The Role of differentiation state in reprogramming of somatic cells into induced pluripotent stem cells

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

by
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from Berlin

2010

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Tag der Disputation 27.4. 2010

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1. Introduction

1.1 Mammalian development

Mammalian development begins with the fertilization of the oocyte by the sperm resulting in the formation of the diploid zygote. Following cleavage divisions the blastocyst forms after 3 days post fertilization (dpf) in mice and 5 dpf in humans. The blastocyst consists of the inner cell mass (ICM) cells and an outer layer of cells, the trophoblast (Figure 1).

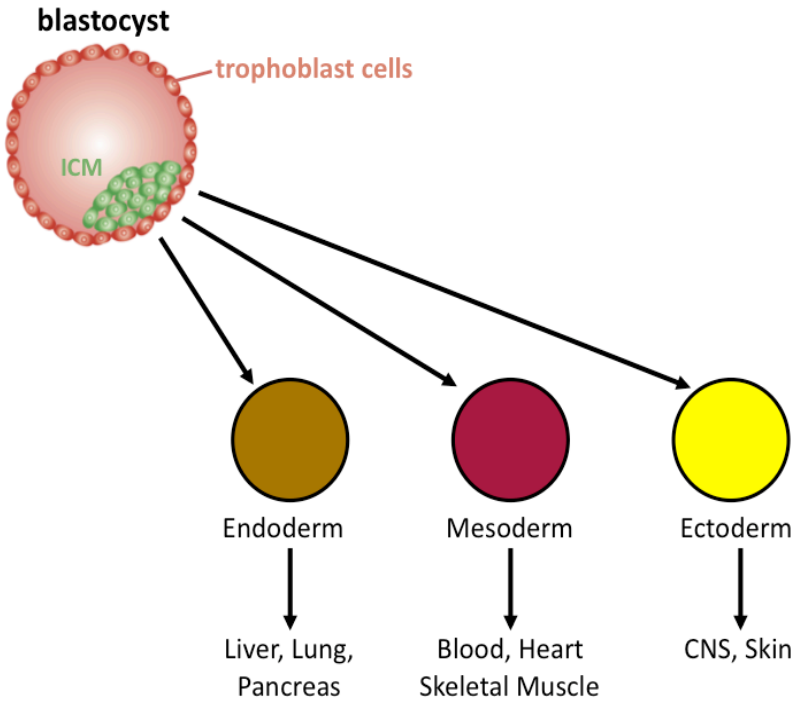

Figure 1: The blastocyst and its developmental potential.
The inner cell mass (ICM) cells of the blastocyst can give rise to all three germ layers (endoderm, mesoderm, ectoderm) and generate an embryo *in vivo*.

Trophoblast cells are the first committed cells of the early embryo and mediate the implantation of the blastocyst into the endometrium of the uterus. They later develop into extra-embryonic tissues such as the placenta, but never

contribute to the developing embryo (Winkel and Pedersen, 1988). In contrast, **ICM** cells, which lie within the blastocyst cavity, will form the epiblast and have the ability to differentiate into the three germ layers during gastrulation (Figure 1).

The **ectoderm** develops in such tissues as: the epidermis, sensory organs, hair, teeth, nails, and the nervous system.

The **endoderm** develops into the inner linings of the gastrointestinal tract, the liver, pancreas and respiratory tract and associated structures.

The **mesoderm** gives rise to muscles, connective tissue, bone, and the urogenital and circulatory systems.

1.2 Classification of cells and their developmental potential

During mammalian development, cells become increasingly specialized and restricted in their developmental potential. Development begins with the formation of the zygote and ends with the establishment of hundreds of specialized cell types. It is common to classify individual cell populations according to their specific developmental potential:

Totipotent cells have the capacity to differentiate into all embryonic and extra-embryonic cell types and thus can construct a complete, viable organism. In mammals, only the zygote and the first cleavage stage blastomeres are totipotent (Amabile and Meissner, 2009; Hochedlinger and Plath, 2009).

Pluripotent cells have the potential to form all cell types of the embryo, but lack the ability to form extra-embryonic tissues such as the placenta. They exist *in vivo* only for a short period of time that includes later blastomeres, ICM cells of the blastocyst and the ICM derived epiblast. Under appropriate culture conditions explanted ICM cells give rise to pluripotent embryonic stem (ES) cells.

Additional pluripotent cell types can however be derived from various stages of development and propagated *in vitro*. Examples are embryonic carcinoma (EC) cells, embryonic germ (EG) cells, epiblast stem (EpiS) cells, ES-like cells and induced pluripotent stem (iPS) cells (Hochedlinger and Plath, 2009) (see also Chapter 1.3.3, 1.5.3 and 1.5.5).

Multipotent cells are more restricted in their developmental potential compared to pluripotent cells; however, they retain the potential to form multiple different cell types within one lineage (Godin and Cumano, 2002; Orkin, 2000b). This group includes most adult stem cells such as intestinal stem cells, skin stem cells, neural stem cells and hematopoietic stem cells (HSCs) (Orkin, 2000a; Orkin and Zon, 2008a, b; van der Flier and Clevers, 2009). For example, HSCs in the bone marrow can give rise to all cell types of the hematopoietic lineage, and continue in their ability to self-renew and differentiate long after the embryo has developed into an adult (Morrison et al., 1995; Morrison and Weissman, 1995). However, HSCs do not contribute to other lineages (Wagers and Weissman, 2004).

Multipotent stem cells act as a repair system for the body by replacing lost or damaged cells, and thereby maintaining the normal turnover of regenerative organs by generating precursors and restocking specialized cells. An approach, which is becoming increasingly used in practice, is to replace damaged cells with healthy cells of patients still possessing healthy stem cells (autologous cells) in an undamaged part of the body. The autologous stem cells residing in the limbus of the eye have been successfully used to replace damaged or injured corneas (Homma et al., 2004; Ueno et al., 2007). Also, skin stem cells are utilized for regenerating skin epidermis in burn victims (Fuchs et al., 2004; Sekhon et al., 2004; Tumbar et al., 2004). Further, HSCs are taken from the bone marrow of patients before chemotherapy or radiation treatment. When chemotherapy or

radiation is done, the patient gets their stem cells back in a so-called "rescue" transplant (Pavletic et al., 2005).

Where no autologous stem cells are available or exist, alternative approaches for cell replacement therapy may provide an alternative solution in the future (Chapter 1.5).

Unipotent cells are capable of sustaining only one cell type or cell lineage. Examples include differentiated cells such as hepatocytes (Sekhon et al., 2004), committed progenitors like common lymphoid progenitors (CLPs) (Kondo et al., 1997) and certain adult stem cells such as spermatogonial stem cells, which exclusively differentiate into sperm (Guan et al., 2006).

1.3 Pluripotent stem cells

1.3.1 ES cells and pluripotency

Because of the potential of ES cells to develop into any cell type of the body and thus their possible utility for medical applications, a lot of emphasis has been placed on understanding their fundamental biology. ES cells represent an extremely attractive model system for studying gene function during development and disease. In particular, the use of homologous recombination, including conditional and inducible gene targeting technologies such as the Cre/lox and FLPe/frt systems in combination with other genetic tools (drug inducible systems, shRNA mediated gene silencing, fluorescent reporter proteins) make them useful instruments to induce precise mutations in a spatio-temporal manner (Nagy, 2000; Novak et al., 2000; Sauer and Henderson, 1988a, b; Sternberg and Hamilton, 1981; Zhu et al., 1995). Subsequently, the genetically altered ES cells

can be used to derive mouse lines, where the exact genomic manipulation is perpetuated allowing for the analysis of their function *in vivo*. Pluripotent cell lines remain an essential tool in molecular and developmental biology to this day.

In 1981 the first mouse ES cells were derived by explanting the ICM cells of a blastocyst, (Evans and Kaufman, 1981; Martin, 1981) (Figure 2). In 1998 ES cells lines were generated from human blastocysts (Thomson et al., 1998). Furthermore, human and mouse ES cells can also be derived from morula stage embryos and, although less efficiently, from 8-cell stage blastomeres (Chung et al., 2006).

ES cells can only be maintained in an undifferentiated proliferative state by precise and defined culture conditions. In order to maintain mouse ES cell identity *in vitro* the presence of the cytokine Leukemia Inhibitory Factor (LIF) is required, whereas human ES cells require the presence of the basic Fibroblast Growth Factor (bFGF) (Amit et al., 2000; Smith et al., 1988; Williams et al., 1988). Upon withdrawal of LIF or bFGF, ES cells can differentiate into complex structures called embryoid bodies (EBs) that contain cells from each of the three germ layers. Additional support for mouse and human ES cell culture is normally provided by so-called feeder cells (irradiated or mitomycin C treated mouse embryonic fibroblasts (MEFs) or human fibroblasts). The exact factors that feeder cells produce that prevent the differentiation of ES cells are unknown.

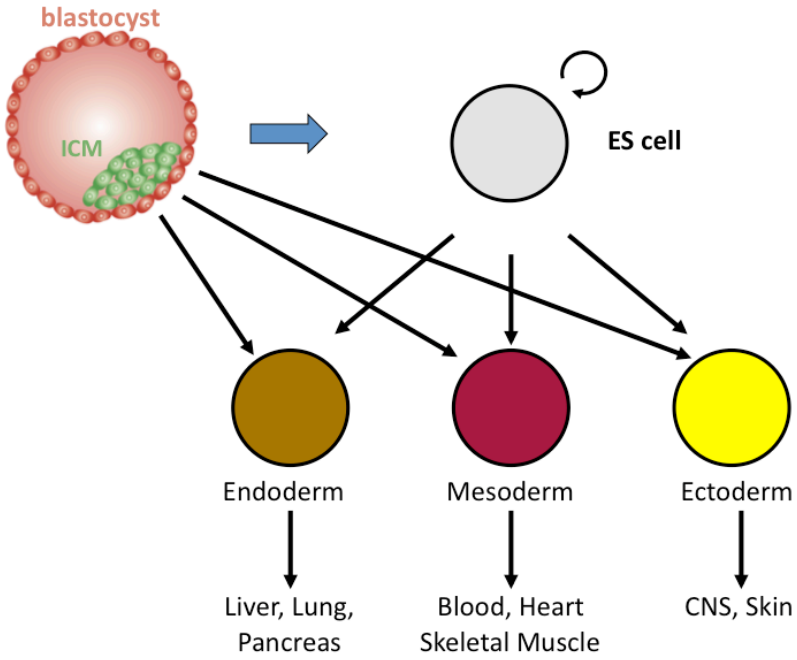

Figure 2: ICM and ES cells are pluripotent.
The inner cell mass (ICM) of the blastocyst can give rise to all three germ layers and generate an embryo *in vivo,* or when explanted into culture, can give rise to ES cells, which can self-renew and retain the potential to differentiate into all somatic cell types.

ES cells have three defining properties: Self-renewal, pluripotency and formation of germline-competent chimeras.

Self-renewal by mitotic cell divisions is a feature of stem cells and enables ES cells to indefinitely expand *in vitro*, while maintaining their pluripotent state.

Pluripotency as described above is the potential to differentiate into all three germ layers (Figure 1 and 2). Various tests can be used to demonstrate pluripotency of human and mouse ES cells. These include with increasing stringency immunostaining for essential pluripotency markers, teratoma formation and germline-competent chimera formation and tetraploid embryo complementation assays.

The subcutaneous injection of ES cells into immunodeficient mice causes the development of **teratomas** after several weeks. These ES cell derived

tumours produce a wide variety of differentiated cell types representing all three germ layers. Although teratoma formation is considered the minimal requirement in mouse pluripotent cells, it is to date the most stringent *in vivo* test that can be used for pluripotent human cells.

A more stringent test for pluripotency in the mouse is the formation of a **germline-competent** adult chimera achieved through blastocyst injection (Gardner, 1978; Rossant et al., 1978). Specifically, after donor ES cells are injected into a diploid blastocyst, some of these cells will be incorporated into the host ICM. By implanting the blastocyst into a surrogate mother, the injected ES cells will contribute to normal development and all lineages of the developing mouse (chimera). Because host-derived cells in the chimera may complement for cell autonomous defects, testing for germline contribution will determine if the injected donor ES cells can generate functional germ cells and thus normal offspring (Bradley et al., 1984; Solter, 2006) (Figure 3).

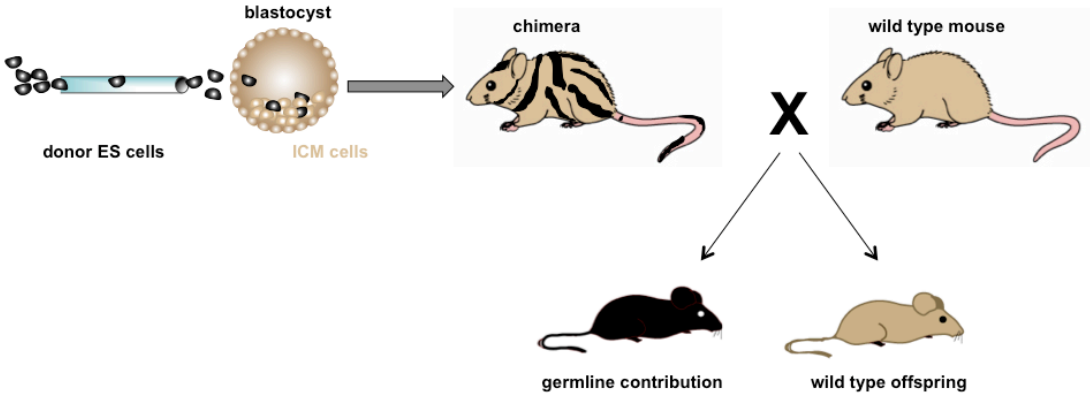

Figure 3: Generation of germline-competent chimeras.
Injection of ES cells into mouse blastocysts and generation of a chimeric mouse (black coat color originating from donor ES cells). Mating of a chimeric mouse with a wild type mouse results partly in germline transmission of the injected donor ES cells (indicated by black coat color).

The most stringent test for pluripotency is **tetraploid embryo complementation**, where ES cells are injected into a tetraploid (4n) host blastocyst, generated by fusing a diploid 2-cell embryo. Tetraploid cells cannot contribute to somatic lineages of the embryo and therefore the embryo has to be exclusively derived from the injected ES cells (Eggan et al., 2001; Nagy et al., 1990).

1.3.2 Pluripotency factors

The pluripotency of ES cells is regulated by a set of core transcription factors including Oct-4, Sox2 and Nanog (Figure 4). These essential genes activate self-renewal and pluripotency networks and block differentiation into specialized cells.

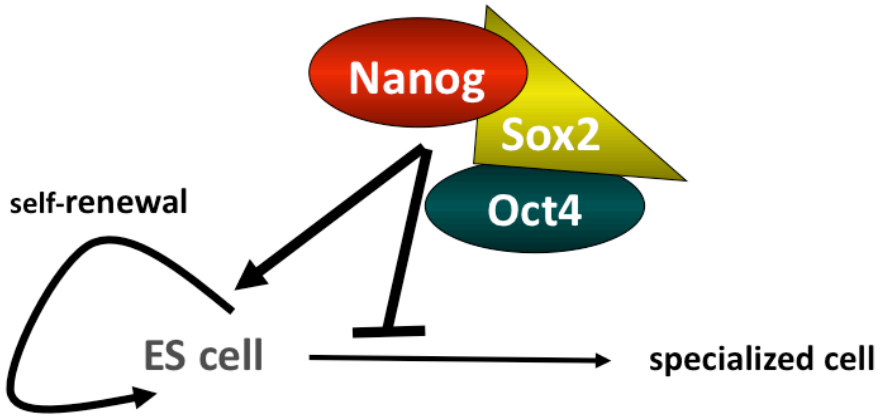

Figure 4: The key regulators of pluripotency.
The three transcriptions factors Nanog, Sox2 and Oct4 are essential for maintaining the pluripotent state of ES cells by inhibiting differentiation and maintaining their self-renewal.

Oct-4 or Pou5f1 (Octamer-binding transcription factor-4 or POU class 5 homeobox 1) is expressed in unfertilized oocytes, ES cells and primordial germ cells (PGCs) (Nichols et al., 1998; Okamoto et al., 1990; Scholer et al., 1989a; Scholer et al., 1989b), whereas **Nanog** ('Tir nan Og'-celtic for land of the ever

young) is specific for pluripotent cells such as the ICM and ES cells (Chambers et al., 2003; Mitsui et al., 2003). The transcription factor **Sox2** (sex-determing region Y-box2) is expressed in ES cells, trophoblast stem cells as well as in neural stem and progenitor cells (Avilion et al., 2003; Masui et al., 2007; Pevny et al., 1998).

Gene disruption of any of the individual transcription factors in ES cells causes loss of pluripotency and is followed by an improper differentiation into trophectoderm (loss of Oct-4 and Sox2) or extra-embryonic endoderm (loss of Nanog) resulting in embryonic lethality *in vivo* (Avilion et al., 2003; Mitsui et al., 2003; Nichols et al., 1998; Ying et al., 2002). Although, inactivation of Sox2 leads to ES cell differentiation, forced overexpression of Oct4 can rescue this phenotype (Masui et al., 2007). Also overexpression of Nanog can maintain ES cells in an undifferentiated state independently of the cytokine LIF when Oct4 and Sox2 expression is sustained (Mitsui et al., 2003). These rescue experiments can be explained by the notion that the transcription factors Oct4, Sox2 and Nanog can bind to their own promoters as well as to the promoters of each of the two other transcription factors. For example, Oct4 and Sox2 co-occupy and activate Oct4 and Nanog expression (Boyer et al., 2005; Kuroda et al., 2005; Loh et al., 2006; Okumura-Nakanishi et al., 2005).

