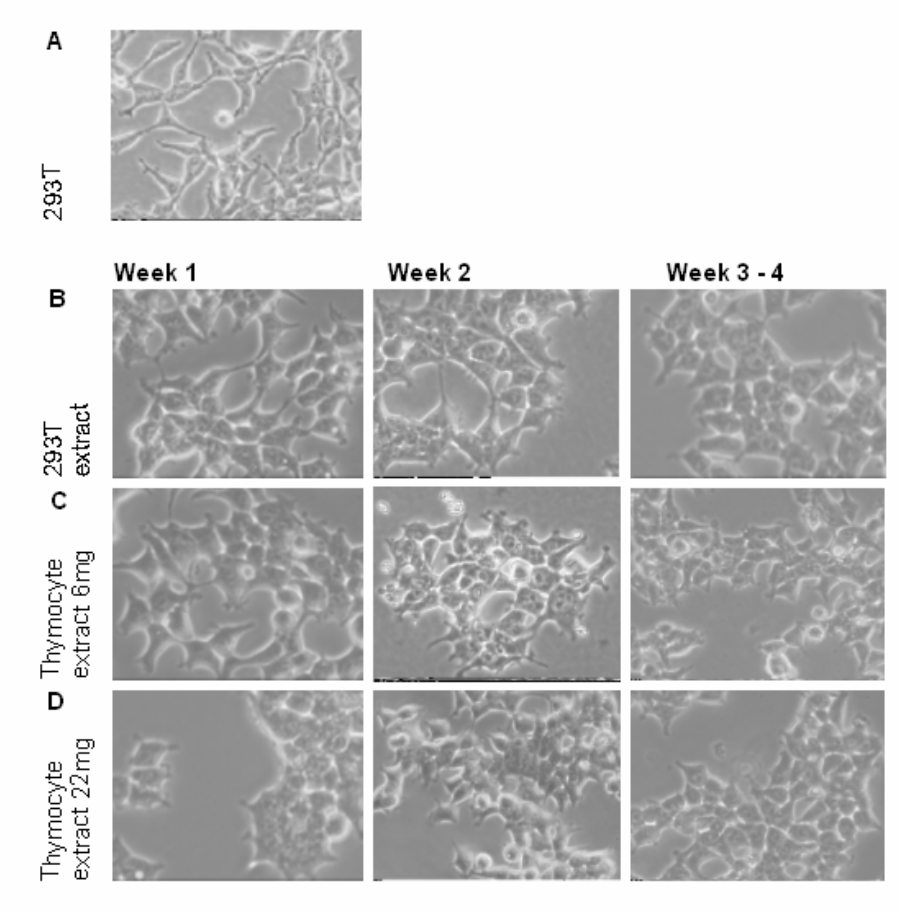

Figure 9. Morpology of 293T cells and of 293T cells treated with thymocyte or 293T cell extract and cultured for up to four weeks. (A) Untreated 293T cells. (B) 293T cells treated with 293T extract. (C,D) 293T cells treated with thymocyte extract of low protein concentration (C; 6 mg) and of high protein concentration (D; 22 mg). Magnification, (A) 100x, (B-D) 200x.

untreated 293T cells ("level 1"). Cells were analyzed by QRT-PCR at 8, 13 and 31 days after extract treatment. As the primers designed lie within exons (see above), total RNA was purified and digested with DNase to reduce the extent of putative contamination with genomic DNA. Relative expression levels of *RAG1* and *RAG2* in thymocyte and 293T extract-treated cells are shown in Figures 10A and 10B. 293T cells exposed to thymocyte extracts of low protein concentration (6 mg/ml) or to 293T extract showed less than 2-fold upregulation of *RAG1* expression relative to untreated 293T cells at all times (Fig. 10A, left and right panels). *RAG2* expression also remained relatively unaltered under these conditions, with a ~2-fold

