Stem Cell Biology and Regenerative Medicine

Açelya Yilmazer *Editor*

In Vivo Reprogramming in Regenerative Medicine

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To my parents, Reyhan and Ali Ulvi, and the love of my life, Süleyman, and to Zeki Yaman, you all made it worthwhile.

Preface

This new volume, as a part of Springer's Stem Cells in Regenerative Medicine series, is dedicated to review the current progress on different approaches of in vivo reprogramming technology. Leaders in the field discuss how in vivo reprogramming can be used for tissue repair and regeneration in different organs, including the pancreas (Chap. 2), liver (Chap. 3), and heart (Chap. 4). Recent studies on in vivo cell reprogramming toward pluripotency are also reviewed; examples are given in order to show its potential in regenerative medicine (Chaps. 5 and 6). In each chapter, the regenerative potential of different in vivo reprogramming approaches is discussed in detail. Examples from various animal models are given, and the regenerative potential of in vivo reprogramming is compared to that of cell transplantation studies. In the last chapter, current challenges of these preclinical studies are discussed, and hypotheses and suggestions are given in order to improve the current strategies. Future directions are pointed out for the transition of in vivo reprogramming technology to clinical settings. This volume is among the first books in literature which specifically focuses on the in vivo reprogramming technology in regenerative medicine, and these proposed chapters collectively cover one of the most important and exciting topics of regenerative medicine.

Based on my experiences on gene therapy and cellular reprogramming, I believe this volume will attract attention from researchers working in different fields. By exploring the preclinical studies on animal models discussed in this book, researchers and clinicians can direct the future of in vivo reprogramming approaches to clinical settings. Researchers, graduate students, and postdoctoral fellows will find food for thought in this insightful guide presenting the collective knowledge of leaders in the stem cell field. It can be also used as a source volume for graduate courses covering the regenerative medicine field.

Ankara, Turkey — Açelya Yilmazer

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Abbreviations

AAV	Adeno-associated virus
AP	Alkaline phosphatase
Arx	Aristaless-related homeobox
BMP	Bone morphogenetic protein
Brn4	POU domain, class 3, transcription factor 4
CF	Cardiac fibroblast
ciCMs	Chemical-induced cardiomyocyte-like cells
CPA1	Carboxypeptidase A1
DAPT	*N*-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1, 1-dimethylethyl ester
DNA	Deoxyribonucleic acid
ECCS	Embryonic carcinoma cells
EMT	Epithelial-mesenchymal transition
ESCs	Embryonic stem cells
Etv5	Translocation-Ets-leukemia virus 5
Ezh2	Enhancer of zeste homolog 2
FGF	Fibroblast growth factor
Foxo1	Forkhead box protein O1
FoxP4	Forkhead box protein P4
G26d	Gestational day 26
GABA	γ-Aminobutyric acid
GATA	GATA transcription factor
GHMT	Combination of Gata4, Hand2, Mef2c, and Tbx5
GMT	Combination of separate transcription factors Gata4, Mef2c, and Tbx5
GSIS	Glucose-stimulated insulin secretion
H3K27me3	Trimethylation of histone H3 at lysine 27
H3K4me2	H3K4 dimethylation
H3K4me3	Trimethylation of histone H3 at lysine 4
HCF	Human cardiac fibroblasts
HDF	Human dermal fibroblasts