In addition to this auto regulatory circuit, Oct4, Sox2 and Nanog co-bind many of the same target genes, which fall into two classes of genes: The first are actively expressed genes important for pluripotency and the second are silent developmental and lineage specification genes that are activated later during development (Boyer et al., 2005; Chambers et al., 2003; Jaenisch and Young, 2008; Loh et al., 2006; Masui et al., 2007; Mitsui et al., 2003; Nichols et al., 1998; Niwa et al., 2005). Thus, Oct4, Sox2 and Nanog are essential regulators of early development and ES cell identity.

1.3.3 Other types of pluripotent cells - EC, EG, EpiSC and ES-like cells

In addition to ES cells, which originate from the ICM, pluripotent cells can be derived from other sources and stages of development. For example, **EC** cells can be isolated from teratocarcinomas, a tumor that occasionally occurs in the gonads of fetuses and originates from PGCs (Andrews, 2002; Kleinsmith and Pierce, 1964). **EG** cells can be isolated from PGCs explanted in culture (Matsui 1992). Later stage epiblast cells (5.5 dpf in mouse) can give rise to pluripotent **EpiSC** *in vitro* (Brons et al., 2007; Tesar et al., 2007). Finally, it is possible to isolate **ES-like** cells from neonatal and adult mouse testis (Guan et al., 2006; Kanatsu-Shinohara et al., 2004).

Like ES cells, EG, EC, EpiSC, and ES-like cells can give rise to teratomas and chimeras. Despite the phenotypic similarities of these cells, functional and molecular differences exist that might reflect their different cellular origin (details in Chapter 1.5.3).

1.4 Epigenetic regulation in pluripotent cells

Mammalian development is a unidirectional process from the totipotent zygote over the pluripotent ICM cells to more restricted progenitors, and eventually fully differentiated cells (Surani et al., 2007). The outcome is that all cells of an organism are genetically homogeneous but functionally heterogeneous due to the differential expression of genes, which is the result of reversible epigenetic changes that are gradually imposed on the genome during development (Amabile and Meissner, 2009; Hochedlinger and Plath, 2009; Wakayama et al., 1998; Wilmut et al., 1997; Wilmut et al., 1998).

Epigenetic explains how the gene expression state of a cell is changed without altering the DNA sequence (by applying modifications that affect DNA transcription). Therefore, an adult stem cell and a differentiated cell within one organism have the same genome but a different epigenome. The two best-studied epigenetic components are **DNA methylation** and **histone modifications**.

Most of the genome is highly **DNA methylated** with the exception of CpG islands. CpG islands are areas of the genome rich in cytosine and guanine dinucleotides and are typically localized close to promoter regions of most human and mouse genes. The addition of methyl groups to the DNA converts cytosine to 5-methylcytosine, which is read as a repressive mark directly by the transcriptional machinery or indirectly through Methyl-Binding-Domain (MBD) containing factors (Dodge et al., 2002).

Histone modification provides another way of transcriptional regulation: The DNA itself is wrapped around so-called histone proteins. Chromatin refers to the structure formed by both DNA and the four different core histones (two each forming an octamer). The level of chromatin compaction affects gene transcription. Histone modifications of the amino terminal tails include methylation, acetylation, phosphorylation and ubiquitation (Goldberg et al., 2007).

Regarding the epigenetic of pluripotency the methylation status of the 4^{th} and the 27^{th} lysine (K) residue on histone 3 (H3), H3K4 and H3K27, respectively, is of particular interest. Methylation (mono, di and tri, abbreviated: me1, me2, me3) at H3K4 correlates with gene activation and accessible chromatin, whereas methylation at H3K27me3 correlates with gene silencing (Bernstein et al., 2006). In ES cells, most developmental genes are inactive and methylated on both H3K4 and H3K27, which have been termed **"bivalent domains"**. Although not unique to ES cells, they contain the largest number of bivalent domains and often

resolve these upon differentiation. In somatic cells bivalent domains frequently change into H3K4me3 only, H3K27me3 only or no modification at all. This bivalent structure indicates that ES cells are poised to alter the gene expression status rapidly to induce cellular differentiation processes.

Interestingly, many of the transcriptionally silenced developmental genes in ES cells are co-occupied by the pluripotency factors Oct4, Nanog and Sox2 and regulatory proteins such as the polycomb repressive complexes (PRCs). The two regulatory factors, the PRC and the trithorax-group (trxG) proteins act antagonistically. TrxG proteins maintain the active state of gene expression, while PcG proteins counteract this activation with a repressive function by catalyzing methylation of H3K27me3 for example in bivalent domains. This suggests that silencing seems to be the default state (Beisel et al., 2007; Sauvageau and Sauvageau, 2008; Schmitt et al., 2005; Schuettengruber et al., 2007).

1.5 Different strategies to reprogram somatic cells

The generation of viable cloned animals such as Dolly the sheep demonstrated for the first time that the developmental state of somatic cells is not irreversibly fixed. This reversal of the differentiated state of a somatic cell to one that is characteristic of the undifferentiated, embryonic state is defined as **“nuclear reprogramming”** (Figure 5).

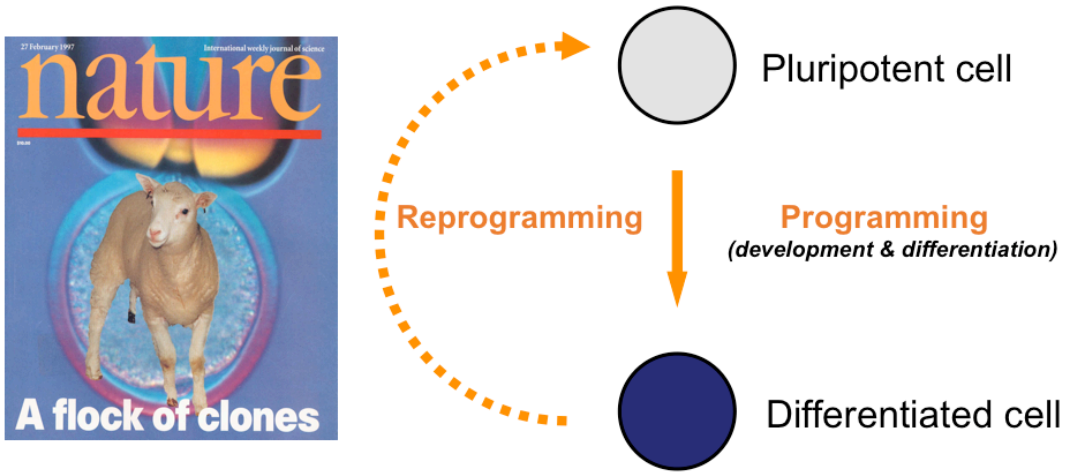

Figure 5: Developmental programming is a reversible process.
The differentiation process of a pluripotent cell into a more restricted mature cell is reversible as shown by nuclear transfer experiments.

In the following sections, a brief review of the current literature for the various approaches to achieve nuclear reprogramming will be described. There are four different recognized approaches to achieve nuclear reprogramming: nuclear transfer (NT) (Chapter 1.5.1), cell fusion (Chapter 1.5.2), culture-induced reprogramming (Chapter 1.5.3) and direct reprogramming (Chapter 1.5.5) (Figure 6).

1.5.1 Reprogramming by nuclear transfer

Reprogramming by NT describes a procedure whereby the donor somatic cell is transferred into an enucleated unfertilized oocyte, resulting in reprogramming of the somatic cell nucleus. Upon transfer into a surrogate mother, a cloned animal such as Dolly can be generated (Wilmut et al., 1997; Wilmut et al., 1998). The factors required for either reprogramming or embryonic development are unknown, but present in the cytoplasm of the unfertilized occyte (Egli et al., 2007).

Nuclear transfer is also referred to as ‘reproductive cloning’ in distinction to ‘therapeutic cloning’ where the reconstructed embryo is explanted into culture and can give rise to nuclear transfer derived embryonic stem (NT-ES) cells (Figure 6A).

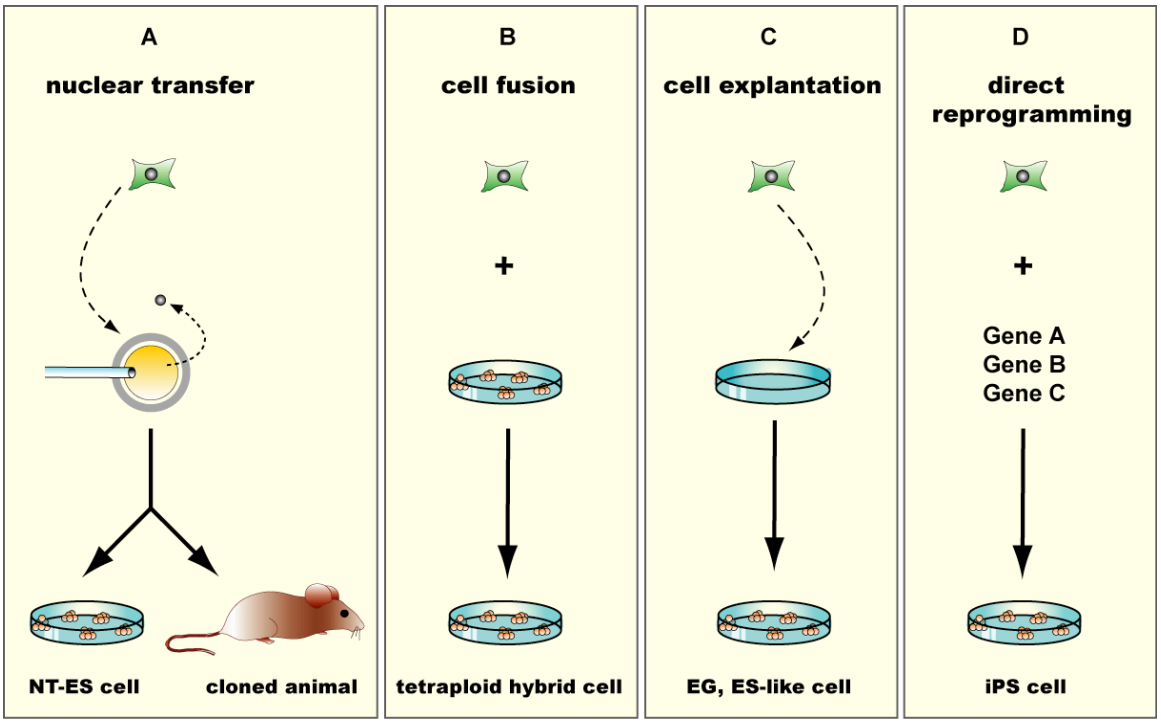

Figure 6: Different approaches to induce nuclear reprogramming
Illustration of the four major strategies used for studying nuclear reprogramming (**A**) Nuclear transfer (NT) involves the injection of a somatic nucleus into an enucleated oocyte which, upon transfer into a surrogate mother, can give rise to a cloned animal (reproductive cloning) or, upon explantation in culture, can give rise to NT-ES cells (therapeutic cloning). (**B**) Cell fusion of a somatic cell with pluripotent ES cells results in the generation of tetraploid hybrids that show all features of pluripotent ES cells. (**C**) Explantation of germ cells in culture selects for immortal cell lines like embryonic germ cells (EG) or ES-like cells that have regained pluripotency. (**D**) Introduction of a defined set of genes into somatic cells yields induced pluripotent stem (iPS) cells (Figure taken and modified from (Eminli et al., 2006 review)).

Generally, the process of ES cell derivation seems to select for cells that have undergone complete reprogramming to pluripotency or may assist the completion through extended time in culture. Based on molecular

characterization one can conclude that ES cell lines derived by NT are expected to have the same therapeutic potential as ES cell lines derived from fertilized embryos (Brambrink et al., 2006; Hochedlinger and Jaenisch, 2002b, 2003, 2006).

The possibility of generating patient-specific (genetically matched) stem cells through nuclear reprogramming is of great therapeutic potential. These cells could produce new populations of functional cells to replace the lost or non-functional cells in many diseases without immune-rejection. Indeed, by combining therapeutic cloning with gene therapy, genetic disorders have already been successfully treated in murine proof of principal experiments (Rideout et al., 2002).

Most adult tissues represent a heterogeneous population of cells containing multipotent stem cells, progenitor cells and terminally differentiated cells. When Dolly and other mammals were initially cloned from adult cells, there were no genetic markers available that could unambiguously prove the differentiation state of the donor cell. The derivation of monoclonal mice from mature B and T cells by NT used the genetic rearrangements of the differentiation-associated immunoglobulin and T cell receptor genes as genetic markers to retrospectively verify the differentiation state of the donor nucleus (Hochedlinger and Jaenisch, 2002a). Similarly, the cloning of mice from genetically labeled postmitotic olfactory neurons demonstrated that terminal differentiation does not restrict the potential of a nucleus to support the development of an animal (Eggan et al., 2004; Li et al., 2004).

Interestingly, terminally differentiated cells seem to be less efficiently reprogrammed than immature cells. It was demonstrated that cloning of mice from pluripotent ES cells and blastomeres was more efficient in creating cloned animals compared to cloning from adult cells such as fibroblasts (Eggan et al., 2001; Rideout et al., 2001; Rideout et al., 2000; Wakayama et al., 1999). Moreover, the generation of NT-ES cells is more efficient from neural stem cells

than from neurons (Blelloch et al., 2006; Inoue et al., 2006).

In line with these results, direct cloning of mice from skin stem cells yielded higher number of cloned animals than from differentiated keratinocytes (Li et al., 2007). Together, these observations suggested that the state of differentiation, possibly through differences in the epigenetic state of the donor cell, might affect the reprogramming efficiency of cloned animals. Therefore, the genome of adult stem and progenitor cells seems to be more amenable to reprogramming by NT than that of differentiated cells. This important fact will be later discussed in the context of direct reprogramming by defined factors (Chapter 1.5.5).

NT has been a unique tool for functionally testing nuclear potency and for distinguishing between genetic and epigenetic alterations of various donor cells (Hochedlinger and Jaenisch, 2002a, b; Hochedlinger et al., 2004; Li et al., 2004; Li et al., 2003a). However, any medical applications are constrained by the inefficiency of the cloning process, the failure to generate human NT-ES cells to date, and ethical concerns surrounding the use of human embryos.

Therefore, more tractable approaches are needed for generating patient-specific pluripotent cells and for dissecting reprogramming at the cellular, molecular and biochemical level.

1.5.2 Reprogramming by cell fusion

Epigenetic reprogramming of murine somatic nuclei back to an undifferentiated state can also be achieved by fusing the membranes of a pluripotent cell for example an EC, EG or ES cell, with a differentiated somatic cell by using a fusogen, such as polyethylene glycol (PEG) or by a short electric pulse (electro fusion) (Matveeva et al., 1998; Miller and Ruddle, 1976; Tada et al., 1997) (Figure 6B). The resulting pluripotent hybrid cells share many features with the

parental cell, indicating that the pluripotent phenotype is dominant over that of the differentiated cell (Tada et al., 2001). This reprogramming potential seems also to be conserved in human ES cells (Cowan et al., 2005; Yu et al., 2006). Therefore, cell fusion experiments indicated that ES cells, like oocytes or zygotes used for NT experiments, must contain the necessary reprogramming factors.

An important question arising from fusion experiments is, whether the ES cell nucleus or cytoplasm is required for the reprogramming process. Fusion experiments between neuronal cells and the nuclear compartment (karyoblast) or the cytoplasmic compartment (cytoblast) from ES cells indicated that nuclear factors are essential for molecular reprogramming (Byrne et al., 2003; Do and Scholer, 2004).

The key pluripotency regulators Nanog, Oct4 and Sox2 seem to be crucial for reprogramming. Indeed, overexpression of the *Nanog* gene in ES cells increased the reprogramming efficiency in hybrids up to 200 fold (number of hybrid colonies) when fused with neural stem cells (Silva et al., 2006). Interestingly, there was hardly any effect when fibroblasts were used as fusion partners, or when Nanog was ectopically expressed in the somatic cell partner prior to fusion. This suggests that the differentiation state and the cellular context are critical for Nanog's effect on reprogramming.

In experiments where some of the ES-cell derived chromosomes were selectively eliminated, hybrid cells remained pluripotent and demonstrating that the ES cell genome is not continuously required for maintaining pluripotency of the hybrids (Matsumura et al., 2007). While these experiments have shown that proteins present in a pluripotent cell are effectors of reprogramming, the resulting tetraploidy of the hybrid cell is a major limitation for potential clinical applications (Cowan et al., 2005; Silva et al., 2006).

1.5.3 Culture-induced reprogramming

EC cells, pluripotent cells derived from teratocarcinomas, were the first such cells discovered in adult mammals (Kleinsmith and Pierce, 1964). The stem cells of these tumors arise spontaneously from **PGCs** during gonad formation. In normal development PGCs reside in the embryonic genital ridge and normally differentiate into oocytes or sperm (Andrews, 2002; Stevens and Little, 1954) (Figure 7A and B).

Under special *in vitro* culture conditions PGCs can reprogram into pluripotent **EG** cells and acquire properties similar to those of ES cells (Figure 6C and 7A). When PGCs were isolated from the genital ridge and injected into host blastocysts, they did not contribute to any tissue in the animal (Durcova-Hills et al., 2006b). In contrast, EG cells derived from explanted PGCs and EC cells isolated from teratocarcinomas contributed to chimeric animals (Durcova-Hills et al., 2006a, b; Labosky et al., 1994a, b; Stewart et al., 1994; Stewart and Mintz, 1982).

PGCs represent embryonic unipotent germ cells. This raised the question of whether pluripotent cells can also be derived from adult germ cells such as spermatogonial stem cells. Indeed, spermatogonial stem cells from neonatal (Kanatsu-Shinohara et al., 2004) and adult (Guan et al., 2006) testis cells were shown to give rise to **ES-like** cells when exposed to a specific combination of growth factors albeit at an extremely low frequency (1 in 1.5 $x10^7$ neonatal and 1 in 7.5 x 10^7 adult testis cells) (Figure 6C and 7C). ES-like cells derived from neonatal and adult testis expressed all markers of pluripotent cells and gave rise to germline-competent chimeric animals.