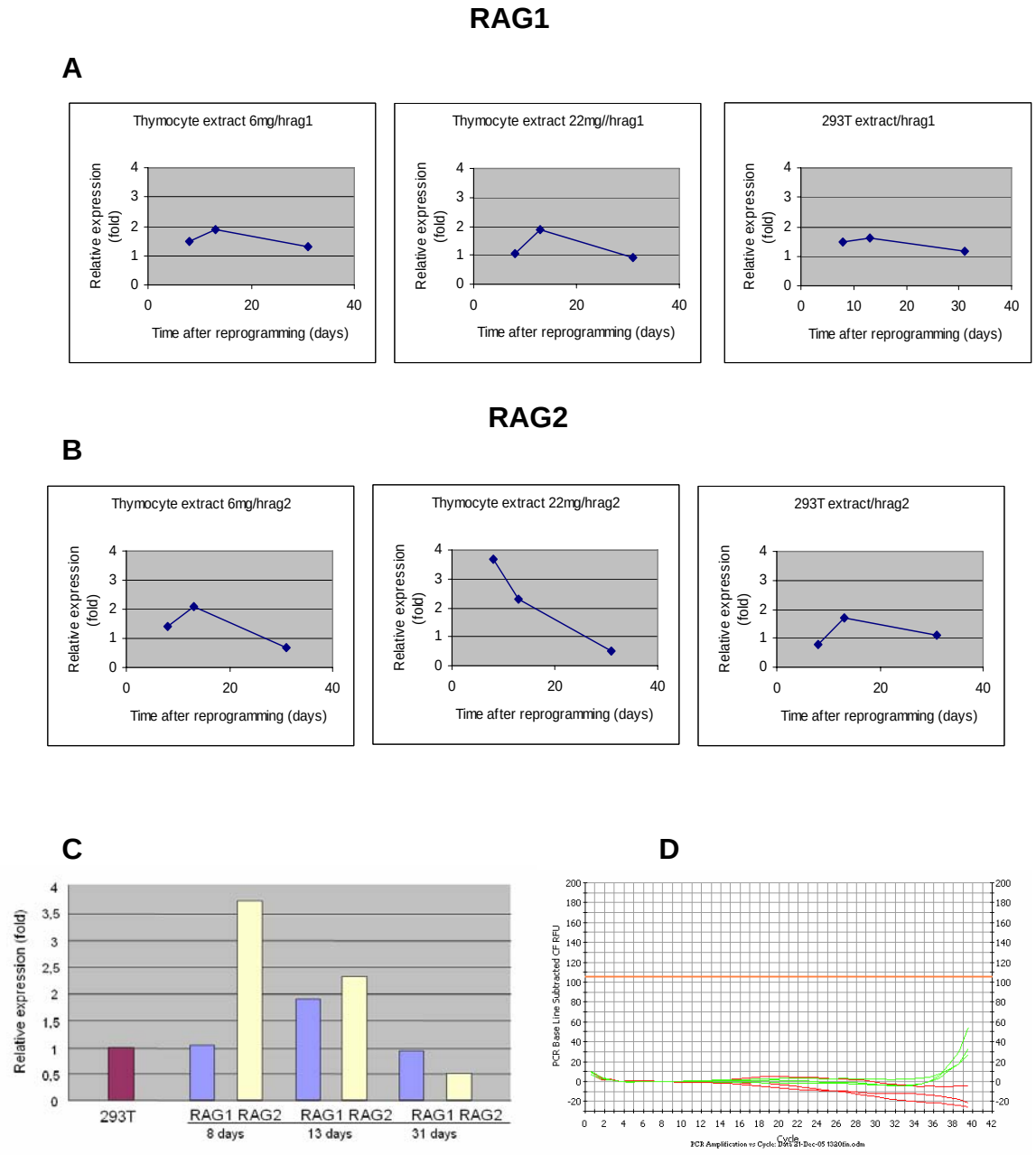

Figure 10. Quantitative RT-PCR analysis of human *RAG1* and *RAG2* expression in extract-treated 293T cells relative to input 293T cells ("level 1"). Relative expressions (fold) of (A) *RAG1* and (B) *RAG2*, in 293T cells treated with thymocyte extract of low and high protein concentration (6 mg and 22 mg, respectively) and with 293T extract. Expression levels were normalized with human GAPDH in triplicate analyses. No significant up regulation of human *RAG1* or *RAG2* were observed in 293T cells exposed to thymocyte (6 mg/ml) and 293T extracts. 293T cells treated with thymocytes extract (22 mg/ml) showed up regulation of *RAG2* 8 days after extract treatment. (C) Relative expression of human *RAG1* (blue bars) and *RAG2* (yellow bars) in thymocyte extract (22 mg) treated cells at indicated time points after extract treatment. Expression levels in extract treated cells were relative to untreated 293T cells (purple bar, level 1 for both human *RAG1* and *RAG2*). Human *RAG2* was up regulated approximately 3.7-fold after 8 days. (D) QRT-PCR analysis with pig RAG1 and RAG2 (red and green, respectively) on thymocyte extract (22 mg/ml) treated 293T cells, 8 days after extract treatment. No pig transcripts of extract origin were detected in mRNA preparation of extract treated 293T cells.

upregulation of *RAG2* by 13 days and expression levels close to baseline at other time points (Fig. 10B, left and right panels). Similarly, treatment with high-protein extracts (22 mg/ml) caused a ~2-fold upregulation of *RAG1* expression by 13 days, and only minor alterations at other time points (Fig. 10A, middle panel). In contrast, *RAG2* upregulation was clearly detected 8 days after extract treatment (3.7-fold upregulation; Fig. 10B, middle panel). After 13 days, *RAG2* levels were 2.3- fold upregulated compared to baseline and by 31 days expression had returned close to that of untreated 293T cells (Fig. 10B, middle panel). *RAG1* and *RAG2* expression levels in 293T cells exposed to thymocyte extract of high protein concentration relative to input 293T cells are summarized in Figure 10C.

We determined in a previous experiment that thymocyte extracts did not contain any detectable *RAG1* or *RAG2* transcripts (see Fig. 7), ruling out the possibility of contamination of permeabilized 293T cells with RAG transcripts of extract origin. To further rule out this unlikely possibility, extract-treated 293T cells were analyzed by QRT-PCR using pig RAG1 and RAG2 primers, which are specific for pig transcripts and do not detect human products (see Fig. 4). As expected, the results indicate that no pig *RAG1* or *RAG2* mRNAs were detected in extract (22 mg/ml protein) treated cells 8 days after

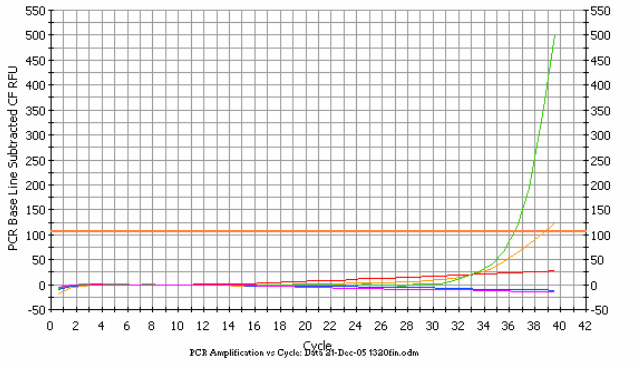

Figure 11. Genomic control for thymocyte extract treated 293T cells. None of the primer pairs used gave products on the genomic control for thymocyte extract treated cells. hGAPDH (orange), hRAG1 (red), hRAG2 (green), pRAG1 (blue) and pRAG2 (pink). Pig RAG2 was detected at cycle 36 (green line) and was considered to be non-specific.

treatment (Fig. 10D). These data strongly suggest that upregulation of *RAG2* detected in 293T cells exposed to thymocyte extract results from induction of the 293T cell genome. The peak of *RAG2* expression observed at day 8 decreased to baseline within 23 days. Thus, induction of *RAG2* expression was transient and not sustained over the four weeks examined. This observation is reminiscent of previous data reported in other similar extract-based systems (Gaustad et al., 2004; Håkelien et al., 2004 see Discussion).