HFFs	Human foreskin fibroblasts
HMG	High-mobility group protein
HMT complex	MLL/SET1 histone methyltransferase complex
Hnf	Hepatocyte nuclear factor
HNGMT	Combination of Hand2, Nkx2-5, Gata 4, Mef2c, and Tbx5
HTV injection	Hydrodynamic tail vein injection
i^2PS cells	In vivo induced pluripotent stem cells
iCM	Induced CM-like cell
ICM	Inner cell mass
iCPC	Induced cardiac progenitor cells
Id	DNA-binding protein inhibitor ID
iPSCs	Induced pluripotent stem cells
Isl1	Insulin gene enhancer protein1
Lhr1(Nr5a2)	Liver receptor homolog-1/nuclear receptor subfamily 5, group A, member
LIF	Leukemia inhibitor factor
MafA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MEFs	Mouse embryonic fibroblasts
MGT	Polycistronic vector carrying Mef2c, Gata4, and Tbx5 sequentially
MI	Myocardium infarction
miRNAs	MicroRNAs
MM3-GHT	Chimeric Mef2c together with Gata4, Tbx5, and Hand2
MPCs	Multipotent progenitor cells
Myt1	Myelin transcription factor 1
NECA	5′-Nethylcarboxamidoadenosine
NeuroD1	Neurogenic differentiation 1
Ngn3	Neurog3
Nkx2.2	Nirenberg and Kim 2 homeobox 2
Nkx6.1	Nirenberg and Kim 6 homeobox 1
OSKM	*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*
Osr	Odd-skipped related genes
Pax4	Paired box 4
Pax6	Paired box 6
pDNA	Plasmid DNA
Pdx1	Pancreas and duodenum homeobox 1
PNM	Pdx1,Ngn3,MafA
PP	Polypeptide
PPAR	Peroxisome proliferator-activated receptors
PRC1	Polycomb repressive complex 1
Prox1	Prospero homeobox protein 1
Pse	Putative sulfate exporter
Ptf1a	Pancreas transcription factor 1 subunit alpha
RORC	RAR-related orphan receptor C
rtTA	The Tet activator

SCNT	Somatic cell nuclear transfer
scRNA-seq	Single cell RNA sequencing analysis
SHH	Sonic hedgehog
Snail1	Snail family transcriptional repressor
SOX	Sex-determining region Y-box
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TGF	Transforming growth factor
TGIF2	Homeobox protein TGIF2
TTR	Liver-specific promoter
VEGF	Vascular endothelial growth factor
WBSCR14	WBS critical region gene 14
WT	Wild type
WY14643	Pirinixic acid
Xbp1	X-box binding protein 1

Chapter 1
Introduction to In Vivo Cell Reprogramming Technology

Cansu Gurcan, Hadiseh Taheri, and Açelya Yilmazer

1.1 Introduction

With the continuous increase in life expectancy and the prevalence of life-threatening diseases, there is an urgent need to explore new therapeutic approaches beyond the usual small molecules and known surgical interventions. Regenerative medicine is an emerging field that pursues restoration of the damaged or degenerated tissues rather than just compensation of their impaired functions. Although this field is still in its infancy, a wide range of materials have already been explored as scaffolds and matrixes that assist the engineering of the tissue to be regenerated [1, 2]. In parallel, extensive research has been carried out seeking for suitable cell sources to populate the tissues to be restored and compensate the biological functions of the dysfunctional endogenous cells. Stem cells represent an invaluable candidate for cell-based therapies, given their capacity to self-renew and differentiate into several cell types [3]. To date, different stem cell types have been investigated as sources of customized cells for regenerative therapies in pre-clinical and clinical studies. Embryonic, adult and induced pluripotent stem (iPS) cells are the different stem cell types that are studied in various clinical and preclinical applications [4].

The ultimate goal of regenerative medicine is to replace lost or damaged cells. This can be achieved by using the process of dedifferentiation, transdifferentiation

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and reprogramming. While natural regeneration occurs in certain non-mammalian and mammalian vertebrate species, these three processes can also be induced in human cells experimentally [5]. Nuclear transplantation experiments in amphibians established that the genome of differentiated cells remains constant and can be reprogrammed into an embryonic state [6]. Later, the generation of iPS cells from somatic cells showed that the cell fate could be manipulated by simply introducing a few key factors [7]. These groundbreaking discoveries have completely changed our view of the developmental biology and therefore awarded by Nobel Prize of Medicine in 2012. This conceptual revolution has inspired researchers to reprogram and transdifferentiate variety of cells in vitro and in vivo with regenerative medicine purposes. However, transplantation of these in vitro generated cells has several limitations such as safe and efficient production in cell culture; long-term survival, and functional integration after transplantation. In vivo reprogramming, which makes use of endogenous cells for regeneration purpose, emerged as an approach to circumvent cell transplantation.

In the process of in vivo reprogramming, cells switch to another cell type within the living organism. In one of the first in vivo reprogramming studies, Zhou et al. reported that alpha pancreatic cells can be converted into endocrine insulin-positive beta cells with efficiencies reaching 20% in only 3 days [8]. This encouraging finding was followed by many in vivo cardiomyocyte reprogramming studies. In 2012, different groups from United States and Japan showed that viral transfection with cardiac transcription factors (GATA4, Mef2c and Tbx5) led to the conversion of cardiac fibroblasts into functional beating cardiomyocytes [9–11]. Similarly, expression of cardiac-enriched miRNA molecules such as miR1, miR133 and miR208 reprogrammed cardiac fibroblasts into functional cardiomyocyte-like cells [12].