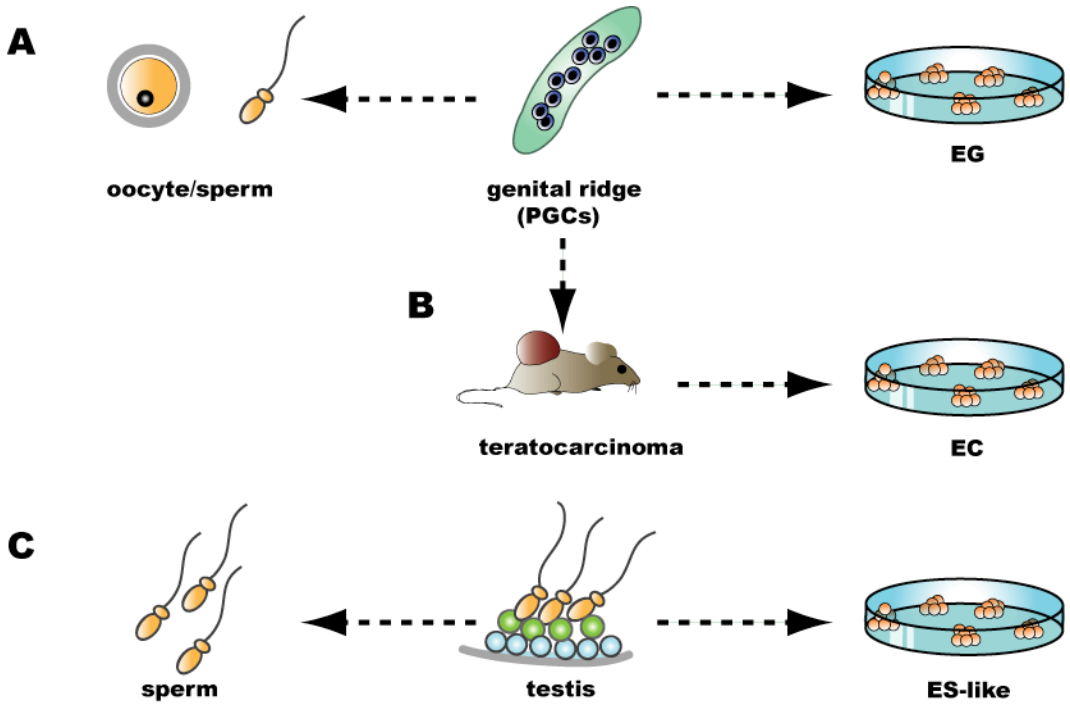

Figure 7: Culture induced reprogramming
(**A**) PGCs (primordial germ cells) of the genital ridge give rise to oocytes and spermatozoa *in vivo* and can give rise to embryonic germ (EG) cells *in vitro*. (**B**) In teratocarcinomas derived from PGCs of the genital ridge, pluripotent embryonic carcinoma (EC) cells are found that resemble EG cells. (**C**) Spermatogonial stem cells from newborn and adult testes normally differentiate into spermatozoa *in vivo* but occasionally give rise to pluripotent ES-like cells in culture (Figure taken and modified from (Eminli et al., 2006 review)).

In PGCs, the parental imprints are erased and sequentially re-established in a male or female specific pattern during subsequent gametogenesis (Hajkova et al., 2002; Lee et al., 2002; Yamazaki et al., 2005). Thus, ES-like cells derived from testis cells and EG cells derived from PGCs have an unbalanced imprinting status. Therefore these types of pluripotent cells are more limited in their application potential compared to ES cells. For example, it has been shown that loss of imprinting by genetic manipulation of DNA methylation results in tumorigenesis in mice (Holm et al., 2005) and consequently, therapeutic applications of pluripotent cells derived from adult testis may be problematic.

It is possible that the major epigenetic changes that occur during gametogenesis make embryonic and adult germ cells more amenable to epigenetic reprogramming in contrast to somatic cells. In addition, germ cells already express Oct4 and other proteins important for maintaining ES cell identity (Kanatsu-Shinohara et al., 2004), which may facilitate the reprogramming process and suggests that multipotent adult stem cells may also have a broadened potency to convert into cell types of other lineages.

1.5.4 "Trans-differentiation" / "Lineage-conversion"

Lineage-conversion or trans-differentiation describes the direct conversion of one somatic cell type into another by overexpression or deletion of individual transcription factors; a transition that normally does not occur *in vivo*. The first study on transcription factor mediated direct reprogramming demonstrated that overexpression of the myogenic transcription factor MyoD was sufficient to convert fibroblasts into myoblasts (Davis et al., 1987). Pax5 deletion from mouse B cells results in their de-differentiation into progenitors, which are then able to give rise to multiple hematopoietic cell types (Cobaleda et al., 2007b). The overexpression of transcription factor CEBPα (CCAAT enhancer binding protein alpha) can reprogram B and T cells into macrophages *in vitro* (Laiosa et al., 2006; Xie et al., 2004). The stable direct conversion (without passing through a pluripotent state) of one differentiated cell type into another has also been shown *in vivo* by overexpressing three defined transcription factors in adult pancreatic exocrine cells thereby converting them into insulin producing β cells (Zhou et al., 2008). It remains unclear, however, if transdifferentiation involves an initial de-differentiation stage of cells for example into less-differentiated progenitor cells as it occurs during reprogramming of adult cells into pluripotent cells.

1.5.5 Direct reprogramming by defined transcription factors into induced pluripotent stem (iPS) cells

In 2006, Takahashi and Yamanaka converted somatic cells into pluripotent stem cells by the introduction of defined transcription factors (Figure 6D). Based on the results from cell fusion experiments that ES cells must contain reprogramming factors, they exposed mouse fibroblasts to a pool of 24 retrovirally-expressed genes (selected based on high expression in ES cells or the early embryo) to screen for emerging colonies with ES cell characteristics (Takahashi and Yamanaka, 2006). Some of these genes were previously identified as ES cell-specific and were combined with a selected set of other candidate genes, known to contribute to long-term maintenance of the ES cell phenotype and their rapid proliferation in culture (Mitsui et al., 2003). In order to distinguish merely transformed fibroblast colonies from truly “reprogrammed” ES cell-like colonies, thereafter called induced pluripotent stem cells (iPS), the authors selected for cells that had reactivated the ES cell specific, but not essential, gene Fbx15 driving a neomycin-resistance cassette (neo^R), and obtained iPS colonies in 0.02% of infected fibroblasts. After selectively omitting individual viruses from the initial set of 24, the authors were able to narrow down the minimal set of genes required for the induction of pluripotency to the four transcription factors Oct4, Sox2, c-Myc and Klf4 (Takahashi and Yamanaka, 2006) (Figure 8).

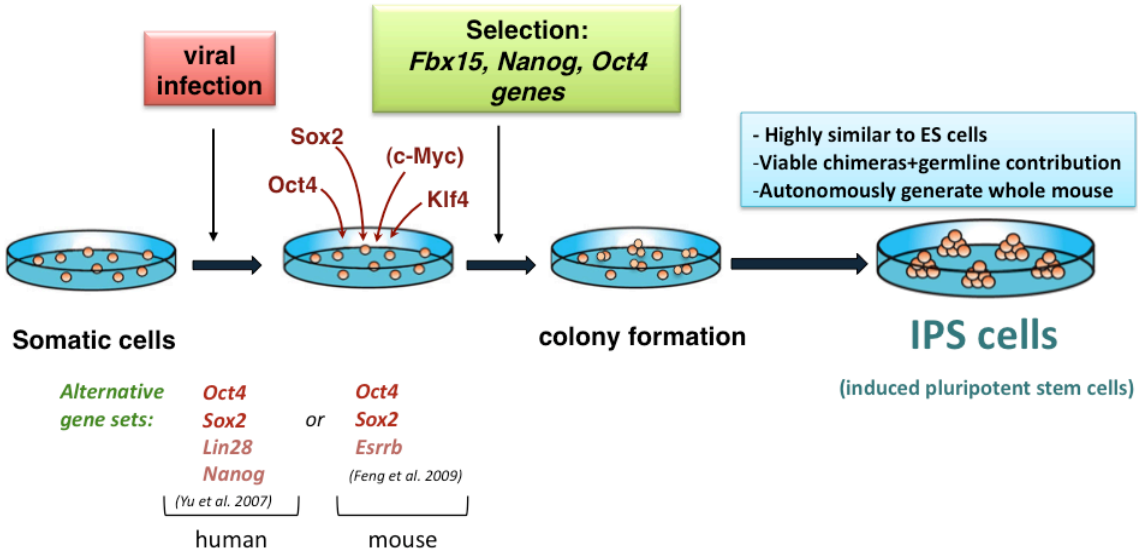

Figure 8: Steps involved in direct reprogramming to pluripotency.
Viral introduction of the four transcription factors Oct4, Sox2, Klf4 and optionally c-Myc into somatic cells, followed by selection for reactivated pluripotency reporter genes and subsequent iPS cell formation.

Oct4 and Sox2 are both master regulators of ES cell pluripotency (see Chapter 1.3.2) and c-Myc and Klf4 has been implicated in promoting the pluripotent state of ES cells (Cartwright et al., 2005; Jiang et al., 2008). c-Myc is ubiquitously expressed and plays a role in cell cycle regulation, proliferation and differentiation and is generally low or absent in quiescent cells (Kastan et al., 1989a; Kastan et al., 1989b; Laurenti et al., 2008; Murphy et al., 2005; Schmidt, 1999; Wilson et al., 2008). Another function of c-Myc is that it regulates chromatin modifications that may allow the reprogramming factors increased access to genes necessary for reprogramming (Fernandez et al., 2003; Frank et al., 2003; Li et al., 2003b). Krueppel-like factor (Klf4) is a transcription factor expressed in a variety of tissues including the epithelium of the intestine, kidney and skin (Garrett-Sinha et al., 1996; Segre et al., 1999; Shields et al., 1996). In addition, forced expression of Klf4 in ES cells can block differentiation and promote self-renewal (Jiang et al., 2008; Li et al., 2005). Its role in direct

reprogramming is still unclear but depending on the target genes it can both silence and activate transcription (Rowland and Peeper, 2006). Thus, c-Myc together with Klf4 possibly allows for an immortal growth potential phenotype associated with ES cells (Takahashi et al., 2007).

The first generation iPS cells generated by Takahashi and Yamanaka were selected for the reactivation of the Fbx15 gene (Figure 8). These Fbx15^{+} iPS cell colonies formed differentiated teratomas, but did not support development of the viable term chimeras. And were therefore not fully reprogrammed. Similarly, gene expression analyses revealed that only part of the ES cell transcriptome was reactivated and expressed. Methylation analyses of promoters including the pluripotency genes Nanog and Oct4 demonstrated an epigenetic pattern that was intermediate between that of fibroblasts and ES cells (Takahashi and Yamanaka, 2006). It is crucial that cells are fully reprogrammed, because aberrantly reprogrammed cells may result in an impaired ability to differentiate and may therefore increase the risk of teratoma formation after directed differentiation.

In subsequent experiments, iPS cells were produced by selecting for reactivation of the essential pluripotency genes Nanog or Oct4. This resulted in iPS cells, which were functionally and molecularly indistinguishable from ES cells (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007b) (Figure 8). Those established iPS cell lines showed silencing of the introduced retroviral genes when the reprogramming process was completed (moloney viruses are rapidly silenced in ES cells due to the *de novo* methyltransferases Dnmt3a/b) (Okano et al., 1998), reactivation of the silent X chromosome in female cells, the re-establishment of DNA and histone methylation pattern characteristic of ES cells (Maherali et al., 2007; Wernig et al., 2007b) and the ability to autonomously generate a whole adult mouse by tetraploid embryo complementation – the most stringent test for pluripotency (see also Chapter 1.3.1) (Kang et al., 2009; Zhao et al., 2009).

In parallel over the last years human somatic cells have been reprogrammed into iPS cells with the same four factors (Lowry et al., 2008; Takahashi et al., 2007) or a different combination that substitutes c-Myc and Klf4 with Nanog and Lin28 (Yu et al., 2007) (Figure 8).

Mice lacking either c-Myc or Klf4 still survive until birth, indicating that other factors compensate their function during normal development to maintain pluripotency (Ghaleb et al., 2007; Malynn et al., 2000; McConnell et al., 2007). Indeed, recent evidence indicated that omission of c-Myc from the reprogramming cocktail gives rise to iPS cells from fibroblasts, albeit at extremely low frequencies and with delayed kinetics (Nakagawa et al., 2008; Wernig et al., 2007a). Recently, it has been shown that the orphan nuclear receptor Esrrb (Estrogen-related receptor beta) functions in conjunction with Oct4 and Sox2 to mediate reprogramming in mouse MEFs without exogenous Klf4 and c-Myc (Figure 8). In ES cells, Essrb targets many genes involved in self-renewal and pluripotency, and therefore could act as a general activator-enhancing transcription of common target genes of Oct4 and Sox2 during reprogramming (Feng et al., 2009).

It seems that different combinations of transcription factors are possible to convert a somatic cell into a functional iPS cell and that Oct4 and Sox2 are essential for reprogramming while c-Myc and Klf4 may enhance the process.

1.5.6 Potential clinical and pharmaceutical applications of iPS cells

At least some iPS cells seem to have the same developmental potential as ES cells (Maherali et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007b). The possibility that customized patient derived iPS cell might one day be used as autologous transplantable sources to cure genetic and degenerative diseases without the complication of immune rejection, make them a promising tool. Recently it has been demonstrated in the mouse that iPS cells

derived from skin cells of sickle cell mice were able to restore normal blood function upon repair of the underlying genetic defect and transplantation into diseased mice (Hanna et al., 2007).

In human, neurodegenerative diseases such as Alzheimer's and Parkinson's, where acetylcholine- or dopamine-secreting cells of the brain are destroyed, respectively or other diseases such as diabetes, which results in the destruction of insulin-producing beta cells would greatly benefit from patient-specific stem cells that could provide an inexhaustible source of the lost cells.

Different groups have already generated iPS cells from various human degenerative diseases such as ALS (amyotrophic lateral sclerosis) (Figure 9), SMA (spinal muscular atrophy) and Parkinson disease (Dimos et al., 2008; Ebert et al., 2009; Park et al., 2008a; Soldner et al., 2009). Similarly, disease-corrected human iPS cells from patients with Fanconi anemia could be differentiated into blood progenitors, which are disease-free in short-term repopulation assays in mice (Raya et al., 2009).

The ability to produce pluripotent cells from patients with genetic diseases, and then differentiate those patient-specific iPS into the actual cell type affected by the disease is useful for drug screening but also to solve the underlying mechanisms of disease pathogenesis (Figure 9). For example, in patients with familial dysautonomia (FD) the mechanism of neuron loss was not understood owing to the lack of an appropriate model system. Patient-specific FD-iPS cell lines were recently used to identify neurogenic differentiation and migration phenotypes *in vitro*. These studies demonstrated that a tissue specific splicing defect of the transcription regulator IKBKAP (transcriptional elongation) caused this condition and could be corrected with the candidate compound kinetin (Lee et al., 2009).

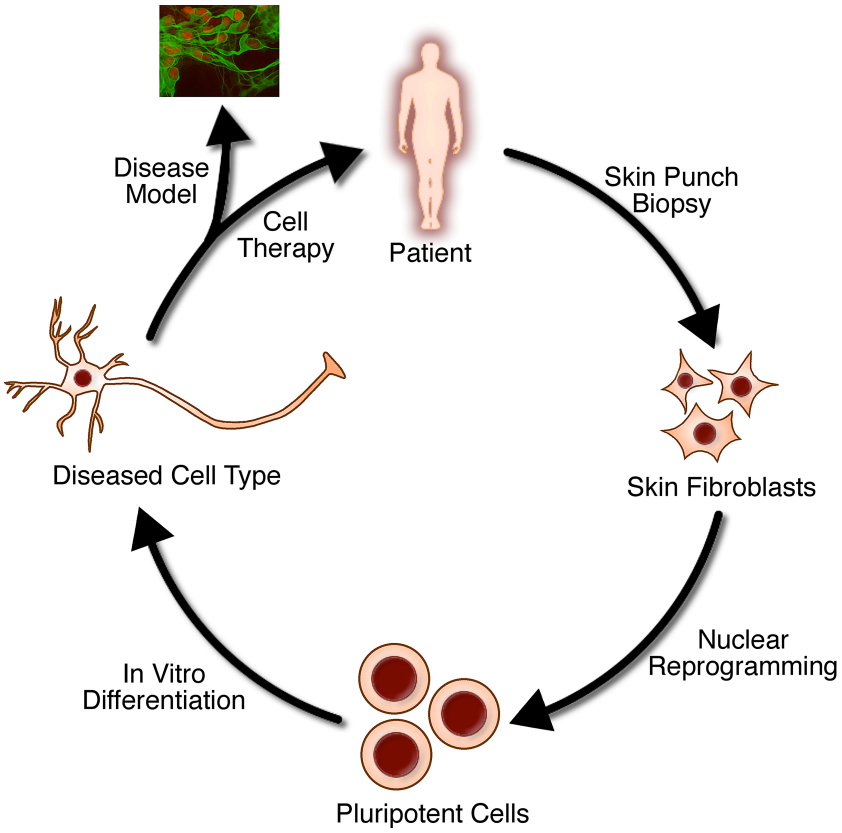

Figure 9: Generation of patient-specific iPS cells for potential medical applications. iPS cells from epidermal fibroblast of ALS (amyotrophic lateral sclerosis) patient were generated and differentiated into motor neurons. This provided an inexhaustible *in vitro* supply for elucidating why motor neurons degenerate in ALS patients (Figure adopted from Dimos et al. 2008).