Lastly, to determine whether the mRNA preparation analyzed in the previous experiment contained any genomic DNA, cDNA synthesis without reverse transcriptase was used to prepare a genomic DNA control for total RNA isolated from thymocyte extract-treated cells (8 days after extract treatment). The RNA preparation was found not to contain any genomic DNA (Fig. 11). This observation reinforced our conclusion that *RAG2* was upregulated in cells exposed to thymocyte extract of high protein concentration, at least by 8 days after extract treatment.

Discussion

This thesis evaluates the capacity of cellular extracts of pig thymocytes to induce the expression of two lymphoid-specific genes, *RAG1* and *RAG2*, in human epithelial 293T cells. As such, RAG1 and RAG2 are used as markers for functional changes in 293T cells, which may be qualified as transdifferentiation events. Real time RT-PCR analysis provided a quantitative method to detect alterations in *RAG1* and *RAG2* expression levels from relatively small amounts of total mRNA. Furthermore, we investigated whether extract treatment affected the morphology of 293T cells. Results from QRT-PCR analysis show upregulation of *RAG2* in 293T cells exposed for 50 min to thymocyte extract of high protein concentration, but no clear upregulation of *RAG1*. Moreover, thymocyte extract treatment showed not to affect the morphology of 293T cells.

Homology between human and porcine *RAG* sequences: implications for transcriptional regulation of *RAG1* and *RAG2*

Earlier studies have shown conservation of *RAG1* and *RAG2* genes in variety of species that carry out V(D)J recombination (Oettinger et al., 1990; Schatz et al., 1989). Based on these reports, we expected to find a high homology between human and porcine *RAG1* and *RAG2* coding sequences. The sequence alignment by two-sequence BLAST (Tatusova and Madden, 1999), that assigns the homology of two sequences by local alignment using a BLAST engine, showed homology between human and pig *RAG1* and *RAG2* coding sequences to be 88% and 91%, respectively. Although little is known about transcriptional regulators or other mechanisms that regulate *RAG1* and *RAG2* gene expression, we assumed from the high coding sequence homology and *RAG* gene conservation between species that the *RAG1* and *RAG2* genes would be regulated by similar factors in different

species. Further, to attempt to determine if these genes have similar regulation patterns in human and pig, we could have determined the homology between pig and human *RAG1* and *RAG2* regulatory regions and looked for conserved regions that might be involved in regulation of these genes. However, earlier reprogramming studies involving two species have shown that it is possible to use extract of one species (rat) to reprogram cells of another species (human) (Gaustad et al., 2004). Thus, we hypothesized that pig thymocyte extract could be used to reprogram 293T cells.

Extracts

Several studies have shown that treatment of donor cell type (e.g., 293T cell) with a 'target' cell type (e.g., Jurkat cell) extract favors the establishment of a target cell- specific, although perhaps not complete, transcriptional program (Gaustad et al., 2004; Håkelien et al., 2002; Håkelien et al., 2005; Taranger et al., 2005). Although extracts are believed to contain factors essential for establishment of new transcriptional program in reprogrammed cells, the nature of these factors remains to be identified. Nonetheless, preliminary studies indicate that in the Jurkat T cell or embryonal carcinoma systems (Håkelien et al., 2002; Taranger et al., 2005), DNase or RNase treatment of extracts does not apparently affect phenotypic or gene expression changes in extract-treated cells. To which extent this contention can be generalized to complete morphological and functional reprogramming, however, is uncertain. Furthermore, trypsin, protease or heat treatment of extracts abolish gene expression induced by extract exposure (Taranger et al., 2005). In addition, recent studies from our laboratory and others have shown that the ATPase chromatin remodeling factor Brg1, a component of the SWI/SNF chromatin remodeling complex (Khavari et al., 1993; Klochendler-Yeivin et al., 2002) was essential for induction of expression of the embryonic transcription factor *OCT4* (Hansis et al., 2004; Taranger et al., 2005). Lastly,

there is a dose-dependent requirement for exogenous energy sources in the extract, as ATP-depletion, or substitution of ATP or GTP with non-hydrolyzable analogues abolishes reprogramming of gene expression (Taranger et al., 2005).

Our work was based on earlier studies showing reprogramming capacity of human primary T cells or Jurkat cell extracts to induce transdifferentiation in 293T cells (Håkelien et al., 2002; Håkelien et al., 2005; Landsverk et al., 2002). The thymus is the organ where T cells differentiate and mature from T cell progenitors, or thymocytes. Because thymocytes undergo TCR gene rearrangement during maturation, they contain factors essential for these functions. Consequently, thymocyte extracts would be expected to contain components required for activating the *RAG1* and *RAG2* genes.

To support this view, our results indicate that 293T cells exposed to thymocyte extract can upregulate *RAG2* expression (*RAG1* expression is not upregulated in our study, however one cannot exclude the possibility that this event is impossible). This result suggests therefore two possibilities. First, *RAG* gene-activating factors are taken up by the permeabilized 293T cells and imported into the nucleus where they activate their target genes (the extract supports active nuclear-cytoplasmic transport; Landsverk et al., 2002). Alternatively, and not exclusively, *RAG* gene transcriptional regulators are being activated and/or induced to be synthesized by 293T cells in order to induce endogenous *RAG1* and *RAG2* expression. The latter view is supported by the time interval required to detect *RAG2* upregulation in our current system; however, we have at present no experimental evidence to conclude one way or the other. Only RT-PCR analysis using species-specific primers suggests that upregulation/activation of endogenous *RAG2*, at least, occurs in 293T cells upon treatment with thymocyte extract.

RAG2 induction in our system was shown to be due to extract treatment and not caused by *RAG2* mRNA of extract origin or genomic DNA contamination. In addition,

because no upregulation was detected in 293T cells exposed to low protein thymocyte extract, our results also suggest that protein concentration in the extract is an essential factor for successful reprogramming events.

Induction of gene expression in thymocyte extract

Three macro- or microarray-based gene expression analyses of extract-treated 293T cells strongly suggest that whole cell extracts, such as those developed here, are more efficient at inducing transcription or upregulation of transcription than gene repression or downregulation (Håkelien et al., 2002; Håkelien et al., 2005; Taranger et al., 2005). This observation may presumably be due to nature of extract. The system used in our laboratory supports the maintenance of a reorganized (e.g., hyperacetylated chromatin; Landsverk et al., 2002) yet decondensed interphase chromatin structure. This is in contrast to mitotic cell extract-based protocols developed that promote a condensed and repressive chromosome configuration (Sullivan et al., 2004).