Different organs/tissues have been investigated as a target for in vivo reprogramming. Figure 1.1 summarizes the different tissues and related cell types that have been reported to be reprogrammed in vivo. Different studies in this field are summarized below and further details are discussed in the following chapters.

1.2 Spinal Cord

Damage to any part of the spinal cord or nerves at the end of the spinal canal results in spinal cord injury (SCI). This damage to spinal cord causes paralysis and neurological dysfunction. Paralysis is a condition that negatively affects the psychological state of the patient, which in turn reduces the quality of life. Modern advances in surgical interventions and management of injuries involving the spinal column and underlying cord have drastically reduced mortality rates and extended lifespan of SCI patients. By the mid twentieth century, mortality from traumatic SCI has been reduced to less than half of the rates, however, despite this improved survival SCI patients continue to have significant morbidity [13, 14]. In vivo reprogramming could provide a new treatment option for irreversible neural loss and glial scar formation in SCI (Table 1.1) [15, 16].

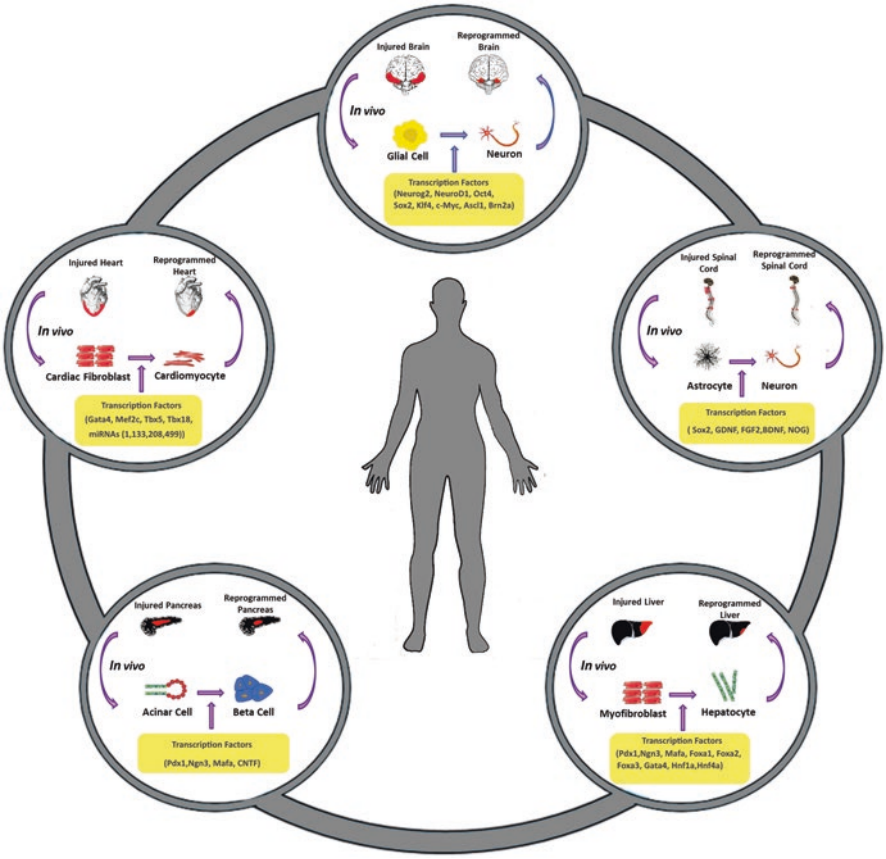

Fig. 1.1 In vivo reprogramming technologies have been applied in various organs

Table 1.1 Different studies involving in vivo reprogramming for spinal cord injury

	Disease model	Reprogrammed cells	Reprogrammed protocol	References
In vivo reprogramming for spinal cord	-Spinal cord injury in NSG mice	-Endogenous astrocytes to neurons	-Lentiviral vector -Trancription factor (SOX2)	[14]
	-Spinal cord injury	-Astrocytes to neurons	-Lentiviral vector -Trancription factor (SOX2) -Neurotrophic factors (GDNF,FGF2,BDNF,NOG)	[16]