1.5.7 Development of a "secondary" iPS system

A major bottleneck of direct reprogramming is its very low efficiency ranging from 0.001% and 0.1% (Amabile and Meissner, 2009; Hochedlinger and Plath, 2009). This could be the result of the low rate of quadruple (Oct4, Sox2, Klf4 and c-Myc) infected cells and further the possible need for multiple infections per factor and/or the need for a specific stoichiometry. The infection of somatic cells results

in a heterogeneous population of cells, where some cells receive none, others one or two copies and some multiple copies of the viral vectors. Only a few somatic cells receive the right quantity of transgenes with functional integration, allowing full reprogramming into iPS cells (Figure 10).

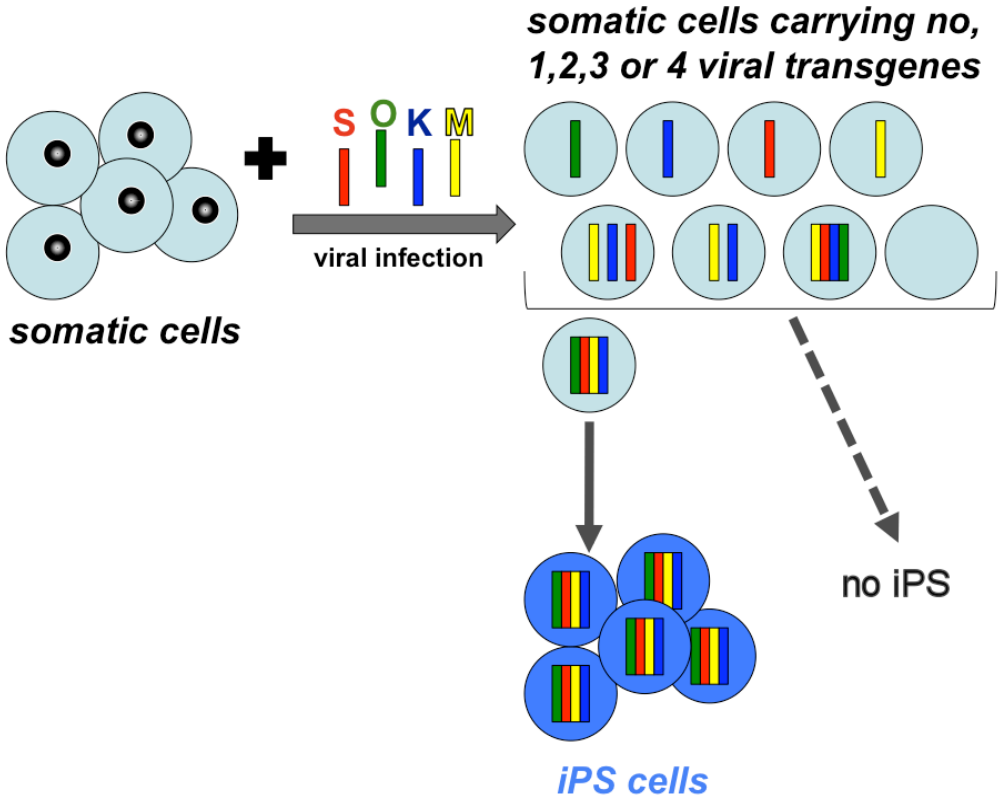

Figure 10: Direct infection results in a heterogenous transgene expression
After direct infection of somatic cells only a small number of cells take up all four viruses at the correct stoichiometry and will reprogram into iPS cells.

To evaluate the reprogramming efficiency more accurately, a cell population with homogenous expression of viral transgenes would be required. The development of so-called “secondary systems” has enabled the generation of iPS cells without the need for direct infection and overcomes these limitations. In this approach, somatic cells are first infected with doxycycline-inducible versions of the reprogramming factors (Figure 11 and 12).

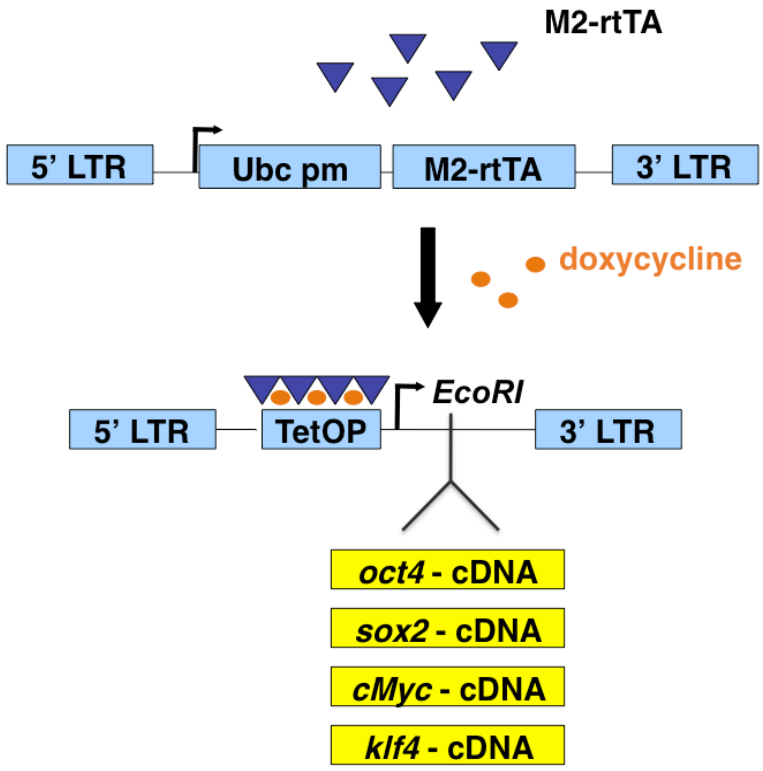

Figure 11: Doxycyline-inducible lentiviral system
Schematic drawing of the lentiviral vector constitutively expressing the reverse tetracycline-controlled transactivator (M2-rtTA) and an inducible lentivirus containing a doxycycline-controllable promoter (TetOP) and a unique EcoRI restriction site to insert separately the cDNAs for c-Myc, Klf4, Oct4 and Sox2. The lentiviral vector is flanked by LTRs (long terminal repeats). The transactivator M2-rtTA is under the control of an ubiquitin promotor (Ubc pm).

Adding doxycycline to this mixed population of infected (primary) cells enables the generation of "primary" iPS cells. Subsequent injection of these iPS cells into blastocysts generates chimeras or "reprogrammable mice", in which every cell derived from the injected iPS cell clone has the same functional viral integrations. Finally, isolation of transgenic cells and re-exposure of these cells to doxycycline then results in homogeneous re-expression of the factors and the

generation of “secondary” iPS cells (Stadtfeld et al., 2008b; Wernig et al., 2008) (Figure 12).

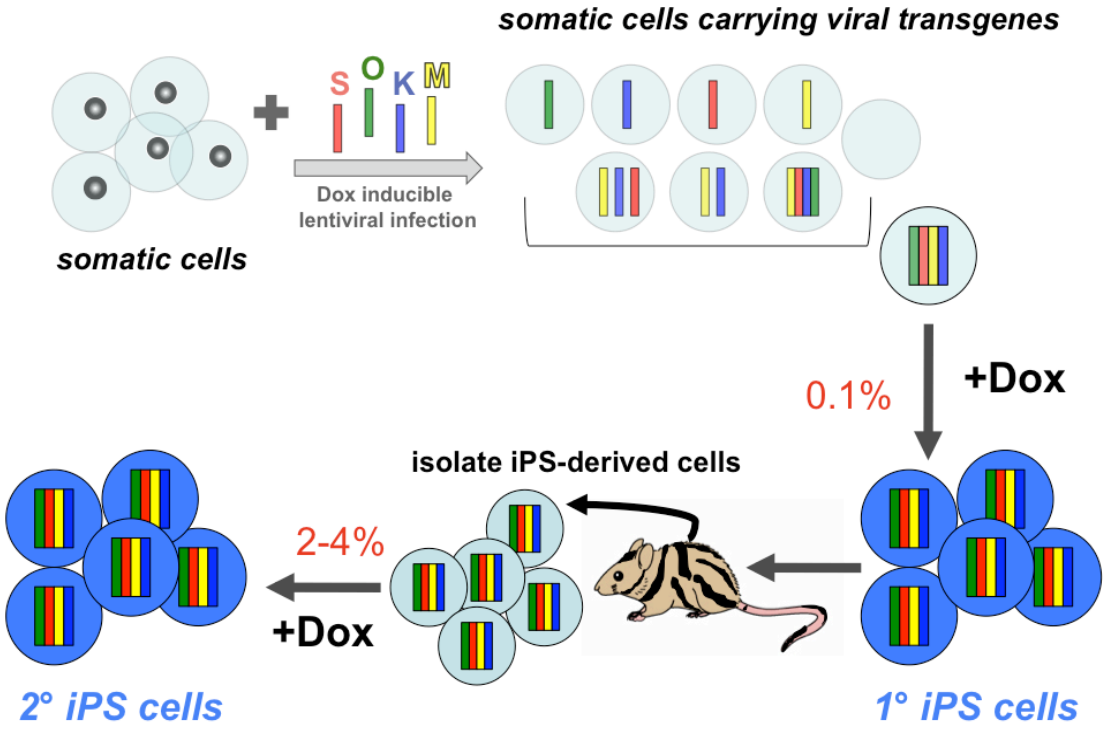

Figure 12: The secondary system.
Direct infection of somatic cells such as mouse fibroblasts with doxycycline inducible lentiviruses results in 0.1% primary iPS cells. Subsequent generation of chimeras (“reprogrammable mice”), isolation of fibroblasts and exposing those to doxycycline *in vitro* results in the formation of secondary iPS cells showing a higher reprogramming efficiency between 2-4%.

Although secondary iPS cells derived from mouse fibroblasts showed a substantial (20-40-fold) increase in their reprogramming efficiency, the overall efficiency is still far from the expected 100% in a population where every cell has the right functional combination of factors (Wernig et al., 2008). Therefore, the technical difficulties resulting from direct infection cannot alone explain the low reprogramming efficiency. Other factors must influence the efficiency of reprogramming by providing a barrier for efficient conversion of adult cells into pluripotent iPS cells.

2. Aims of this thesis

When I began working on reprogramming only Yamanaka's original study had been published on the generation of iPS cells from mouse fibroblasts (mesodermal lineage). The next three studies, including one from our lab, also used mouse fibroblast cells (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007b). It was therefore unclear whether cells originating from other germ layers (ectoderm and endoderm) could be reprogrammed into pluripotent cells.

Aim 1 of this thesis investigated **(i)** if somatic cells of the ectodermal lineage are equally amenable to reprogramming into iPS cells as the previously used mesodermal cells by isolating cells of the well-defined neuroectodermal lineage; **(ii)** if cells such as Neural Progenitor Cells (NPCs), which already express the reprogramming factor Sox2 endogenously, can be reprogrammed with fewer factors (minus Sox2) and thus with less genetic manipulation.

The results from the secondary system described above have shown that the low infection efficiency of somatic cells (with the right combination of factors) does not explain the overall low reprogramming efficiency. One possible barrier to reprogramming is the differentiation state of the starting cell population. This has been already shown to affect the reprogramming efficiency in the context of alternative reprogramming strategies including NT and cell fusion.

Therefore, **Aim 2** of this thesis investigated if the differentiation stage of a somatic cell influences its reprogramming potential into iPS cells. To address this question, cells from various differentiation stages in the well-defined hematopoietic lineage were isolated and compared for their ability and efficiency to generate iPS cells.

3. List of publications

1.) **Eminli, S.,** Utikal, J., Arnold, K., Jaenisch, R., and Hochedlinger, K. (2008)

Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression.

Stem Cells *26*, 2467-2474.

2.) **Eminli, S.**, Foudi, A., Stadtfeld, M., Maherali, N., Ahfeldt, T., Mostoslavsky, G., Hock, H., and Hochedlinger, K. (2009).

Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells.

Nature Genetics *41*, 968-976.

3.1 Publication 1

1.) **Eminli, S.,** Utikal, J., Arnold, K., Jaenisch, R., and Hochedlinger, K. (2008)

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Stem Cells *26*, 2467-2474.

doi: 10.1634/stemcells.2008-0317

The original article will be available online at http://www3.interscience.wiley.com/journal/121628346/abstract

3.2 Publication 2

Eminli, S., Foudi, A., Stadtfeld, M., Maherali, N., Ahfeldt, T., Mostoslavsky, G., Hock, H., and Hochedlinger, K. (2009).

Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells.
Nature Genetics *41*, 968-976.

doi:10.1038/ng.428

The original article will be available online at http://www.nature.com/ng/journal/v41/n9/abs/ng.428.html

3.3 Additional data for *Nature Genetics* manuscript Eminli et al. 2009

We wanted to further substantiate the conclusion that hematopoietic stem and progenitor cells reprogram more efficiently than their differentiated progeny (Aim 2). We therefore repeated the relevant experiments with another, recently developed reprogramming system (Stadtfeld et al., 2010). This viral free system was generated by placing a doxycycline-inducible polycistronic cassette encoding the four reprogramming factors Oct4, Sox2, Klf4 and c-Myc (Figure 13) (Sommer et al., 2009) into the collagene locus of ES cells (Figure 14). The ES cells also express the tetracycline-dependent transactivator (M2-rtTA) from the ROSA26 locus (see also Figure 11 in the introduction part).

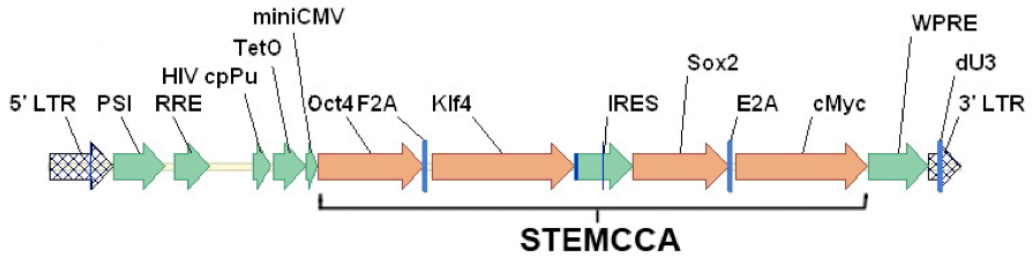

Figure 13: The polycistronic expression cassette (STEMCCA)
The doxycyline-inducible (TetO) polycistronic cassette encodes the four reprogramming factors (Oct4, Klf4, Sox2 and c-Myc) (Figure adopted from Sommer 2008).

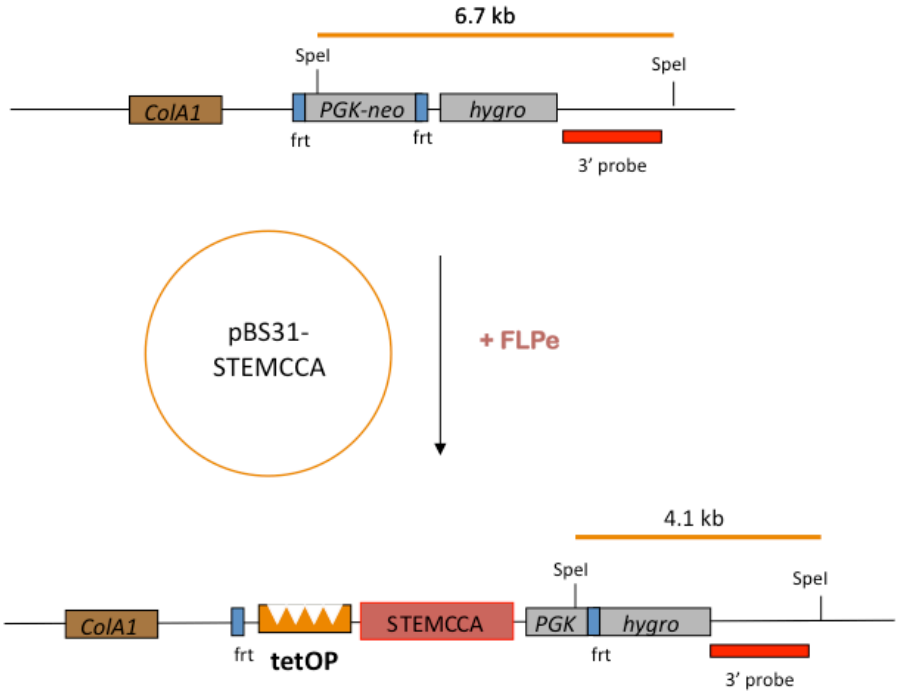

Figure 14: Targeting of the doxycycline-inducible-STEMCCA plasmid into the collagen locus of M2rtTA-ES cells.

The generation of a reprogrammable mouse carrying this inducible construct in all its tissues allowed us to isolate the same hematopoietic cell populations as used before, which could be converted into iPS cells upon addition of doxycycline. The resulting reprogramming efficiencies showed that all immature hematopoietic cell types reprogrammed at high efficiency whereas all mature hematopoietic cells reprogrammed at low efficiency (Figure 15 and Figure 16).

cell type	cytokines	wells plated	iPS colonies (Oct4 GFP+,dox independent)	% efficiency	cell viability on day 5
LT-HSCs	KL,T,FL,3,6	120	41	31.4%	91%
ST-HSCs	KL,T,FL,3,6	180	45	25%	68.5%
CLP	KL,FL,7	180	36	20%	56%
pro-B	KL,FL,7	180	9	5%	52%
GMP	KL,T,FL,3,11,E,GM	180	102	56.6%	100%
CD3-T cells	ConA,2	360	-	-	-
IgM-B cells	C,L,7	360	-	-	-
granulocytes	GM, G	360	-	-	-
macrophages II	M	360	2	-	63%

Figure 15: Reprogramming potential of different hematopoietic cell types into iPS cells with the ES cell based reprogramming system determined by single-cell per well assay.