How could one, then, promote enhanced reprogramming of gene expression? Conceivably, 293T cells, or any cell to be reprogrammed, may be induced to first undergo a step of nuclear breakdown (mitotic-like) and chromosome condensation in a mitotic extract (Sullivan et al., 2004). Presumably owing to protein phosphorylation events, this step promotes the dissociation of transcriptional regulators from the donor cell (Sullivan et al., 2004). As a result, gene activity would be reduced. In a second step, cells harboring condensed chromosomes may be triggered to undergo nuclear reconstitution (such as that occurring upon exit from mitosis), with associated chromatin decondensation and activation of novel genes as a result of exposure to reprogramming molecules of extract origin. Although this two-step reprogramming has not formally been tested, collaborative work between our laboratory and Hematech LLC (a private corporation based in Sioux

Falls, SD, USA) has shown that purified human somatic nuclei can be induced to undergo chromosome condensation in extracts of metaphase II bovine oocytes. Further, upon reentry of extracts into interphase (promoted by exogenous calcium), the nuclei decondense, reform a nuclear envelope and replicate DNA (P. Collas and P. Kasinathan; personal communication). Thus, it would in principle be possible to carry out a two-step reaction to enhance reprogramming of gene expression.

Perhaps the most argumentative rationale to support this view is the successful reprogramming of nuclear function in somatic nuclei following transplantation into enucleated metaphase II oocytes. Interestingly, transplanted nuclei invariable undergo a step of breakdown and chromatin condensation in the oocyte before being reformed with a completely new morphology (pronucleus-like) after activation of the recipient oocyte in order to promote embryo development (Lemaitre et al., 2005; Sullivan et al., 2004).

Why *RAG2* and not *RAG1*? Insights into chromatin organization

The two lymphoid-specific genes *RAG1* and *RAG2* are located on the same chromosome and only 8 kilobases apart from each other in human (Oettinger et al., 1990). During the T cell (or B cell) development, *RAG* genes are transcribed convergently to produce products that mediate V(D)J recombination of antigen receptor genes (Oettinger et al., 1990). Furthermore, little is still known about the mechanisms that regulate *RAG1* and *RAG2* expression. Tissue- and stage-specific regulation of the *RAG1* gene is thought to be controlled through changes in chromatin structure and by cis-acting elements outside the *RAG1* promoter region (ranging from -2.7 kb to +50 kb) (Kitagawa et al., 1996; Kurioka et al., 1996). Also *Rag2* gene expression is shown to be regulated by alterations of chromatin structure, in addition to regulation by cis-regulatory elements (Wei et al., 2002). A lymphoid cell line-specific enhancer (D3) has been found at the distal promoter region of

Rag2. The C/EBP transcriptional regulator is predicted to bind to this enhancer and therefore might functions as transcriptional factor responsible for enhancer activity (Wei et al., 2002). *Rag2* promoter activity has also been activated by Pax-5 (in B cells) and GATA-3 (in T cells) transcriptional factors in lymphoid cell lineages (Kishi et al., 2000).

Coordinate transcription of *RAG1* and *RAG2* genes during V(D)J recombination suggests that common regulatory elements might be involved in controlling the expression of these two genes. However, no such elements have yet been identified. Nevertheless, regulation of chromatin structure is shown to be involved in gene expression control of both *RAG1* and *RAG2* (Kitagawa et al., 1996; Kurioka et al., 1996; Wei et al., 2002). As we have only observed upregulation of the *RAG2* gene in our experiments, we hypothesize that chromatin structure has been changed to enable *RAG2* activation or upregulation. Furthermore, as no significant changes of *RAG1* expression was observed, this presumes that *RAG1* transcriptional control regions are either repressed by chromatin structure or by binding of specific inhibitory regulatory elements, or that *RAG1*-activating factors are absent or present below a threshold concentration. However, to prove these predictions, we would need to further investigate the chromatin structure of *RAG1* and *RAG2* genes as a result of thymocyte extract treatment.

Chromatin structure can be altered by chromatin remodeling complexes that use the energy of ATP hydrolysis to change the structure of nucleosomes. Furthermore, chromatin structure can be affected by covalent modification of N-terminal tails of histones, for example by acetylation, methylation, phosphorylation and ubiquitination. Acetylation of histone H3 on lysine 9 (H3K9) is generally associated with trancriptionally active chromatin (Sims, III et al., 2003). In contrast, H3K9 methylation is associated with a repressed chromatin state and gene inactivation (Sims, III et al., 2003). There is now evidence showing that some activation of chromatin remodeling complexes and acetylation

of histones can be induced in 293T cell by extract treatment (Håkelien et al., 2004; J.A. Dahl and C.K. Taranger in our laboratory; unpublished data). Thus, to determine if induction of *RAG2* expression detected in 293T cells exposed to thymocyte extract was due to changes in chromatin structure, it would be interesting to investigate the *RAG2* gene regulatory regions for histone acetylation or methylation. Chromatin immunoprecipitation (ChIP) procedures suitable for investigation of the H3K9 and H3K4 modification are being used in our laboratory. Thus, in the future, ChIP can be used to further determine the chromatin organization status of the *RAG1* and *RAG2* gene promoters before and after thymocyte extract treatment. In addition, because gene expression is also largely regulated by DNA methylation status in the promoter and/or enhancer regions (Doerfler, 2005; Fuks, 2005), another possibility is to assess DNA methylation changes occurring after extract treatment. Interestingly, such procedures (e.g., bisulfite sequencing analysis) are also being routinely used in our laboratory. Therefore, a logical follow up of the present work would consist in an epigenetic analysis of (mis)regulation of *RAG1* and *RAG2* genes in 293T cells as a result of thymocyte extract treatment.