1.3 Brain

Brain damage including traumatic and acute brain injuries can occur due to accidents or biological complications. Traumatic brain injury (TBI) is the leading cause

Table 1.2 Different studies involving in vivo reprogramming for brain injury

	Disease model	Reprogrammed cells	Reprogrammed protocol	References
In vivo reprogramming for brain	-Damaged brain	-Non-neuronal cells to generate new neurons	-Retroviral vector -Combination of growth factors and transcription factor (Neurog2)	[25]
	-Neural conversion	-Fibroblast to functional neurons	-Lentiviral vector -Neural conversion factors (Ascl1,Brn2a,Myt1l)	[26]
	-Healthy adult mouse brain	-Adult astrocytes to neurons	-Lentiviral vector -Transcription factor (Sox2)	[24]
	-Brain injury or Alzheimer' Disease model	-Reactive glial cells (reactive astrocytes) to functional neurons	-Retroviral vector -Transcription factor (NeuroD1)	[20]
	-Injured adult cerebral cortex	-NG2 glia into induced doublecortin (DCX) + neurons	-Retroviral vector -Transcription factors (Sox2, Ascl1)	[21]
	-Healthy adult mouse brain	-Adult astrocytes to neurons	-Lentiviral vector -Transcription factor (Sox2)	[23]
	-Traumatic brain injury (TBI)	-Reprogramming reactive glia into iPSCs	-Retroviral vector -Transcription factors (Oct4, Sox2,Klf4,c-Myc)	[17]

of death in children and young adults. Furthermore, because of the considerable damage and limited therapeutic approaches, TBI is a serious public health problem [17]. The renewal capacity of brain cells is very low; therefore brain injury generally results in irreversible damages. With the advances in cellular therapy, these irreversible damages are likely to be treated, as suggested in various regenerative medicine studies [18, 19]. Among these novel treatment technologies is in vivo reprogramming. Through this approach, somatic cells can be transformed into functional neurons (Table 1.2). Non-neural cells (such as astrocytes, glial cells, etc.) can be converted into new neurons by introduction of exogenous factors and have been applied in various disease models for brain [15, 17, 20–26].

1.4 Pancreas

Maintaining a certain level of blood-glucose level is important for homeostasis. The imbalance of the blood-glucose level can cause many problems in daily life. If this condition is not treated, diabetes can occur. Diabetes is a complex metabolic disorder characterized by loss or dysfunction of pancreatic β-cells mass. There are two

Table 1.3 Different studies involving in vivo reprogramming for pancreas

	Disease model	Reprogrammed cells	Reprogrammed protocol	References
In vivo reprogramming for pancreas	-Animal model	-Adult pancreatic exocrine cells to beta-cells	-Adenoviral vector -Transcription factors (Ngn3, Pdx1, MafA)	[8]
	-Diabetic	-Sox9+ cells in the liver to insulin-secreting ducts	-Adenoviral vector -Transcription factors (Pdx1, Ngn3, MafA)	[32]
	-Hyperglycemic mice	-Acinar cells to beta-like cells	-Combination of the cytokines EGF(epidermal growth factor) and CNTF (ciliary neurotrophic factor)	[29]
	-Models of pancreatitis transgenic mice	-Pancreatic acinar cells to endocrine cells (including beta cells)	-Transcription factor (Pdx1) -Adenoviral vector	[31]
	-Murine model	-Pancreatic exocrine cells to beta cells	-Adenoviral vector -Transcription factors (Ngn3,Pdx1,MafA)	[30]

types of diabetes: type 1 and type 2. Type 1 diabetics are unable to produce insulin. Type 2 diabetics have insulin resistance and impaired insulin secretion. Diabetes is one of the most prevalent chronic conditions worldwide [27]. With diabetes rates rising globally, it is required to better integrate of all aspects of diabetes treatment and care for improved population outcomes [28]. Therefore there is an urgent need to develop novel treatment strategies to overcome the limitations of current treatment options. In vivo reprogramming promises a treatment without the need for organ or islet transplantation. Through in vivo reprogramming, pancreatic non-β cells can be transformed into β-cells (Table 1.3). This method provides us with new possibilities for the treatment of diabetes [8, 29–32].