Reprogramming efficiencies of different cell types after single cell sorting into 96-well plates. Reprogramming efficiencies were determined by counting Oct4 GFP^+ colonies at day 18, 3 days after doxycycline withdrawal. Cell viabilities of individual populations were determined by scoring uninduced cells sorted in 96-well or terasaki plates. “LT-HSC” denotes $CD34^-$ long-term HSCs, “ST-HSCs” denotes $CD34^+$ short-term HSC, “Macrophage II” denotes macrophages arising from total BM cultures for 5 days in ES medium with M-CSF. KL, Kit-ligand; T, TPO; FL, Flt3-ligand; E, EPO; GM, GM-CSF; G, G-CSF; M, M-CSF; C, CpG; L, LPS; 3, IL-3; 6, IL-6; 7, IL-7; 11, IL-11.

Therefore, our results have been verified with an ES cell-derived mouse model, which is free of viral integrations and independent from the origin of the primary somatic cell. Thus, these data corroborated our previous findings that the reprogramming potential inversely correlates with differentiation stage (Eminli et al 2009) (Figure 15 and 16 and also compare to Figure 4B in manuscript).

cell type	cytokines	% efficiency Viral-based system	% efficiency ESC-based system
LT-HSCs	KL,T,FL,3,6	16.1%	31.4%
ST-HSCs	KL,T,FL,3,6	n.d.	25%
CLP	KL,FL,7	15.7%	20%
pro-B	KL,FL,7	7.6%	5%
GMP	KL,T,FL,3,11,E,GM	25%	56.6%
CD3-T cells	ConA,2	0.02%	0.02%
IgD-B cells	C,L,7	0.036%	0.02%
granulocytes	GM, G	0.12%	0.04%
macrophagesII	M	0.35%	0.35%

Figure 16: Resulting reprogramming efficiencies of different hematopoietic cell types into iPS cells with the ES cell (ESC) based reprogramming system and the viral-based system.
Reprogramming efficiencies of different cell types were determined by counting Oct4-GFP$^+$ colonies. LT-HSCs, ST-HSCs, CLPs, pro-B cells, GMPs and macrophages were sorted as single cells into 96-wells, whereas CD3$^+$ T cells, IgD$^+$ B cells and granulocytes were plated on 10cm dishes. Granulocytes (1.5x10^3 cells per 10cm dish), spleen IgD$^+$ mature B cells and spleen mature CD3$^+$ T cells (1x10^6 cells per 10cm dish).

4. Erklärung über Eigenanteil an den Publikationen

Die vorliegende Doktorarbeit besteht aus zwei wissenschaftlichen Veröffentlichungen. In Zusammenarbeit mit meinem Betreuer Prof. Dr. Konrad Hochedlinger entwickelte ich die Grundkonzepte und die initialen Grundideen der Experimente.

Die Planung und die Etablierung des experimentellen Aufbaus, sowie die Auswertung der Ergebnisse und die Verfassung der Manuskripte erfolgte durch meine Person, mit Unterstützung von Prof. Dr. Konrad Hochedlinger. Er betreute die Arbeit und half durch kritische Überarbeitung der Manuskripte.

Ich war an allen experimentellen Aspekten dieser zwei Projekte beteiligt und wurde teilweise von anderen Wissenschaftlern in neuen Techniken angelernt oder unterstützt. Der von mir zu diesen zwei Manuskripten beigetragene experimentelle Eigenanteil wird im Folgenden dargelegt.

1. Publikation:

Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression.
Eminli S, Utikal J, Arnold K, Jaenisch R, Hochedlinger K. ***Stem Cells.*** 2008

Mein experimenteller Eigenanteil beinhaltet im Einzelnen die Bereitstellung von Studienmaterial, u.a. Virusproduktion und Virusinfektionen, sowie zu einem Grossteil die Extraktion und Kultivierung von primären neuralen Vorläuferzellen aus Maushirnen und deren Umwandlung in induzierten pluripotente Stammzellen (iPS Zellen) sowie darauffolgende Kultivierung. Die Weiteranalyse (Teratomainduktion, Produktion von Chimären via Blastozyteninjektion, u.a.) wurde von mir alleine durchgeführt.

Jochen Utikal unterstützte mich bei der Kultivierung von neuralen Vorläuferzellen

und der Weiterkultivierung von iPS Zellen, sowie mit der molekularen Analyse dieser Zellen.

2. Publikation

Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells.

Eminli S, Foudi A, Stadtfeld M, Maherali N, Ahfeldt T, Mostoslavsky G, Hock H, Hochedlinger K. ***Nat Genet.*** 2009

Mein experimenteller Eigenanteil beinhaltet die Bereitstellung von Studienmaterial wie u.a. die Extraktion und Kultivierung von verschiedenen hematopoetischen Zellen. Ich habe die Virusproduktion und Virusinfektionen, sowie deren Umwandlung in induzierte pluripotente Stammzellen (iPS Zellen) und deren darauffolgende Kultivierung zu einem Grossteil übernommen (90%). Die Weiteranalyse wie u.a. Teratomainduktion und die Produktion von Chimären ("reprogrammierbare Mäuse"/sekundäre Mäuse und tertiäre Mäuse) via Blastozysteninjektion wurde komplett von mir durchgeführt. Die zusätzliche Datengeneration, Datenanalyse und -interpretation (beschrieben im Chapter 3.3) wurden ebenfalls von mir alleine durchgeführt.

Adlen Foudi aus dem Hock Labor hat mich unterstützt und angelernt in der Extraktion von primären hematopoietischen Zellen aus den spezifischen Mausorganen (Knochenmark, Blut, Lymphknoten, Thymus sowie Milz). Zusätzlich wurde mir von ihm die Isolation durch fluorescence activated cell sorting (FACS) beigebracht, so dass ich die weiteren Experimente selbstständig durchführen konnte.

5. Discussion

Takahashi and Yamanaka were the first who generated iPS cells in 2006, by ectopic expression of the four transcription factors Oct4, Sox2, c-Myc and Klf4 in murine fibroblasts (Takahashi and Yamanaka, 2006). Since then, iPS cells proved to be highly similar to ES cells (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007b). Like ES cells, iPS cells can differentiate into virtually all cell types making them very attractive for basic research and clinical applications. In addition, iPS cells circumvent two of the major obstacles that limit the use of human ES cells: (i) the ethical concern over the use of embryos to isolate ES cells and (ii) the generation of patient-specific stem cells without the risk of immune rejection.

Transcription factor-mediated reprogramming is therefore likely to have a tremendous impact on regenerative medicine and will enable essential studies eventually leading to the treatment of many diseases. However, before safe therapeutic interventions based on iPS cell technology can be devised, the biological mechanisms underlying reprogramming have to be better understood.

This thesis makes several relevant contributions towards this goal and addresses among others the following key questions in the reprogramming field: Are all cell types equally amenable to reprogramming? Which cell type can be reprogrammed with fewer factors and thus with less genetic manipulations? Does the differentiation state of the donor cell directly influence the reprogramming efficiency? What is the ideal starting cell type for efficient and safe reprogramming?

5.1 Somatic cells from all three germ layers can be reprogrammed into iPS cells *(Aim 1.1 published in Eminli et al. 2008)*

Initial studies used mouse fibroblasts, which originate from the mesodermal lineage, as the starting population for reprogramming into iPS cells. It remained unclear whether cells from the ectodermal and endodermal lineage could also be reprogrammed by the four factors.

Mouse fibroblast cultures represent a heterogeneous population of cells, possibly including muscle cells, hematopoietic cells, endothelial cells and probably even mesenchymal stem cells. All of these can be propagated under the same culture conditions used for reprogramming (S.E. unpublished data). Consequently, the exact cell type that gave rise to the initial iPS cell lines across all previous experiments was unknown.

Subsequent experiments showed that iPS cells can be generated from explanted liver and stomach tissues, which belong to the endodermal lineage (Aoi et al., 2008). However, these cultures very likely contained non-epithelial cell types such as mesenchymal and hematopoietic cells, which may have been the selective donors for reprogrammed cells.

Given that mesodermal (fibroblasts) and endodermal (liver) cells had been reported to be reprogrammed into iPS cells in previous experiments, we set out using a more defined system to test the questions: (i) whether cells of the third germ layer (ectoderm) are equally amenable to reprogramming into iPS cells and (ii) whether ectodermal cells require the same combination of factors as mesodermal and endodermal cells. Addressing these questions is important for drawing conclusions about the generality of factor-induced reprogramming across cell types from all three germ layers and for identifying cell types that may require fewer factors.

Thus, we isolated a well-defined ectodermal cell type, namely Neural Progenitor Cells (NPCs). NPCs are characterized by high expression of the

specific neural progenitor marker Nestin and high levels of the transcription factor Sox2 (Lendahl et al., 1990; Pevny and Placzek, 2005; Pevny et al., 1998; Yaworsky and Kappen, 1999). Another experimental advantage of using NPCs is that they require culture in serum-free conditions, which selects against many non-neuronal cell types. Across experiments the purity of the starting NPC population was confirmed by isolating NPCs from mice carrying two different reporter constructs: (i) a Sox2-GFP reporter and (ii) a Nestin-Cre/R26-EYFP lineage-tracing system, which genetically marks NPCs. We isolated NPCs from neonatal mice, expanded them in serum-free medium and infected them with the four reprogramming factors.

Indeed, as shown in Figure 1A-D of our *Stem Cells* publication (Eminli et al. 2008), we could generate stable NPC-derived four factor iPS cells, which re-expressed specific pluripotency markers and showed demethylation of the promoters of essential pluripotency genes. Both confirming the successful reprogramming and re-establishment of an embryonic like phenotype.

The data of this first part of aim 1 unequivocally demonstrated that in addition to mesoderm- and endoderm-derived iPS cells, cells of the ectodermal lineage are equally amenable to reprogramming into iPS cells with the same four factor-combination.

Since our publication, transcription factor-induced reprogramming of somatic cells into iPS cells has further evolved and also been achieved in several other cell types from all three germ layers (Eminli et al., 2009; Stadtfeld et al., 2008a; Takahashi and Yamanaka, 2006; Utikal et al., 2009). In addition iPS cells were generated from different species such as rat (Li et al., 2009; Liao et al., 2009), monkey (Liu et al., 2008) and human (Lowry et al., 2008; Park et al., 2008b; Park et al., 2008c; Takahashi et al., 2007) with the same four factors. So far, these results confirmed that direct reprogramming into iPS cells seems to be a universal process.

5.2 Sox2 is dispensable for the reprogramming of NPCs into iPS cells *(Aim 1.2 published in Eminli et al. 2008)*

The second part of aim 1 was to determine if the factor requirement is depended on the cellular context of a cell. More specifically we asked, if cells that endogenously express a subset of the four factors no longer require these to be exogenously expressed in order to reprogram them into iPS cells. For instance, as described above, NPCs express high levels of the transcription factor Sox2, one of the four reprogramming and essential pluripotency genes (Pevny and Placzek, 2005; Pevny et al., 1998; Takahashi and Yamanaka, 2006).

We therefore hypothesized that these cells might not require exogenous Sox2 for reprogramming into iPS cells and thus represent a “primed” starting population.

To confirm the purity of the starting NPC population, NPCs were again isolated from mice carrying either the Sox2-GFP reporter or the Nestin-Cre/R26-EYFP lineage-tracing system.

As shown in Figure 2C and 4B in our *Stem Cells* publication (Eminli et al. 2008), exogenous expression of Sox2 is not required to reprogram NPCs into stable pluripotent cell lines. These three-factor iPS cells expressed markers of ES cells and contributed to high degree adult chimeras. In contrast, infection of tail-tip fibroblasts with just three factors in the absence of Sox2 was insufficient to produce iPS cells.

Interestingly, the attempt to generate iPS cells with the complete set of four factors resulted in a substantially lower number of iPS cells. This suggests that elevated levels of Sox2 might be detrimental and that the specific stochiometric balance of the four reprogramming factors is critical during reprogramming.

All of the established iPS cell lines produced until 2008 contained multiple viral integration sites in their genome. Lenti- and retroviral vectors often randomly

integrate into endogenous genes and can result in improper gene activation and tumor formation (Hochedlinger et al., 2005; Okita et al., 2007). If fewer exogenous factors (in the case of reprogramming NPCs) and consequently less genetic manipulations are required to generate a pluripotent cell from a somatic cell, direct reprogramming would become immediately safer and therefore closer to eventual clinical applications.

More recently, it has been shown that it is feasible to generate transgene-free iPS cells (Kaji et al., 2009; Okita et al., 2008; Soldner et al., 2009; Stadtfeld et al., 2008c; Woltjen et al., 2009). iPS cell formation through the repeated infection with adenoviruses (Stadtfeld et al., 2008c) or transfection with plasmids carrying the four factors (Okita et al., 2008) into mouse hepatocytes and MEFs, respectively, produced several iPS cell lines with no integrations into the host genome. Other recent approaches successfully used excisable lentiviruses (Soldner et al., 2009) or transposons followed by transgene removal using Cre-mediated excision or re-expression of transposase, respectively (Kaji et al., 2009; Woltjen et al., 2009).

Recent advances of using small molecules, which partially act by altering chromatin modifications and DNA methylation or by inhibiting key signaling pathways, were able to replace individual reprogramming factors (Huangfu et al., 2008a; Huangfu et al., 2008b; Ichida et al., 2009; Mikkelsen et al., 2008; Shi et al., 2008; Silva et al., 2009). It seems that some of the small molecules, which are able to replace individual reprogramming factors such as Sox2 act by indirectly inducing other important pluripotency genes like Nanog, suggesting that multiple routes can lead to reprogrammed cells (Ichida et al., 2009).

In summary, the findings of aim 1 suggest that less genetic manipulations are required to reprogram NPCs into a pluripotent state compared to fibroblasts or other mature cell types. This finding is an important step forward for the use of iPS cells and its eventual translation into regenerative medicine.

Consistent with the murine results in my study and further extending the

question of factor requirement, it has been shown that human and mouse NPCs can give rise to iPS cells when only exogenous Oct4 is expressed (Kim et al., 2009a; Kim et al., 2009b). Moreover, even skin-derived neural crest derivatives from adult skin, were recently reprogrammed into iPS cells with just Oct4 and Sox2 (Hunt et al., 2009; Tsai et al.). This sets the stage for identifying small molecule compounds that regulate Oct4 in the context of reprogramming NPCs. In addition it might be used to identify general mechanisms that can be translated, to other easy obtainable cell types. This could avoid the requirement for ectopic expression of Oct4 and therefore eliminate the necessity of using viral transgenes all together.

5.3 Differentiation state determines reprogramming potential of hematopoietic cells into iPS cells *(Aim 2 published in Eminli et al. 2009)*

Based on the current literature it can be assumed that most, if not all, murine cell types can be reprogrammed into iPS cells by introducing the factor combination Klf4, Oct4, c-Myc and Sox2.

However, a recent manuscript suggested that these four factors were insufficient to reprogram terminally differentiated B cells; whereas immature progenitor B cells could readily give rise iPS cells (Hanna et al., 2008). The authors found that a fifth transcription factor, CEBPalpha, had to be overexpressed for reprogramming the mature B cells into a pluripotent state. This work suggested that terminally differentiated lymphocytes might be refractory to reprogramming by just four factors.

Interestingly, previous nuclear transfer (NT) and cell fusion experiments suggested that less differentiated cells generally reprogram more easily and more efficiently than fully differentiated cells. For example, the cloning of mice

from neural stem cells and skin stem cells was found to be more efficient than that from mature neurons and keratinocytes, respectively (Blelloch et al., 2006; Li et al., 2007).

Contradictory results have been reported in other NT experiments using hematopoietic cells (Inoue et al., 2006; Sung et al., 2006). The study by Sung et al. suggested in contrast to what would be expected that cloning efficiency increased with more advanced differentiation state. Specifically, mature granulocytes were found to be more amenable to reprogramming than hematopoietic progenitor and stem cells. Notably, the efficiency was only measured in terms of blastocyst formation and not development of the cloned embryos beyond this stage. This question was never settled in NT experiments. Using the advance of iPS technology it was now possible to revisit this and ask, how does the differentiation stage of the starting cell influence the efficiency of reprogramming into iPS cells?

The second aim of my thesis therefore focused on the question of whether the differentiation state of the somatic donor cell directly influences the reprogramming potential. Specifically, I hypothesized that immature cells are more amenable to reprogramming than mature cells, because they are developmentally, and therefore possibly also epigenetically, closer to pluripotent cells and may as a result reprogram more efficiently.

To test the hypothesis that an adult progenitor cells is indeed easier to reprogram than a differentiated cell, we used the well-defined hematopoietic system. On top of this differentiation hierarchy is the quiescent, most undifferentiated multipotent HSCs, which gives rise to various progenitor cells and ultimately all the terminally differentiated cells of the myeloid and lymphoid lineage. All cell types in the hematopoietic system are well defined by their surface markers, allowing for their prospective isolation by fluorescence-activated cell sorting (FACS), making this lineage ideally suited for the purposes of Aim 2. This made it feasible to compare and interpret reprogramming potentials from

various differentiation stages within the same lineage. We initially used a virus based system secondary system to reprogram hematopoietic cells into iPS cells (see introduction part Chapter 1.5.7).

In order to determine the reprogramming potential of different blood cells, nine different primitive hematopoietic cell populations including HSCs, a mixed cell population consisting of HSC and progenitors (HSC/Ps), myeloid progenitors (consisting of common myeloid progenitors (CMP), megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs)), common lymphoid progenitors (CLPs), immature pro-B cells and pro-T cells as well as their differentiated progenies, B cells, T cells, macrophages and granulocytes were included in this study.