Transient versus long term gene expression in extract-treated cells

Changes in gene expression patterns in extract-treated cells have been shown in our laboratory to be either short-lived (1-3 weeks; (Gaustad et al., 2004; Håkelien et al., 2004)) or more long-lived (at least several months; (Håkelien et al., 2002; Håkelien et al., 2005; Taranger et al., 2005)). The transient upregulation of *RAG2* detected in our study is consistent with these observations of Håkelien et al. (2004) and Gaustad et al. (2004). *RAG2* expression was detected by 8 days of extract-treatment and decreased to baseline by 23 days.

RAG1 and *RAG2* genes are lymphoid cell specific and thought to be solely expressed in immature T cells (and B cells). RAG1 and RAG2 are essential for T cell development and therefore were thought to be reliable markers of nuclear reprogramming. However, *RAG1* and *RAG2* were shown to be expressed also in untreated 293T cells (see Results). Fortunately, the level of expression observed was much lower than in thymocytes. From the differences in expression levels we rationalized that these genes could be used as a marker for transdifferentiation in our work. Furthermore, *RAG1* and *RAG2* are shown to be expressed at low levels in naïve $CD4^+$ T cells, activated/memory $CD4^+$ T cells and germinal center T cells in human tonsils (Li et al., 2002). These observations suggest that *RAG1* and *RAG2* are not exclusively expressed in immature T cells, but also expressed at lower levels in peripheral T cells.

Transient upregulation of *RAG2* in 293T cells may simply be a consequence of largely incomplete reprogramming. This view is supported by the lack of morphological reorganization of cellular architecture after extract treatment (see above). Alternatively, transient *RAG2* upregulation may be accounted for by the 'two-wave' expression pattern of *RAG1* and *RAG2* in vivo. In thymocytes, *RAG1* and *RAG2* are upregulated at the TN stage, after which their expression is downregulated and again reinduced in the DP stage. Thus, *RAG1* and *RAG2* expression is not sustained, but altered in a way that correlates the rearrangements of TCRαβ genes in developing thymocytes. Furthermore, *RAG1* and *RAG2* expression is terminated upon positive selection and generally not detected in cells after this stage of development. As such, we expected to detect *RAG1* and *RAG2* expression that would alter and finally be repressed after a period of time. The challenge that lies in the detection of gene expression pattern that alters over time is to manage to detect the 'tops' where these genes are most actively expressed. We have analyzed expression of *RAG1* and *RAG2* at 8, 13 and 31 days after extract treatment. Further, we might assume that the

expression levels of these genes could have altered at other time points and therefore it is possible that we have not been able to detect the 'actual' upregulation of *RAG1* and *RAG2*.

Would it be possible to detect TCR gene rearrangement in 293T cells treated with thymocyte extract? It would have been interesting to investigate the extract treated 293T cells for TCR gene rearrangements. The central portion of the RAG1 protein contains nonamer (conserved sequence of 9 nt that is part of RSS) binding domain (NBD) through which initial recognition of the RSS proceeds (Aidinis et al., 1999; Difilippantonio et al., 1996). RAG2 has shown no apparent affinity for the RSS; however, it interacts with RAG1 and may have a role in coordinating multiple protein-protein interactions at the RSS (Aidinis et al., 2000; Corneo et al., 2000). RAG1 alone has also been shown to inefficiently induce V(D)J recombination in fibroblasts, but when cotrasfected with RAG2 the frequence of recombination was increased 1000-fold (Oettinger et al., 1990). Thus, based on these data we might expect that both RAG1 and RAG2 are equally needed for successful TCR gene rearrangements through V(D)J recombination. Clearly, if rearrangements of TCR genes had been detected in extract-treated cells, these would have indicated *RAG1* and *RAG2* expression in extract-treated cells at other time points than selected. Furthermore, as we have only observed *RAG2* upregulation in the 293T cells, we did not expect to find any TCR rearrangement. Clearly, rearrangement of TCR genes remains to be investigated in our experimental system.

Reprogramming a somatic cell to induce *RAG1* and *RAG2* expression with subsequent TCR gene rearrangement and TCR molecule assembly would potentially have tremendous medical benefits. Mutation in either *RAG1* and *RAG2* genes has been shown to cause immune deficiencies in human (e.g., Omenn disease; Villa et al., 1998; Villa et al., 2000). Moreover, reprogramming a somatic cell type towards T lymphocytes could be used to enhance immune system of cancer patients with low level of T lymphocytes. Related

reprogramming technologies might also be useful to re-educate the thymus in order to make the immune system tolerant to exogenous (transplanted) cells or organs, or to instruct the immune system not to attack self-antigens in the case of autoimmune diseases.

Conclusion

This thesis provides a first step towards determining the capacity of pig thymocyte extracts to reprogram nuclear function in non-lymphoid cells. Upregulation of *RAG2* was observed in 293T cells exposed to thymocyte extract, indicating that, presumably, some nuclear reprogramming events have taken place. *RAG2* upregulation, however, was transient, suggesting that nuclear reprogramming was incomplete (a most likely possibility); alternatively, the pattern of *RAG2* expression may be accounted for by the nature of *RAG1* and *RAG2* gene expression profiles in maturing thymocytes. At this stage of the project, more work is clearly required to (i) improve the reprogramming effects of thymocyte extracts, (ii) enhance the extent and duration of *RAG2* upregulation, (iii) promote *RAG1* expression in extract-treated cells. Only then will one be able to investigate any functional significance of inducing the RAG enzymes, by determining whether TCR gene rearrangement takes place. Therefore, additional evidence is needed to establish a proof-of-concept that TCR gene rearrangement can be manipulated experimentally in non-thymocytic cells.

Reference List

Aidinis,V., Bonaldi,T., Beltrame,M., Santagata,S., Bianchi,M.E., and Spanopoulou,E. (1999). The RAG1 homeodomain recruits HMG1 and HMG2 to facilitate recombination signal sequence binding and to enhance the intrinsic DNA-bending activity of RAG1-RAG2. Mol. Cell Biol. *19*, 6532-6542.

Aidinis,V., Dias,D.C., Gomez,C.A., Bhattacharyya,D., Spanopoulou,E., and Santagata,S. (2000). Definition of minimal domains of interaction within the recombination-activating genes 1 and 2 recombinase complex. J. Immunol. *164*, 5826-5832.

Ardavin,C., Wu,L., Li,C.L., and Shortman,K. (1993). Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. Nature. *362*, 761-763.

Besmer,E., Mansilla-Soto,J., Cassard,S., Sawchuk,D.J., Brown,G., Sadofsky,M., Lewis,S.M., Nussenzweig,M.C., and Cortes,P. (1998). Hairpin coding end opening is mediated by RAG1 and RAG2 proteins. Mol. Cell. *2*, 817-828.

Bhandoola,A. and Sambandam,A. (2006). From stem cell to T cell: one route or many? Nat. Rev. Immunol. *6*, 117-126.

Blau,H.M. and Blakely,B.T. (1999). Plasticity of cell fate: insights from heterokaryons. Semin. Cell Dev. Biol. *10*, 267-272.

Blau,H.M., Chiu,C.P., and Webster,C. (1983). Cytoplasmic activation of human nuclear genes in stable heterocaryons. Cell. *32* , 1171-1180.

Brandle,D., Muller,C., Rulicke,T., Hengartner,H., and Pircher,H. (1992). Engagement of the T-cell receptor during positive selection in the thymus down-regulates RAG-1 expression. Proc. Natl. Acad. Sci. U. S. A. *89*, 9529-9533.

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

Burke,B. and Gerace,L. (1986). A cell free system to study reassembly of the nuclear envelope at the end of mitosis. Cell. *44*, 639-652.

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

Cameron,L.A. and Poccia,D.L. (1994). In vitro development of the sea urchin male pronucleus. Dev. Biol. *162*, 568-578.

Carleton,M., Haks,M.C., Smeele,S.A., Jones,A., Belkowski,S.M., Berger,M.A., Linsley,P., Kruisbeek,A.M., and Wiest,D.L. (2002). Early growth response transcription factors are required for development of CD4(-)CD8(-) thymocytes to the CD4(+)CD8(+) stage. J. Immunol. *168*, 1649-1658.

Carlson,B.M. (2005). Some principles of regeneration in mammalian systems. Anat. Rec. B New Anat. *287*, 4-13.

Cibelli,J.B., Campbell,K.H., Seidel,G.E., West,M.D., and Lanza,R.P. (2002). The health profile of cloned animals. Nat. Biotechnol. *20*, 13-14.

Cibelli,J.B., Stice,S.L., Golueke,P.J., Kane,J.J., Jerry,J., Blackwell,C., Ponce de Leon,F.A., and Robl,J.M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science. *280*, 1256-1258.

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

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

Collas,P., Le Guellec,K., and Tasken,K. (1999). The A-kinase-anchoring protein AKAP95 is a multivalent protein with a key role in chromatin condensation at mitosis. J. Cell Biol. *147*, 1167-1180.

Collas,P. and Poccia,D.L. (1995). Formation of the sea urchin male pronucleus in vitro: membrane-independent chromatin decondensation and nuclear envelope-dependent nuclear swelling. Mol. Reprod. Dev. *42*, 106-113.

Corneo,B., Moshous,D., Callebaut,I., de Chasseval,R., Fischer,A., and de Villartay,J.P. (2000). Three-dimensional clustering of human RAG2 gene mutations in severe combined immune deficiency. J. Biol. Chem. *275*, 12672-12675.

De Robertis,E.M. and Gurdon,J.B. (1977). Gene activation in somatic nuclei after injection into amphibian oocytes. Proc. Natl. Acad. Sci. U. S. A. *74*, 2470-2474.

Difilippantonio,M.J., McMahan,C.J., Eastman,Q.M., Spanopoulou,E., and Schatz,D.G. (1996). RAG1 mediates signal sequence recognition and recruitment of RAG2 in V(D)J recombination. Cell. *87*, 253-262.

Doerfler,W. (2005). On the biological significance of DNA methylation. Biochemistry (Mosc.). *70*, 505-524.

Fuks,F. (2005). DNA methylation and histone modifications: teaming up to silence genes. Curr. Opin. Genet. Dev. *15*, 490-495.

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

Gellert,M. (1992). Molecular analysis of V(D)J recombination. Annu. Rev. Genet. *26:*425-46.

Gill,J., Malin,M., Sutherland,J., Gray,D., Hollander,G., and Boyd,R. (2003). Thymic generation and regeneration. Immunol. Rev. *195:*28-50.

Groettrup,M., Ungewiss,K., Azogui,O., Palacios,R., Owen,M.J., Hayday,A.C., and von Boehmer,H. (1993). A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. Cell. *75*, 283-294.

Gunsilius,E., Gastl,G., and Petzer,A.L. (2001). Hematopoietic stem cells. Biomed. Pharmacother. *55*, 186-194.

Gurdon,J.B. (1962). Adult frogs derived from the nuclei of single somatic cells. Dev. Biol. *4:*256-273.

Gussoni,E., Bennett,R.R., Muskiewicz,K.R., Meyerrose,T., Nolta,J.A., Gilgoff,I., Stein,J., Chan,Y.M., Lidov,H.G., Bonnemann,C.G., Von Moers,A., Morris,G.E., Den Dunnen,J.T., Chamberlain,J.S., Kunkel,L.M., and Weinberg,K. (2002). Long-term persistence of donor nuclei in a Duchenne muscular dystrophy patient receiving bone marrow transplantation. J. Clin. Invest. *110*, 807-814.

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

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

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

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

Harish,M.S., Nagur,M., and Badami,S. (2001). Antihistaminic and mast cell stabilizing activity of Striga orobanchioides. J. Ethnopharmacol. *76*, 197-200.

Harris,H. and Watkins,J.F. (1965). Hybrid cells derived from mouse and man: artificial heterokaryons of mammalian cells from different species. Nature. *205:*640-6.

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

Hiom,K. and Gellert,M. (1997). A stable RAG1-RAG2-DNA complex that is active in V(D)J cleavage. Cell. *88*, 65-72.

Khavari,P.A., Peterson,C.L., Tamkun,J.W., Mendel,D.B., and Crabtree,G.R. (1993). BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. Nature. *366* , 170-174.