Our studies demonstrated that immature cells of the hematopoietic lineage are more amenable to reprogramming than differentiated cell types, providing the first direct link between differentiation state and reprogramming efficiency into iPS cells. The efficiencies at which progenitors converted into iPS cells was up to 2 orders of magnitude higher (7-28%) than that of differentiated blood cell types (0.02-0.6%) and also constitutes the highest reprogramming efficiencies reported thus far.

Importantly, we can conclude from our results that all isolated hematopoietic cell types, including terminally differentiated B an T cells, could be reprogrammed with the same four factors and do not require manipulation of additional genes as suggested by Hanna and colleagues (Hanna et al., 2008). Our observation that C/EBPα expression enhances but is not required for the successful reprogramming of mature B cells into iPS cells may provide an explanation for the previously reported failure to generate iPS cells from mature B cells. Moreover, clone-to-clone variation and the resulting differences in expression levels of the four factors might explain these results (Stadtfeld et al., 2010).

Interestingly we could show that the differentiation state of a cell not only influenced the reprogramming efficiency but also the kinetics of the process. Our experiments indicate that progenitors reprogram twice as fast as differentiated cells. For example, myeloid progenitors reprogrammed after only 6 days of transgene expression in contrast to mature granulocytes, which required at least 12 days of transgene expression. Importantly, reprogramming was independent of the proliferation rate of cells. That is, quiescent (9% cells cycling) and cytokine-activated (41% cells cycling) HSCs reprogrammed equally efficient, whereas mature B and T cells reprogrammed least efficiently irrespective of their proliferation rate.

Consistently, we found that immature cells such as skeletal muscle precursor (SMP) cells, which have a very low cell cycling rate (4%) (Cerletti et al., 2008) reprogrammed more efficiently (28%) compared to differentiated myoblasts (60% cycling rate) (Tan and Eminli et al. *in preparation*).

Integration site and cell type dependent viral silencing may limit the secondary system, we therefore wished to confirm our results with an independent system. Specifically, we used a transgenic mouse line generated from ES cells carrying the four reprogramming factors in a defined locus under doxycycline control (Stadtfeld et al., 2010). The generation of a “reprogrammable mouse” allowed us to isolate the same hematopoietic cell populations as used with the virus-based system (Eminli et al., 2009). The resulting reprogramming efficiencies of immature and mature hematopoietic cells were consistent with the ones obtained from the iPS cell derived secondary system chimeras (Eminli et al., 2009). Immature cells showed a very high reprogramming efficiency in contrast to more mature cell types (these additional data are presented in Chapter 3.3).

In summary, the ES cell-derived mouse model, which is free of viral integrations and should be less variable, supported the notion that the differentiation state of somatic cells influences their reprogramming potential into iPS cells.

Various groups have recently derived iPS cells from mature skin cells of patients with different neurodegenerative diseases for therapeutic applications and disease modeling (Dimos et al., 2008; Ebert et al., 2009; Soldner et al., 2009) (see also Chapter 1.5.6 in the introduction). Although it has been shown that epigenetic changes and the loss of telomerase activity is reversed in adult mouse and human iPS cells (Marion et al., 2009; Stadtfeld et al., 2008b; Takahashi et al., 2007), pre-existing mutations cannot be corrected during the reprogramming process. Progenitor cells have likely accrued fewer genetic changes than differentiated cells and may thus be safer source to produce high quality patient-specific iPS cells (Trifunovic, 2006; Trifunovic et al., 2004).

We therefore, as a proof-of-principle experiment, wanted to extend our prior murine results and reprogram human $CD34^+$ umbilical cord blood (UCB) cells into iPS cells. UCB is blood that remains in the placenta and in the attached umbilical cord after childbirth. It is often collected and stored in UCB banks because it contains CD34+ progenitor cells. The use of these cells for therapeutic applications could represent an alternative and readily accessible source of stem cells. Moreover, if UCB iPS cells could be established from existing UCB samples, it would represent a wide panel of HLA (Human Leukocyte Antigen) haplotypes. Thus, selecting donors homozygous for common haplotypes would provide a perfect match for successful transplantation for a large percentage of the population.

As we have shown in Supplementary Figure 6 in Eminli et al 2009, human iPS derived from UCB cells emerged unusually fast and were already transgene-independent on day 12 compared to 30 days in previously used human cell types (Huangfu et al., 2008b; Maherali et al., 2008; Takahashi et al., 2007). This is also consistent with the accelerated reprogramming kinetics that we observed in the

hematopoietic mouse stem and progenitor cells. These data therefore suggest, that an increased efficiency to reprogramming may be a common attribute of human and mouse hematopoietic stem and progenitor cells. As demonstrated by our work of reprogramming NPCs, progenitor cells need less exogenous manipulation because they share some features with ES cells, such as the capacity to differentiate into different cell types and certain transcriptional regulators like Sox2 (Eminli et al., 2008; Galan-Caridad et al., 2007). Therefore, identifying the molecular differences between immature and mature cell populations could further teach us about the epigenetic and transcriptional barriers that seem inherent to the nuclear reprogramming process and could lead to human iPS cells which require less genetic manipulations.

Indeed, shortly after our initial publication (Eminli et al., 2009) it was reported that human UCB cells could be reprogrammed into iPS cells by just introducing Oct4 and Sox2. The authors showed that human hematopoietic stem and progenitor cells express a set of pluripotency-associated genes, Oct4 and Nanog, albeit at much lower levels than human ES, and their promoters seem to have much lower levels of the repressive histone marks H3K27 and H3K9 compared to fibroblasts cells (Giorgetti et al., 2009). Thus, the inherent epigenetic state of theses important pluripotency genes in stem and progenitor cells may facilitate the required epigenetic changes to pluripotency.

In addition, both human immature hematopoietic cells and juvenile keratinocytes, seem to express high levels of c-Myc and Klf4 compared to mature fibroblasts (Aasen et al., 2008; Feinberg et al., 2007; Giorgetti et al., 2009). These high endogenous levels of reprogramming factors in immature cell types may allow them to reprogram faster, more efficiently and with less exogenous factors compared to human mature fibroblast cells. These recent observations are consistent with our initial finding that factor requirement is dependent on the cellular context (Eminli et al., 2009).

High levels of endogenous Klf4 could favor the reprogramming process in these cell types. Overexpression of Klf4 in ES cells has been shown to prevent their differentiation and this may in part be through its role of regulating the pluripotency factor Nanog (Jiang et al., 2008).

Similarly, the transcription factor c-Myc has been implicated in promoting the self-renewal of ES cells (Cartwright et al., 2005). Because human and mouse stem and progenitor cells express high c-Myc levels it could partly explain the increased reprogramming potential in these cells (Feinberg et al., 2007; Giorgetti et al., 2009; Kastan et al., 1989a; Kastan et al., 1989b). Specifically, it is known that loss of c-Myc in HSCs prevents their differentiation into progenitors but their proliferation capacity is not affected. Therefore, c-Myc seems to control the balance between stem cell self-renewal and differentiation by regulating the interaction between HSCs and their niche (Wilson et al., 2008). If forced c-Myc expression induces HSC differentiation, it may explain why we observed that the reprogramming efficiency of dormant HSCs was lower than that of certain progenitor cells, although HSCs are on top of the differentiation hierarchy (see Figure 4 in Eminli et. al 2009).

In summary, our data provided several new insights into the process of direct reprogramming. In particular, we showed that cells that already endogenously express a subset of the four factors can be reprogrammed with fewer factors and thus with less exogenous manipulation. Furthermore, the reprogramming of NPCs was the first demonstration that cells from the ectodermal lineage can also be reprogrammed to iPS cells, indicating that cells from all three germ layers are equally amenable to reprogramming into iPS cells. Finally, we established the first direct link between the differentiation state of cells and their reprogramming potential using cells of the hematopoietic system.

5.4 Perspectives

Global gene expression profiles of NT ES cell lines derived from different donor cell types are indistinguishable from those of fertilization-derived ES cell lines, indicating that the reprogrammed NT ES cells might be as useful for therapeutic applications as fertilization-derived ES cells (Brambrink et al., 2006). It will be important to determine in the future whether transcription factor induced reprogramming is as faithful as reprogramming by NT, which could affect their therapeutic utility.

The use of iPS cells generated with the different techniques discussed so far still comprises major safety concerns for potential clinical applications such as custom-tailored cell therapy and might complicate disease modeling. Therefore, several questions need to be resolved before applying iPS cells in a clinical setting becomes feasible.

First, iPS cells need to be generated without integrating transgenes, especially considering the oncogenes c-Myc and Klf4, since the reactivation of these genes causes tumors in mice (Markoulaki et al., 2009; Okita et al., 2007). Therefore, transplanting patient-specific iPS cells after their directed differentiation into the desired cell type maybe harmful (leading to tumor formation in the patient), possibly due to remnant undifferentiated cell, the residual activity or reactivation of the transgenes.

Second, iPS cells made from different cell types may have varying propensities to differentiate into mature cell types. Consistent with this idea is the observation that iPS cell lines derived from mouse adult fibroblasts differentiate less efficiently *in vitro* and are more prone to develop tumors upon transplantation compared to immature mouse embryonic fibroblasts (Miura et al., 2009). However, the underlying molecular mechanism for this observation remains elusive. Another unresolved question is whether iPS cells derived from different cell types retain an epigenetic memory of the cell they were derived

from. For example, is there a trend of iPS cells to preferentially differentiate into cell lineages of their origin? This needs to be addressed before patient-specific iPS cells can be faithfully used for disease modeling and thus for investigating the underlying mechanism of disease pathogenesis. Further it is crucial to investigate if iPS cells derived from different cell types are molecularly and functional equivalent or not. However, one could also take advantage of any differentiation bias and produce somatic cells that have thus far been difficult to obtain from ES cells, such as pancreatic beta cells or skeletal muscle cells.

Third, a quantitative and qualitative readout for the grade of iPS cells would be crucial. There has been significant progress in identifying cell types that are efficiently reprogrammed and easy to obtain, but currently the most stringent test to measure pluripotency in the human system is teratoma formation. This assay is time-consuming and difficult to interpret, since it could be shown in mouse that incompletely reprogrammed iPS cells are still able to form teratomas but cannot generate germline-competent mice (Takahashi and Yamanaka, 2006). Hence, it will be important to identify markers that allow researchers to prospectively isolate the highest quality iPS cells.

Fourth, another challenge in the iPS cell field is to develop improved differentiation protocols into all functional cell types *in vitro*, which are comparable to and function as well as their counterparts *in vivo*.

Lastly, it remains to be seen if reprogramming by “lineage conversion”, also called trans-differentiation, may be another way to produce patient-specific cells without the need of a pluripotent intermediate or immature cell state. While this approach is quite appealing for generating certain cell types, which are difficult to obtain from iPS and ES cells such as pancreatic beta cells, this approach has its limitation. For example, only closely related cell types within the same tissues have been amenable to trans-differentiation so far (Cobaleda et al., 2007a; Cobaleda et al., 2007b; Davis et al., 1987; Laiosa et al., 2006; Xie et al., 2004; Zhou et al., 2008) (see also introduction part Chapter 5.4). Also, only a

handful of cell type switches have been achieved to date and lastly, it remains unclear if the switched cells are as functional as their *in vivo* counterparts.

Despite rapid progress in the field of direct reprogramming over the last three years and the promise of iPS cells for therapeutic purposes, the molecular and functional properties of these cells are not yet fully understood. It will thus be essential to verify to what extent iPS cells are indistinguishable from ES cells. Until these questions have been resolved, ES cells will remain an important reference and gold standard for pluripotent cells.

6. Summary

Direct reprogramming of somatic cells into induced pluripotent stem (iPS) cells has been achieved by overexpression of defined transcription factor combinations such as c-Myc, Klf4, Oct4 and Sox2. iPS cells are molecularly highly similar to ES cells and can differentiate into virtually any cell type of the body including germ cells. This observation makes iPS cells very attractive for basic research and potential clinical applications.

This PhD thesis consists of two parts; the first part was aimed at studying the role of the somatic cell-of-origin in factor-mediated reprogramming while the second part is aimed at determining whether the differentiation stage of the starting cell affects the efficiency of reprogramming. In the first part, I showed that a defined ectodermal cell type, specifically Neural Progenitor Cells (NPCs), can be reprogrammed into iPS cells, thus demonstrating that cells from all three germ layers are equally amenable to reprogramming into iPS cells by the four factors. Another main conclusion of this part is the observation that cells, which already express one of the four reprogramming factors endogenously, can be reprogrammed with fewer exogenous factors. In particular, NPCs, which express high levels of Sox2, could be reprogrammed into iPS cells in the absence of exogenous Sox2.

The second part of this thesis provided the first direct link between the differentiation stage of cells and their reprogramming potential into iPS cells. By using nine different immature hematopoietic cell populations and their differentiated progenies, I showed that immature cells are more amenable to reprogramming than differentiated cell types. For example, adult hematopoietic stem and progenitor cells converted 300 times more efficiently and twice as fast as terminally differentiated cells into iPS cells. Moreover, I was able to translate

these observations into a human setting by generating the first human umbilical cord blood-derived iPS cells.

In summary, this thesis provides novel biological insights into the role of the somatic cell-of-origin in direct reprogramming and may ultimately facilitate a more efficient and safer generation of patient specific iPS cells.

7. Zusammenfassung

Somatische Zellen können durch die Überexpression von vier verschiedenen Transkriptionsfaktoren, c-Myc, Klf4, Oct4 and Sox2, in sogenannte induzierte pluripotente Stammzellen (iPS Zellen) reprogrammiert werden. Es scheint, dass iPS Zellen und embryonale Stammzellen (ES Zellen) sich in ihren Eigenschaften sehr ähnlich sind. ES Zellen wie auch iPS Zellen haben das Potential, sich in jeden Zelltyp des Körpers zu entwickeln. Diese Eigenschaft macht iPS Zellen zu einem attraktiven Target für die Grundlagenforschung, öffnet aber auch weitreichende Möglichkeiten für potentielle therapeutische Anwendungenszwecke.

Diese Doktorarbeit besteht aus zwei Teilen; der erste Teil befasste sich mit der Rolle des Ausgangzelltyps in Bezug auf Faktoren-vermittelte Reprogrammierung während der zweite Teil untersucht, ob der Differenzierungsgrad einen Einfluss auf die Effizienz von somatischer Zellreprogrammierung hat.

Im ersten Teil dieser Doktorarbeit konnte gezeigt werden, dass ein bestimmter ektodermaler Zelltyp, insbesondere neurale Vorläuferzellen, in iPS Zellen reprogrammiert werden können, und bestätigte damit, dass Zellabkömmlinge von allen drei Keimblättern gleichermaßen zugänglich sind, um mit den selben 4 Faktoren in iPS Zellen reprogrammiert zu werden. Ein weiteres wichtiges Ergebnis des ersten Projekts ist, dass Zellen, die bereits einen der 4 Reprogrammierungsfaktoren endogen exprimieren, weniger exogene Faktoren benötigen, um iPS Zellen zu produzieren. Es konnte gezeigt werden, dass neurale Vorläuferzellen, die vergleichbare Mengen an Sox2 exprimieren wie ES Zellen, auch ohne virale Überexpression von Sox2 in iPS Zellen reprogrammiert werden können.
Im zweiten Teil dieser Doktorarbeit konnte erstmals ein Zusammenhang zwischen dem Differenzierungsgrad somatischer Zellen und deren

Reprogrammierungspotential in iPS Zellen aufgezeigt werden. Zu diesem Zweck wurden 9 verschiedene hämatopoetische unreife Zellpopulationen und ihre differenzierten Abkömmlinge isoliert. Weiter konnte gezeigt werden, dass unreifen Zelltypen ein generell viel höheres Reprogrammierungspotential besitzen als differenzierte Zellen. Beispielsweise konnten hämatopoetische Stamm- und Vorläuferzellen 300-fach effizienter und zweimal schneller in iPS Zellen umgewandelt werden verglichen mit differenzierten Zelltypen. Darüber hinaus konnten wir unsere Beobachtungen auch auf humane Zellen übertragen, indem wir zum ersten Mal aufzeigten, dass menschliches Nabelschnurblut in iPS Zellen umgewandelt werden kann.

Zusammenfassend lieferte diese Doktorarbeit neuartige biologische Erkenntnisse über die Rolle der somatischen Ausgangsszelle im Zusammenhang mit Faktoren-vermittelter Reprogrammierung und mag damit letztendlich die effiziente und sicherere Erzeugung von Patienten-spezifischen iPS Zellen ermöglichen.

8. Bibliography

Aasen, T., Raya, A., Barrero, M.J., Garreta, E., Consiglio, A., Gonzalez, F., Vassena, R., Bilic, J., Pekarik, V., Tiscornia, G.*, et al.* (2008). Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol *26*, 1276-1284.

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

Amit, M., Carpenter, M.K., Inokuma, M.S., Chiu, C.P., Harris, C.P., Waknitz, M.A., Itskovitz-Eldor, J., and Thomson, J.A. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol *227*, 271-278.

Andrews, P.W. (2002). From teratocarcinomas to embryonic stem cells. Philos Trans R Soc Lond B Biol Sci *357*, 405-417.

Aoi, T., Yae, K., Nakagawa, M., Ichisaka, T., Okita, K., Takahashi, K., Chiba, T., and Yamanaka, S. (2008). Generation of Pluripotent Stem Cells from Adult Mouse Liver and Stomach Cells. Science.

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

Beisel, C., Buness, A., Roustan-Espinosa, I.M., Koch, B., Schmitt, S., Haas, S.A., Hild, M., Katsuyama, T., and Paro, R. (2007). Comparing active and repressed expression states of genes controlled by the Polycomb/Trithorax group proteins. Proc Natl Acad Sci U S A *104*, 16615-16620.