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

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

Kishi,H., Wei,X.C., Jin,Z.X., Fujishiro,Y., Nagata,T., Matsuda,T., and Muraguchi,A. (2000). Lineage-specific regulation of the murine RAG-2 promoter: GATA-3 in T cells and Pax-5 in B cells. Blood. *95*, 3845-3852.

Kitagawa,T., Mori,K., Kishi,H., Tagoh,H., Nagata,T., Kurioka,H., and Muraguchi,A. (1996). Chromatin structure and transcriptional regulation of human RAG-1 gene. Blood. *88*, 3785-3791.

Klochendler-Yeivin,A., Muchardt,C., and Yaniv,M. (2002). SWI/SNF chromatin remodeling and cancer. Curr. Opin. Genet. Dev. *12* , 73-79.

Kurioka,H., Kishi,H., Isshiki,H., Tagoh,H., Mori,K., Kitagawa,T., Nagata,T., Dohi,K., and Muraguchi,A. (1996). Isolation and characterization of a TATA-less promoter for the human RAG-1 gene. Mol. Immunol. *33*, 1059-1066.

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

Lemaitre,J.M., Danis,E., Pasero,P., Vassetzky,Y., and Mechali,M. (2005). Mitotic remodeling of the replicon and chromosome structure. Cell. *123*, 787-801.

Li,T.T., Han,S., Cubbage,M., and Zheng,B. (2002). Continued expression of recombination-activating genes and TCR gene recombination in human peripheral T cells. Eur. J. Immunol. *32*, 2792-2799.

Lohka,M.J. and Maller,J.L. (1985). Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts. J. Cell Biol. *101*, 518-523.

Longo,F.J., Mathews,L., and Palazzo,R.E. (1994). Sperm nuclear transformations in cytoplasmic extracts from surf clam (Spisula solidissima) oocytes. Dev. Biol. *162*, 245-258.

Manley,N.R. (2000). Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation. Semin. Immunol. *12*, 421-428.

Martins,S., Eikvar,S., Furukawa,K., and Collas,P. (2003). HA95 and LAP2 beta mediate a novel chromatin-nuclear envelope interaction implicated in initiation of DNA replication. J. Cell Biol. *160*, 177-188.

Matsuzaki,Y., Gyotoku,J., Ogawa,M., Nishikawa,S., Katsura,Y., Gachelin,G., and Nakauchi,H. (1993). Characterization of c-kit positive intrathymic stem cells that are restricted to lymphoid differentiation. J. Exp. Med. *178*, 1283-1292.

McBlane,J.F., van Gent,D.C., Ramsden,D.A., Romeo,C., Cuomo,C.A., Gellert,M., and Oettinger,M.A. (1995). Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. Cell. *83*, 387-395.

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

Miller,S.C., Pavlath,G.K., Blakely,B.T., and Blau,H.M. (1988). Muscle cell components dictate hepatocyte gene expression and the distribution of the Golgi apparatus in heterokaryons. Genes Dev. *2*, 330-340.

Mombaerts,P., Iacomini,J., Johnson,R.S., Herrup,K., Tonegawa,S., and Papaioannou,V.E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. Cell. *68*, 869-877.

Moore,T.A. and Zlotnik,A. (1995). T-cell lineage commitment and cytokine responses of thymic progenitors. Blood. *86*, 1850-1860.

Notarangelo,L.D., Santagata,S., and Villa,A. (2001). Recombinase activating gene enzymes of lymphocytes. Curr. Opin. Hematol. *8*, 41-46.

Oettinger,M.A., Schatz,D.G., Gorka,C., and Baltimore,D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science. *248*, 1517-1523.

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

Pittenger,M.F., Mackay,A.M., Beck,S.C., Jaiswal,R.K., Douglas,R., Mosca,J.D., Moorman,M.A., Simonetti,D.W., Craig,S., and Marshak,D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science. *284*, 143-147.

Polejaeva,I.A., Chen,S.H., Vaught,T.D., Page,R.L., Mullins,J., Ball,S., Dai,Y., Boone,J., Walker,S., Ayares,D.L., Colman,A., and Campbell,K.H. (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. Nature. *407*, 86-90.

Ramakers,C., Ruijter,J.M., Deprez,R.H., and Moorman,A.F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. *339*, 62-66.

Rao,M.S., Dwivedi,R.S., Subbarao,V., Usman,M.I., Scarpelli,D.G., Nemali,M.R., Yeldandi,A., Thangada,S., Kumar,S., and Reddy,J.K. (1988). Almost total conversion of pancreas to liver in the adult rat: a reliable model to study transdifferentiation. Biochem. Biophys. Res. Commun. *156*, 131-136.

Rao,M.S., Dwivedi,R.S., Yeldandi,A.V., Subbarao,V., Tan,X.D., Usman,M.I., Thangada,S., Nemali,M.R., Kumar,S., Scarpelli,D.G., and . (1989). Role of periductal and ductular epithelial cells of the adult rat pancreas in pancreatic hepatocyte lineage. A change in the differentiation commitment. Am. J. Pathol. *134*, 1069-1086.

Reddy,J.K., Rao,M.S., Yeldandi,A.V., Tan,X.D., and Dwivedi,R.S. (1991). Pancreatic hepatocytes. An in vivo model for cell lineage in pancreas of adult rat. Dig. Dis. Sci. *36*, 502-509.

Ringertz,N.R., Carlsson,S.A., Ege,T., and Bolund,L. (1971). Detection of human and chick nuclear antigens in nuclei of chick erythrocytes during reactivation in heterokaryons with HeLa cells. Proc. Natl. Acad. Sci. U. S. A. *68*, 3228-3232.

Saint-Ruf,C., Ungewiss,K., Groettrup,M., Bruno,L., Fehling,H.J., and von Boehmer,H. (1994). Analysis and expression of a cloned pre-T cell receptor gene. Science. *266*, 1208-1212.

Schatz,D.G., Oettinger,M.A., and Baltimore,D. (1989). The V(D)J recombination activating gene, RAG-1. Cell. *59*, 1035-1048.

Scollay,R.G., Butcher,E.C., and Weissman,I.L. (1980). Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. Eur. J. Immunol. *10*, 210-218.

Shinkai,Y., Rathbun,G., Lam,K.P., Oltz,E.M., Stewart,V., Mendelsohn,M., Charron,J., Datta,M., Young,F., Stall,A.M., and . (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell. *68*, 855-867.

Shockett,P.E. and Schatz,D.G. (1999). DNA hairpin opening mediated by the RAG1 and RAG2 proteins. Mol. Cell Biol. *19*, 4159-4166.

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

Sims,R.J., III, Nishioka,K., and Reinberg,D. (2003). Histone lysine methylation: a signature for chromatin function. Trends Genet. *19*, 629-639.

Slack,J.M. and Tosh,D. (2001). Transdifferentiation and metaplasia--switching cell types. Curr. Opin. Genet. Dev. *11*, 581-586.

Soll,D.R. and Waddell,D.R. (1975). Morphogenesis in the slime mold Dictyostelium discoideum. 1. The accumulation and erasure of "morphogenetic information". Dev. Biol. *47*, 292-302.

Sullivan,E.J., Kasinathan,S., Kasinathan,P., Robl,J.M., and Collas,P. (2004). Cloned calves from chromatin remodeled in vitro. Biol. Reprod. *70*, 146-153.

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

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

Takeuchi,I. and Sakai,Y. (1971). Dedifferentiation of the disaggregated slug cell of the cellular slime mold Dictyostelium discoideum. Dev. Growth Differ. *13*, 201-210.

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

Tatusova,T.A. and Madden,T.L. (1999). BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. *174*, 247-250.

Tonegawa,S. (1983). Somatic generation of antibody diversity. Nature. *302*, 575-581.

Tosh,D. and Slack,J.M. (2002). How cells change their phenotype. Nat. Rev. Mol. Cell Biol. *3*, 187-194.

Tsonis,P.A. (2000). Regeneration in vertebrates. Dev. Biol. *221*, 273-284.

Turka,L.A., Schatz,D.G., Oettinger,M.A., Chun,J.J., Gorka,C., Lee,K., McCormack,W.T., and Thompson,C.B. (1991). Thymocyte expression of RAG-1 and RAG-2: termination by T cell receptor cross-linking. Science. *253*, 778-781.

Ulitzur,N., Harel,A., Goldberg,M., Feinstein,N., and Gruenbaum,Y. (1997). Nuclear membrane vesicle targeting to chromatin in a Drosophila embryo cell-free system. Mol. Biol. Cell. *8*, 1439-1448.

van Gent,D.C., McBlane,J.F., Ramsden,D.A., Sadofsky,M.J., Hesse,J.E., and Gellert,M. (1995). Initiation of V(D)J recombination in a cell-free system. Cell. *81*, 925-934.

Vassilopoulos,G., Wang,P.R., and Russell,D.W. (2003). Transplanted bone marrow regenerates liver by cell fusion. Nature. *422* , 901-904.

Villa,A., Bozzi,F., Sobacchi,C., Strina,D., Fasth,A., Pasic,S., Notarangelo,L.D., and Vezzoni,P. (2000). Prenatal diagnosis of RAG-deficient Omenn syndrome. Prenat. Diagn. *20*, 56-59.

Villa,A., Santagata,S., Bozzi,F., Giliani,S., Frattini,A., Imberti,L., Gatta,L.B., Ochs,H.D., Schwarz,K., Notarangelo,L.D., Vezzoni,P., and Spanopoulou,E. (1998). Partial V(D)J recombination activity leads to Omenn syndrome. Cell. *93*, 885-896.

Wakayama,T., Perry,A.C., Zuccotti,M., Johnson,K.R., and Yanagimachi,R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature. *394*, 369-374.

Wang,H.J., Chen,T.M., Cheng,L.F., Cheng,T.Y., and Tung,Y.M. (1995). Human keratinocyte culture using porcine pituitary extract in serum-free medium. Burns. *21*, 503-506.

Wei,X.C., Kishi,H., Jin,Z.X., Zhao,W.P., Kondo,S., Matsuda,T., Saito,S., and Muraguchi,A. (2002). Characterization of chromatin structure and enhancer elements for murine recombination activating gene-2. J. Immunol. *169*, 873-881.

Weissman,I.L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. Cell. *100*, 157-168.

Wiest,D.L., Berger,M.A., and Carleton,M. (1999). Control of early thymocyte development by the pre-T cell receptor complex: A receptor without a ligand? Semin. Immunol. *11*, 251-262.

Wilmut,I., Schnieke,A.E., McWhir,J., Kind,A.J., and Campbell,K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature. *385*, 810-813.

Wilson,A., Held,W., and MacDonald,H.R. (1994). Two waves of recombinase gene expression in developing thymocytes. J. Exp. Med. *179*, 1355-1360.

Wilson,K.L. and Newport,J. (1988). A trypsin-sensitive receptor on membrane vesicles is required for nuclear envelope formation in vitro. J. Cell Biol. *107*, 57-68.

Wu,L., Li,C.L., and Shortman,K. (1996). Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. J. Exp. Med. *184*, 903-911.

Wu,L., Scollay,R., Egerton,M., Pearse,M., Spangrude,G.J., and Shortman,K. (1991). CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus. Nature. *349*, 71-74.

Tables

Table 1. Human (h) and pig (p) RAG1 and RAG2 and human GAPDH QRT-PCR primers.

	L-primer	**R-primer**	**Expected product**
hRAG1	ATGGTCCTGTGGATGGTAAAAC	ATGGGGCACTGCTAAACTTC	**186bp**
hRAG2	ATGGAACTGTTTTTCTTGGCATAC	CTGGATCTTCTGTTGATGTTTGACT	**147bp**
pRAG1	CGTTCGGTTTTCCTTCACA	CCTCCCATCTCCAGCATTAG	**222bp**
pRAG2	CGAGGGAAAAGTATGGGTGT	GCAGGACGGATGTTATTGG	**251bp**
hGAPDH	TCGGAGTCAACGGATTTGGT	TTGCCATGGGTGGAATCATA	**148bp**