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

Blelloch, R., Wang, Z., Meissner, A., Pollard, S., Smith, A., and Jaenisch, R. (2006). Reprogramming Efficiency following Somatic Cell Nuclear Transfer is Influenced by the Differentiation and Methylation State of the Donor Nucleus. Stem Cells.

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

Bradley, A., Evans, M., Kaufman, M.H., and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature *309*, 255-256.

Brambrink, T., Hochedlinger, K., Bell, G., and Jaenisch, R. (2006). ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. Proc Natl Acad Sci U S A *103*, 933-938.

Brons, I.G., Smithers, L.E., Trotter, M.W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A.*, et al.* (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature *448*, 191-195.

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.

Cartwright, P., McLean, C., Sheppard, A., Rivett, D., Jones, K., and Dalton, S. (2005). LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development *132*, 885-896.

Cerletti, M., Jurga, S., Witczak, C.A., Hirshman, M.F., Shadrach, J.L., Goodyear, L.J., and Wagers, A.J. (2008). Highly efficient, functional engraftment of skeletal muscle stem cells in dystrophic muscles. Cell *134*, 37-47.

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

Chung, Y., Klimanskaya, I., Becker, S., Marh, J., Lu, S.J., Johnson, J., Meisner, L., and Lanza, R. (2006). Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. Nature *439*, 216-219.

Cobaleda, C., Jochum, W., and Busslinger, M. (2007a). Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. Nature *449*, 473-477.

Cobaleda, C., Schebesta, A., Delogu, A., and Busslinger, M. (2007b). Pax5: the guardian of B cell identity and function. Nat Immunol *8*, 463-470.

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

Davis, R.L., Weintraub, H., and Lassar, A.B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell *51*, 987-1000.

Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitsumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R.*, et al.* (2008). Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science *321*, 1218-1221.

Do, J.T., and Scholer, H.R. (2004). Nuclei of embryonic stem cells reprogram somatic cells. Stem Cells *22*, 941-949.

Dodge, J.E., Ramsahoye, B.H., Wo, Z.G., Okano, M., and Li, E. (2002). De novo methylation of MMLV provirus in embryonic stem cells: CpG versus non-CpG methylation. Gene *289*, 41-48.

Durcova-Hills, G., Adams, I.R., Barton, S.C., Surani, M.A., and McLaren, A. (2006a). The role of exogenous FGF-2 on the reprogramming of primordial germ cells into pluripotent stem cells. Stem Cells *in press*.

Durcova-Hills, G., Adams, I.R., Barton, S.C., Surani, M.A., and McLaren, A. (2006b). The role of exogenous fibroblast growth factor-2 on the reprogramming of primordial germ cells into pluripotent stem cells. Stem Cells *24*, 1441-1449.

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

Eggan, K., Akutsu, H., Loring, J., Jackson-Grusby, L., Klemm, M., Rideout, W.M., 3rd, Yanagimachi, R., and Jaenisch, R. (2001). Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc Natl Acad Sci U S A *98*, 6209-6214.

Eggan, K., Baldwin, K., Tackett, M., Osborne, J., Gogos, J., Chess, A., Axel, R., and Jaenisch, R. (2004). Mice cloned from olfactory sensory neurons. Nature *428*, 44-49.

Egli, D., Rosains, J., Birkhoff, G., and Eggan, K. (2007). Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. Nature *447*, 679-685.

Eminli, S., Foudi, A., Stadtfeld, M., Maherali, N., Ahfeldt, T., Mostoslavsky, G., Hock, H., and Hochedlinger, K. (2009). Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. Nat Genet *41*, 968-976.

Eminli, S., Jaenisch, R., and Hochedlinger, K. (2006). Strategies to induce nuclear reprogramming. Ernst Schering Found Symp Proc, 83-98.

Eminli, S., Utikal, J., Arnold, K., Jaenisch, R., and Hochedlinger, K. (2008). Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression. Stem Cells *26*, 2467-2474.

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

Feinberg, M.W., Wara, A.K., Cao, Z., Lebedeva, M.A., Rosenbauer, F., Iwasaki, H., Hirai, H., Katz, J.P., Haspel, R.L., Gray, S.*, et al.* (2007). The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation. EMBO J *26*, 4138-4148.

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

Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. (2003). Genomic targets of the human c-Myc protein. Genes Dev *17*, 1115-1129.

Frank, S.R., Parisi, T., Taubert, S., Fernandez, P., Fuchs, M., Chan, H.M., Livingston, D.M., and Amati, B. (2003). MYC recruits the TIP60 histone acetyltransferase complex to chromatin. EMBO Rep *4*, 575-580.

Fuchs, E., Tumbar, T., and Guasch, G. (2004). Socializing with the neighbors: stem cells and their niche. Cell *116*, 769-778.

Galan-Caridad, J.M., Harel, S., Arenzana, T.L., Hou, Z.E., Doetsch, F.K., Mirny, L.A., and Reizis, B. (2007). Zfx controls the self-renewal of embryonic and hematopoietic stem cells. Cell *129*, 345-357.

Gardner, R.L. (1978). The relationship between cell lineage and differentiation in the early mouse embryo. Results Probl Cell Differ *9*, 205-241.

Garrett-Sinha, L.A., Eberspaecher, H., Seldin, M.F., and de Crombrugghe, B. (1996). A gene for a novel zinc-finger protein expressed in differentiated epithelial cells and transiently in certain mesenchymal cells. J Biol Chem *271*, 31384-31390.

Ghaleb, A.M., McConnell, B.B., Nandan, M.O., Katz, J.P., Kaestner, K.H., and Yang, V.W. (2007). Haploinsufficiency of Kruppel-like factor 4 promotes adenomatous polyposis coli dependent intestinal tumorigenesis. Cancer Res *67*, 7147-7154.

Giorgetti, A., Montserrat, N., Aasen, T., Gonzalez, F., Rodriguez-Piza, I., Vassena, R., Raya, A., Boue, S., Barrero, M.J., Corbella, B.A.*, et al.* (2009). Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. Cell Stem Cell *5*, 353-357.

Godin, I., and Cumano, A. (2002). The hare and the tortoise: an embryonic haematopoietic race. Nat Rev Immunol *2*, 593-604.

Goldberg, A.D., Allis, C.D., and Bernstein, E. (2007). Epigenetics: a landscape takes shape. Cell *128*, 635-638.

Guan, K., Nayernia, K., Maier, L.S., Wagner, S., Dressel, R., Lee, J.H., Nolte, J., Wolf, F., Li, M., Engel, W.*, et al.* (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. Nature *440*, 1199-1203.

Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El-Maarri, O., Reik, W., Walter, J., and Surani, M.A. (2002). Epigenetic reprogramming in mouse primordial germ cells. Mech Dev *117*, 15-23.

Hanna, J., Markoulaki, S., Schorderet, P., Carey, B.W., Beard, C., Wernig, M., Creyghton, M.P., Steine, E.J., Cassady, J.P., Foreman, R.*, et al.* (2008). Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell *133*, 250-264.

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

Hochedlinger, K., and Jaenisch, R. (2002a). Monoclonal mice generated by nuclear transfer from mature B and T donor cells. Nature *415*, 1035-1038.

Hochedlinger, K., and Jaenisch, R. (2002b). Nuclear transplantation: lessons from frogs and mice. Curr Opin Cell Biol *14*, 741-748.

Hochedlinger, K., and Jaenisch, R. (2003). Nuclear transplantation, embryonic stem cells, and the potential for cell therapy. N Engl J Med *349*, 275-286.

Hochedlinger, K., and Jaenisch, R. (2006). Nuclear reprogramming and pluripotency. Nature *441*, 1061-1067.

Hochedlinger, K., and Plath, K. (2009). Epigenetic reprogramming and induced pluripotency. Development *136*, 509-523.

Hochedlinger, K., Rideout, W.M., Kyba, M., Daley, G.Q., Blelloch, R., and Jaenisch, R. (2004). Nuclear transplantation, embryonic stem cells and the potential for cell therapy. Hematol J *5 Suppl 3*, S114-117.

Hochedlinger, K., Yamada, Y., Beard, C., and Jaenisch, R. (2005). Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Cell *121*, 465-477.

Holm, T.M., Jackson-Grusby, L., Brambrink, T., Yamada, Y., Rideout, W.M., 3rd, and Jaenisch, R. (2005). Global loss of imprinting leads to widespread tumorigenesis in adult mice. Cancer Cell *8*, 275-285.

Homma, R., Yoshikawa, H., Takeno, M., Kurokawa, M.S., Masuda, C., Takada, E., Tsubota, K., Ueno, S., and Suzuki, N. (2004). Induction of epithelial progenitors in vitro from mouse embryonic stem cells and application for

reconstruction of damaged cornea in mice. Invest Ophthalmol Vis Sci *45*, 4320-4326.

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

Huangfu, D., Osafune, K., Maehr, R., Guo, W., Eijkelenboom, A., Chen, S., Muhlestein, W., and Melton, D.A. (2008b). Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol *26*, 1269-1275.

Hunt, D.P., Jahoda, C., and Chandran, S. (2009). Multipotent skin-derived precursors: from biology to clinical translation. Curr Opin Biotechnol *20*, 522-530.

Ichida, J.K., Blanchard, J., Lam, K., Son, E.Y., Chung, J.E., Egli, D., Loh, K.M., Carter, A.C., Di Giorgio, F.P., Koszka, K.*, et al.* (2009). A small-molecule inhibitor of tgf-Beta signaling replaces sox2 in reprogramming by inducing nanog. Cell Stem Cell *5*, 491-503.

Inoue, K., Ogonuki, N., Miki, H., Hirose, M., Noda, S., Kim, J.M., Aoki, F., Miyoshi, H., and Ogura, A. (2006). Inefficient reprogramming of the hematopoietic stem cell genome following nuclear transfer. J Cell Sci *119*, 1985-1991.

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

Jiang, J., Chan, Y.S., Loh, Y.H., Cai, J., Tong, G.Q., Lim, C.A., Robson, P., Zhong, S., and Ng, H.H. (2008). A core Klf circuitry regulates self-renewal of embryonic stem cells. Nat Cell Biol *10*, 353-360.

Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P., and Woltjen, K. (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature *458*, 771-775.

Kanatsu-Shinohara, M., Inoue, K., Lee, J., Yoshimoto, M., Ogonuki, N., Miki, H., Baba, S., Kato, T., Kazuki, Y., Toyokuni, S.*, et al.* (2004). Generation of pluripotent stem cells from neonatal mouse testis. Cell *119*, 1001-1012.

Kang, L., Wang, J., Zhang, Y., Kou, Z., and Gao, S. (2009). iPS cells can support full-term development of tetraploid blastocyst-complemented embryos. Cell Stem Cell *5*, 135-138.

Kastan, M.B., Slamon, D.J., and Civin, C.I. (1989a). Expression of protooncogene c-myb in normal human hematopoietic cells. Blood *73*, 1444-1451.

Kastan, M.B., Stone, K.D., and Civin, C.I. (1989b). Nuclear oncoprotein expression as a function of lineage, differentiation stage, and proliferative status of normal human hematopoietic cells. Blood *74*, 1517-1524.

Kim, J.B., Greber, B., Arauzo-Bravo, M.J., Meyer, J., Park, K.I., Zaehres, H., and Scholer, H.R. (2009a). Direct reprogramming of human neural stem cells by OCT4. Nature *461*, 649-643.

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

Kleinsmith, L.J., and Pierce, G.B., Jr. (1964). Multipotentiality of Single Embryonal Carcinoma Cells. Cancer Res *24*, 1544-1551.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661-672.

Kuroda, T., Tada, M., Kubota, H., Kimura, H., Hatano, S.Y., Suemori, H., Nakatsuji, N., and Tada, T. (2005). Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. Mol Cell Biol *25*, 2475-2485.

Labosky, P.A., Barlow, D.P., and Hogan, B.L. (1994a). Embryonic germ cell lines and their derivation from mouse primordial germ cells. Ciba Found Symp *182*, 157-168; discussion 168-178.

Labosky, P.A., Barlow, D.P., and Hogan, B.L. (1994b). Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development *120*, 3197-3204.

Laiosa, C.V., Stadtfeld, M., Xie, H., de Andres-Aguayo, L., and Graf, T. (2006). Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU.1 transcription factors. Immunity *25*, 731-744.

Laurenti, E., Varnum-Finney, B., Wilson, A., Ferrero, I., Blanco-Bose, W.E., Ehninger, A., Knoepfler, P.S., Cheng, P.F., MacDonald, H.R., Eisenman, R.N.*, et al.* (2008). Hematopoietic stem cell function and survival depend on c-Myc and N-Myc activity. Cell Stem Cell *3*, 611-624.

Lee, G., Papapetrou, E.P., Kim, H., Chambers, S.M., Tomishima, M.J., Fasano, C.A., Ganat, Y.M., Menon, J., Shimizu, F., Viale, A.*, et al.* (2009). Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. Nature *461*, 402-406.

Lee, J., Inoue, K., Ono, R., Ogonuki, N., Kohda, T., Kaneko-Ishino, T., Ogura, A., and Ishino, F. (2002). Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. Development *129*, 1807-1817.

Lendahl, U., Zimmerman, L.B., and McKay, R.D. (1990). CNS stem cells express a new class of intermediate filament protein. Cell *60*, 585-595.

Li, J., Greco, V., Guasch, G., Fuchs, E., and Mombaerts, P. (2007). Mice cloned from skin cells. Proc Natl Acad Sci U S A *104*, 2738-2743.

Li, J., Ishii, T., Feinstein, P., and Mombaerts, P. (2004). Odorant receptor gene choice is reset by nuclear transfer from mouse olfactory sensory neurons. Nature *428*, 393-399.

Li, L., Connelly, M.C., Wetmore, C., Curran, T., and Morgan, J.I. (2003a). Mouse embryos cloned from brain tumors. Cancer Res *63*, 2733-2736.

Li, W., Wei, W., Zhu, S., Zhu, J., Shi, Y., Lin, T., Hao, E., Hayek, A., Deng, H., and Ding, S. (2009). Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. Cell Stem Cell *4*, 16-19.

Li, Y., McClintick, J., Zhong, L., Edenberg, H.J., Yoder, M.C., and Chan, R.J. (2005). Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. Blood *105*, 635-637.

Li, Z., Van Calcar, S., Qu, C., Cavenee, W.K., Zhang, M.Q., and Ren, B. (2003b). A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. Proc Natl Acad Sci U S A *100*, 8164-8169.

Liao, J., Cui, C., Chen, S., Ren, J., Chen, J., Gao, Y., Li, H., Jia, N., Cheng, L., Xiao, H.*, et al.* (2009). Generation of induced pluripotent stem cell lines from adult rat cells. Cell Stem Cell *4*, 11-15.

Liu, H., Zhu, F., Yong, J., Zhang, P., Hou, P., Li, H., Jiang, W., Cai, J., Liu, M., Cui, K.*, et al.* (2008). Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. Cell Stem Cell *3*, 587-590.

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

Lowry, W.E., Richter, L., Yachechko, R., Pyle, A.D., Tchieu, J., Sridharan, R., Clark, A.T., and Plath, K. (2008). Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc Natl Acad Sci U S A *105*, 2883-2888.

Maherali, N., Ahfeldt, T., Rigamonti, A., Utikal, J., Cowan, C., and Hochedlinger, K. (2008). A high-efficiency system for the generation and study of human induced pluripotent stem cells. Cell Stem Cell *3*, 340-345.

Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R.*, et al.* (2007). Global epigenetic remodeling in directly reprogrammed fibroblasts. Cell Stem Cells, 55-70.

Malynn, B.A., de Alboran, I.M., O'Hagan, R.C., Bronson, R., Davidson, L., DePinho, R.A., and Alt, F.W. (2000). N-myc can functionally replace c-myc in murine development, cellular growth, and differentiation. Genes Dev *14*, 1390-1399.

Marion, R.M., Strati, K., Li, H., Tejera, A., Schoeftner, S., Ortega, S., Serrano, M., and Blasco, M.A. (2009). Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells. Cell Stem Cell *4*, 141-154.

Markoulaki, S., Hanna, J., Beard, C., Carey, B.W., Cheng, A.W., Lengner, C.J., Dausman, J.A., Fu, D., Gao, Q., Wu, S.*, et al.* (2009). Transgenic mice with

defined combinations of drug-inducible reprogramming factors. Nat Biotechnol *27*, 169-171.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A *78*, 7634-7638.

Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A.A.*, et al.* (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol *9*, 625-635.

Matsumura, H., Tada, M., Otsuji, T., Yasuchika, K., Nakatsuji, N., Surani, A., and Tada, T. (2007). Targeted chromosome elimination from ES-somatic hybrid cells. Nat Methods *4*, 23-25.

Matveeva, N.M., Shilov, A.G., Kaftanovskaya, E.M., Maximovsky, L.P., Zhelezova, A.I., Golubitsa, A.N., Bayborodin, S.I., Fokina, M.M., and Serov, O.L. (1998). In vitro and in vivo study of pluripotency in intraspecific hybrid cells obtained by fusion of murine embryonic stem cells with splenocytes. Mol Reprod Dev *50*, 128-138.

McConnell, B.B., Ghaleb, A.M., Nandan, M.O., and Yang, V.W. (2007). The diverse functions of Kruppel-like factors 4 and 5 in epithelial biology and pathobiology. Bioessays *29*, 549-557.

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

Miller, R.A., and Ruddle, F.H. (1976). Pluripotent teratocarcinoma-thymus somatic cell hybrids. Cell *9*, 45-55.

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

Miura, K., Okada, Y., Aoi, T., Okada, A., Takahashi, K., Okita, K., Nakagawa, M., Koyanagi, M., Tanabe, K., Ohnuki, M.*, et al.* (2009). Variation in the safety of induced pluripotent stem cell lines. Nat Biotechnol *27*, 743-745.

Morrison, S.J., Uchida, N., and Weissman, I.L. (1995). The biology of hematopoietic stem cells. Annu Rev Cell Dev Biol *11*, 35-71.

Morrison, S.J., and Weissman, I.L. (1995). Heterogeneity of hematopoietic stem cells: implications for clinical applications. Proc Assoc Am Physicians *107*, 187-194.

Murphy, M.J., Wilson, A., and Trumpp, A. (2005). More than just proliferation: Myc function in stem cells. Trends Cell Biol *15*, 128-137.

Nagy, A. (2000). Cre recombinase: the universal reagent for genome tailoring. Genesis *26*, 99-109.

Nagy, A., Gocza, E., Diaz, E.M., Prideaux, V.R., Ivanyi, E., Markkula, M., and Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. Development *110*, 815-821.

Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., and Yamanaka, S. (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol *26*, 101-106.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379-391.

Niwa, H., Toyooka, Y., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, R., and Rossant, J. (2005). Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell *123*, 917-929.

Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C.G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis *28*, 147-155.

Okamoto, K., Okazawa, H., Okuda, A., Sakai, M., Muramatsu, M., and Hamada, H. (1990). A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell *60*, 461-472.

Okano, M., Xie, S., and Li, E. (1998). Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. Nucleic Acids Res *26*, 2536-2540.

Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. Nature *448*, 313-317.

Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., and Yamanaka, S. (2008). Generation of mouse induced pluripotent stem cells without viral vectors. Science *322*, 949-953.

Okumura-Nakanishi, S., Saito, M., Niwa, H., and Ishikawa, F. (2005). Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. J Biol Chem *280*, 5307-5317.

Orkin, S.H. (2000a). Diversification of haematopoietic stem cells to specific lineages. Nat Rev Genet *1*, 57-64.

Orkin, S.H. (2000b). Stem cell alchemy. Nat Med *6*, 1212-1213.

Orkin, S.H., and Zon, L.I. (2008a). Hematopoiesis: an evolving paradigm for stem cell biology. Cell *132*, 631-644.

Orkin, S.H., and Zon, L.I. (2008b). SnapShot: hematopoiesis. Cell *132*, 712.

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

Park, I.H., Lerou, P.H., Zhao, R., Huo, H., and Daley, G.Q. (2008b). Generation of human-induced pluripotent stem cells. Nat Protoc *3*, 1180-1186.

Park, I.H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W., and Daley, G.Q. (2008c). Reprogramming of human somatic cells to pluripotency with defined factors. Nature *451*, 141-146.

Pavletic, S.Z., Smith, L.M., Bishop, M.R., Lynch, J.C., Tarantolo, S.R., Vose, J.M., Bierman, P.J., Hadi, A., Armitage, J.O., and Kessinger, A. (2005). Prognostic factors of chronic graft-versus-host disease after allogeneic blood stem-cell transplantation. Am J Hematol *78*, 265-274.

Pevny, L., and Placzek, M. (2005). SOX genes and neural progenitor identity. Curr Opin Neurobiol *15*, 7-13.

Pevny, L.H., Sockanathan, S., Placzek, M., and Lovell-Badge, R. (1998). A role for SOX1 in neural determination. Development *125*, 1967-1978.

Raya, A., Rodriguez-Piza, I., Guenechea, G., Vassena, R., Navarro, S., Barrero, M.J., Consiglio, A., Castella, M., Rio, P., Sleep, E.*, et al.* (2009). Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature *460*, 53-59.

Rideout, W.M., 3rd, Eggan, K., and Jaenisch, R. (2001). Nuclear cloning and epigenetic reprogramming of the genome. Science *293*, 1093-1098.

Rideout, W.M., 3rd, Hochedlinger, K., Kyba, M., Daley, G.Q., and Jaenisch, R. (2002). Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell *109*, 17-27.

Rideout, W.M., 3rd, Wakayama, T., Wutz, A., Eggan, K., Jackson-Grusby, L., Dausman, J., Yanagimachi, R., and Jaenisch, R. (2000). Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat Genet *24*, 109-110.

Rossant, J., Gardner, R.L., and Alexandre, H.L. (1978). Investigation of the potency of cells from the postimplantation mouse embryo by blastocyst injection: a preliminary report. J Embryol Exp Morphol *48*, 239-247.

Rowland, B.D., and Peeper, D.S. (2006). KLF4, p21 and context-dependent opposing forces in cancer. Nat Rev Cancer *6*, 11-23.

Sauer, B., and Henderson, N. (1988a). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci U S A *85*, 5166-5170.

Sauer, B., and Henderson, N. (1988b). The cyclization of linear DNA in Escherichia coli by site-specific recombination. Gene *70*, 331-341.

Sauvageau, M., and Sauvageau, G. (2008). Polycomb group genes: keeping stem cell activity in balance. PLoS Biol *6*, e113.

Schmidt, E.V. (1999). The role of c-myc in cellular growth control. Oncogene *18*, 2988-2996.

Schmitt, S., Prestel, M., and Paro, R. (2005). Intergenic transcription through a polycomb group response element counteracts silencing. Genes Dev *19*, 697-708.

Scholer, H.R., Balling, R., Hatzopoulos, A.K., Suzuki, N., and Gruss, P. (1989a). Octamer binding proteins confer transcriptional activity in early mouse embryogenesis. EMBO J *8*, 2551-2557.

Scholer, H.R., Hatzopoulos, A.K., Balling, R., Suzuki, N., and Gruss, P. (1989b). A family of octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor. EMBO J *8*, 2543-2550.

Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B., and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. Cell *128*, 735-745.

Segre, J.A., Bauer, C., and Fuchs, E. (1999). Klf4 is a transcription factor required for establishing the barrier function of the skin. Nat Genet *22*, 356-360.

Sekhon, S.S., Tan, X., Micsenyi, A., Bowen, W.C., and Monga, S.P. (2004). Fibroblast growth factor enriches the embryonic liver cultures for hepatic progenitors. Am J Pathol *164*, 2229-2240.

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

Shields, J.M., Christy, R.J., and Yang, V.W. (1996). Identification and characterization of a gene encoding a gut-enriched Kruppel-like factor expressed during growth arrest. J Biol Chem *271*, 20009-20017.

Silva, J., Chambers, I., Pollard, S., and Smith, A. (2006). Nanog promotes transfer of pluripotency after cell fusion. Nature *441*, 997-1001.

Silva, J., Nichols, J., Theunissen, T.W., Guo, G., van Oosten, A.L., Barrandon, O., Wray, J., Yamanaka, S., Chambers, I., and Smith, A. (2009). Nanog is the gateway to the pluripotent ground state. Cell *138*, 722-737.

Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M., and Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature *336*, 688-690.

Soldner, F., Hockemeyer, D., Beard, C., Gao, Q., Bell, G.W., Cook, E.G., Hargus, G., Blak, A., Cooper, O., Mitalipova, M.*, et al.* (2009). Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell *136*, 964-977.

Solter, D. (2006). From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. Nat Rev Genet *7*, 319-327.

Sommer, C.A., Stadtfeld, M., Murphy, G.J., Hochedlinger, K., Kotton, D.N., and Mostoslavsky, G. (2009). Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. Stem Cells *27*, 543-549.

Stadtfeld, M., Brennand, K., and Hochedlinger, K. (2008a). Reprogramming of pancreatic beta cells into induced pluripotent stem cells. Curr Biol *18*, 890-894.

Stadtfeld, M., Maherali, N., Borkent, M., and Hochedlinger, K. (2010). A reprogrammable mouse strain from gene-targeted embryonic stem cells. Nat Methods *7*, 53-55.

Stadtfeld, M., Maherali, N., Breault, D., and Hochedlinger, K. (2008b). Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. Cell Stem Cell *2*, 230-240.

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., and Hochedlinger, K. (2008c). Induced pluripotent stem cells generated without viral integration. Science *322*, 945-949.

Sternberg, N., and Hamilton, D. (1981). Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. J Mol Biol *150*, 467-486.

Stevens, L.C., and Little, C.C. (1954). Spontaneous Testicular Teratomas in an Inbred Strain of Mice. Proc Natl Acad Sci U S A *40*, 1080-1087.

Stewart, C.L., Gadi, I., and Bhatt, H. (1994). Stem cells from primordial germ cells can reenter the germ line. Dev Biol *161*, 626-628.

Stewart, T.A., and Mintz, B. (1982). Recurrent germ-line transmission of the teratocarcinoma genome from the METT-1 culture line to progeny in vivo. J Exp Zool *224*, 465-469.

Sung, L.Y., Gao, S., Shen, H., Yu, H., Song, Y., Smith, S.L., Chang, C.C., Inoue, K., Kuo, L., Lian, J.*, et al.* (2006). Differentiated cells are more efficient than adult stem cells for cloning by somatic cell nuclear transfer. Nat Genet *38*, 1323-1328.

Surani, M.A., Hayashi, K., and Hajkova, P. (2007). Genetic and epigenetic regulators of pluripotency. Cell *128*, 747-762.

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.

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

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

Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L., and McKay, R.D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature *448*, 196-199.

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

Trifunovic, A. (2006). Mitochondrial DNA and ageing. Biochim Biophys Acta *1757*, 611-617.

Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooly, Y.M., Gidlof, S., Oldfors, A., Wibom, R.*, et al.* (2004). Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature *429*, 417-423.

Tsai, S.Y., Clavel, C., Kim, S., Ang, Y.S., Grisanti, L., Lee, D.F., Kelley, K., and Rendl, M. Oct4 and klf4 reprogram dermal papilla cells into induced pluripotent stem cells. Stem Cells *28*, 221-228.

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W.E., Rendl, M., and Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. Science *303*, 359-363.

Ueno, H., Kurokawa, M.S., Kayama, M., Homma, R., Kumagai, Y., Masuda, C., Takada, E., Tsubota, K., Ueno, S., and Suzuki, N. (2007). Experimental transplantation of corneal epithelium-like cells induced by Pax6 gene transfection of mouse embryonic stem cells. Cornea *26*, 1220-1227.

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

van der Flier, L.G., and Clevers, H. (2009). Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol *71*, 241-260.

Wagers, A.J., and Weissman, I.L. (2004). Plasticity of adult stem cells. Cell *116*, 639-648.

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.

Wakayama, T., Rodriguez, I., Perry, A.C., Yanagimachi, R., and Mombaerts, P. (1999). Mice cloned from embryonic stem cells. Proc Natl Acad Sci U S A *96*, 14984-14989.

Wernig, M., Lengner, C.J., Hanna, J., Lodato, M.A., Steine, E., Foreman, R., Staerk, J., Markoulaki, S., and Jaenisch, R. (2008). A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. Nat Biotechnol *26*, 916-924.

Wernig, M., Meissner, A., Cassady, J.P., and Jaenisch, R. (2007a). C-Myc is dispensable for direct reprogramming of mouse fibroblasts. Cell Stem Cell.

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

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., and Gough, N.M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature *336*, 684-687.

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.

Wilmut, I., Young, L., and Campbell, K.H. (1998). Embryonic and somatic cell cloning. Reprod Fertil Dev *10*, 639-643.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118-1129.

Winkel, G.K., and Pedersen, R.A. (1988). Fate of the inner cell mass in mouse embryos as studied by microinjection of lineage tracers. Dev Biol *127*, 143-156.

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

Xie, H., Ye, M., Feng, R., and Graf, T. (2004). Stepwise reprogramming of B cells into macrophages. Cell *117*, 663-676.

Yamazaki, Y., Low, E.W., Marikawa, Y., Iwahashi, K., Bartolomei, M.S., McCarrey, J.R., and Yanagimachi, R. (2005). Adult mice cloned from migrating primordial germ cells. Proc Natl Acad Sci U S A *102*, 11361-11366.

Yaworsky, P.J., and Kappen, C. (1999). Heterogeneity of neural progenitor cells revealed by enhancers in the nestin gene. Dev Biol *205*, 309-321.

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

Yu, J., Vodyanik, M.A., He, P., Slukvin, II, and Thomson, J.A. (2006). Human embryonic stem cells reprogram myeloid precursors following cell-cell fusion. Stem Cells *24*, 168-176.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R.*, et al.* (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917-1920.

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

Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J., and Melton, D.A. (2008). In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature *455*, 627-632.

Zhu, X.D., Pan, G., Luetke, K., and Sadowski, P.D. (1995). Homology requirements for ligation and strand exchange by the FLP recombinase. J Biol Chem *270*, 11646-11653.

9. Abbreviation list

ALS - amyotrophic lateral sclerosis

bFGF - basic fibroblast growth factor

BMF - bone marrow failure

cDNA - complementary DNA

CLP - common lymphoid progenitor

CMP - common myeloid progenitor

EBs - embryoid bodies

EC cells - embryonic carcinoma cells

EG cells - embryonic germ cells

EpiS cells - epiblast stem cells

ES cells - embryonic stem cells

Esrrb - Estrogen-related receptor beta

FACS - Flourescence activated cell sorting

FD - familial dysautonomia

GMP - granulocyte/macrophage progenitors

HSCs - hematopoietic stem cells

ICM - inner cell mass

iPS cells - induced pluripotent stem cells

Klf4 – kruepple-like-factor 4

LIF – Leukemia Inhibitory Factor

LTR – long terminal repeat

MBD - Methyl-Binding-Domain

MEFs - mouse embryonic fibroblasts

MEP - megakaryocyte/erythrocyte progenitors

neo^{R} - neomycin resistance cassette

NPCs - neuronal progenitor cells

NT - nuclear transfer

NT-ES cells- nuclear transfer derived embryonic stem cells

Oct4 - octamer-binding transcription factor 4

PEG - polyethylene glycol

Pou5f1 - Pou class 5 homebox 1

PRC - polycomb repressive complex

PGCs - primordial germ cells

rtTA - reverse tetracycline-controlled transactivator

shRNA - short hairpin ribonucleic acid

SMA - spinal muscular atrophy

SMPs - Skeletal Muscle Precursors

Sox2 - sex-determing region Y-box2

TetOP - doxycycline-controllable promoter

trxG proteins - trithorax-group proteins

Ubc pm - ubiquitin promoter

UCB - umbilical cord blood

10. Publication record

- Kah Yong Tan, **Eminli S**, Hochedlinger K, Amy Wagers. Efficient generation of iPS cells from skeletal muscle stem cells (in preparation).

- Polo J.M., Liu S., Figueroa M.E., Kulalert W., **Eminli S**., Ta K.Y., Apostolou E., Stadtfeld M., Li Y., Natesan S, Wagers A., Melnick A., Evans T., Hochedlinger K. Cell of origin influences molecular and functional properties of murine induced pluripotent stem cells. ***Nature*** 2010 (under review)

- **Eminli S**, Foudi A, Stadtfeld M, Maherali N, Ahfeldt T, Mostoslavsky G, Hock H, Hochedlinger K. (2009). Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. ***Nat Genet.*** *41*, 968 – 976.

- Chen L, Yabuuchi A, **Eminli S**, Takeuchi A, Lu CW, Hochedlinger K, Daley GQ. (2009). Cross-regulation of the Nanog and Cdx2 promoters. ***Cell Res.*** *19*; 1052–1061.

- Meissner A, **Eminli S**, Jaenisch R. (2009). Derivation and manipulation of murine embryonic stem cells. ***Methods Mol Biol.*** *482*, 1064-3745.

- **Eminli S**, Utikal J, Arnold K, Jaenisch R, Hochedlinger K. (2008). Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression. ***Stem Cells.*** *26,* 2467-2474.

- Maherali N, Sridharan R, Xie W, Utikal J, **Eminli S**, Arnold K, Stadtfeld M, Yachechko R, Tchieu J,Jaenisch R, Plath K, Hochedlinger K. (2007). Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. ***Cell Stem Cell.*** *3*, 55-70

- **Eminli S**, Jaenisch R, Hochedlinger K. (2006). Strategies to induce nuclear reprogramming. ***Ernst Schering Found Symp Proc.*** *5*; 83-98.

- **Eminli S**, Seitz H. (2005). Methods for characterization of DNA interactions. ***Genome Express.*** 16-18

11. Danksagung-Acknowledgement

Most importantly I would like to thank Konrad Hochedlinger for giving me the opportunity to work in his lab and for letting me pursue all the different projects.

I am very thankful to my thesis advisor Prof. Dr. Rupert Mutzel, Danke!

I would like to thank my family and friends for their continuous support and patience. Mama, Papa, Erk und Anke ich bin so froh, dass ich Euch hab.
Meinen Mädels will ich nur sagen, ihr seid meine Schätze!

***Especially, I want to thank Alex, mein Sonnenschein, who was there for me every single day during this PhD. Without you I could not have done it - wirklich nicht! More family days are about to come. ***

I am thankful to Katrin Arnold, the best bay mate ever.
I would like to thank Matthias Stadtfeld, Katrin Arnold, Jose Maria Polo for help throughout my PhD and for proof-reading of manuscripts and this dissertation.
Rachael, I am thankful for your friendship and for proof-reading of this dissertation.
Many thanks to my main collaborators Adlen Foudi and Jochen Utikal who contributed significantly to this work.
I would also like to thank Prof. Dr. Hanno Hock and Prof. Dr. David Scadden and my former mentor Prof. Dr. Regine Hengge for their support.

Herr Klitsch, ohne Sie hätte ich nie Bio Leistungsfach gewählt!

Eidesstattliche Erklärung zur Dissertation

Ich versichere hiermit, dass ich die vorliegende Dissertation selstständig verfasst, die für diese Arbeit benutzten Hilfsmittel genannt und die Ergebnisse anderer klar gekennzeichnet habe.

Ich versichere außerdem, dass die vorliegende Arbeit noch nicht einem anderen Prüfungsverfahren zugrunde gelegen hat.
Ich bin damit einverstanden, dass ein Exemplar meiner Dissertation in der Bibliothek ausgeliehen werden kann.

Sarah Eminli,

Boston, den 5.März 2010