1.5 Liver

The liver is the only internal organ in the human body capable of regenerating itself after being damaged. In chronic liver disease, however, damage to the liver over long periods of time leads to the accumulation of scar tissue that limits the ability of the liver to function and repair itself. There is enough evidence from mouse liver regeneration and serial transplantation studies which suggested that adult hepatocytes retain their ability to proliferate in vivo. With the use of in vivo reprogramming technology, the damaged liver can be further repopulated with the reprogrammed hepatic

Table 1.4 Different studies involving in vivo reprogramming for liver

	Disease model	Reprogrammed cells	Reprogrammed protocol	References
In vivo reprogramming for liver	-Diabetic	-Neo-islet to hepatic progenitor cells	-Adenoviral vector -Transcription factor (Ngn3)	[36]
	-Healthy mouse liver	-Hepatic cells to pluripotent stem cells	-Naked plasmid injection -Transcription factors (Oct3/4, Klf4, Sox2, c-Myc)	[35]
	-Chronic liver disease	-Hepatic myofibroblasts into induced hepatocyte-like cell (iHEP)	-Adenoviral vector -Transcription factors (FOXA3,GATA4,HNF1A,HNF4A)	[34]
	-Liver fibrosis	-Myofibroblasts into hepatocyte	-Adeno-associated virus vector (AAV) -Transcription factors (Foxa1,Foxa2,Foxa3,Gata4,Hnf1a,Hnf4a)	[33]

cells. As summarized in Table 1.4, in vivo reprogramming has been applied by reprogramming hepatic cells towards hepatocytes or pluripotent stage [33–36].

1.6 Heart

Heart is a vital organ and there are many external factors affecting it. Stress, unhealthy diet, smoking and genetic predisposition can cause heart attacks. Following myocardial infarction, myocardial mass cell death leads to heart failure. Adult cardiomyocytes have limited regenerative capacity and unfortunately current therapeutic strategies can not restore the loss of myocardium after injury. Recent

Table 1.5 Different studies involving in vivo reprogramming for heart

	Disease model	Reprogrammed cells	Reprogrammed protocol	References
In vivo reprogramming for cardiomyocyte	-Myocardial infarction (MI)	-Cardiac fibroblasts to cardiomyocyte-like cells	-Retroviral vector -Transcription factors (Gata4, Mef2c, Tbx5)	[9]
	-Cardiac injury	-Cardiac fibroblasts to cardiomyocyte	-Lentiviral vector -Mature miRNAs (1, 133, 208, 499)	[12]
	-Cardiac injury	-Cardiac fibroblasts to cardiomyocyte-like cells	-Retroviral vector -Transcription factors (Gata4, Mef2c, Tbx5 and dsRed)	[10]
	-Complete heart block	-Cardiomyocytes into pacemaker cells	-Adenoviral vector -Transcription factor (TBX18)	[39]
	-Cardiac injury	-Non-cardiac myocytes to cardiac myocytes	-Lentiviral vector -Combination of miRNAs (miRNAs 1, 133, 208, and 499; miR combo)	[37]

studies have successfully shown that endogenous fibroblasts residing in the heart can be reprogrammed to cardiomyocyte-like cells after myocardial infarction and heart function can be improved subsequently [9, 12, 37–39]. Therefore, in vivo reprogramming technology has also strong implications for the treatment of heart failure (Table 1.5).

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Chapter 2
In Vivo Reprogramming for Regenerating Insulin-Secreting Cells

Jiaqi Lu and Qiao Zhou

2.1 Introduction

Diabetes Mellitus is a disease that has been identified for more than 2000 years, since Hippocrates first described the diabetic condition as "flesh melts into urine" around 500 BC [1]. In 1788, Cawley associated the diabetic condition with pancreatic tissue degeneration. In 1869, the German pathologist, Paul Langerhans, identified the pancreatic islet as "those small patches of tissue embedded in the sea of acinar tissue". In early 1900s, the structures of the endocrine glands in pancreas and their secretions were revealed. There are four types of cells in the pancreatic islet, including glucagon-secreting alpha cells, insulin-secreting beta cells, somatostatin-secreting delta cells and polypeptide-producing PP cells. Diabetes is caused by the loss or dysfunction of the beta-cells that secret insulin for blood glucose regulation. Overt insulin deficiency is seen in late-stage Type 2 Diabetes (T2D) and in all cases of Type 1 Diabetes (T1D), which is an autoimmune disease in which beta cells are specifically destroyed. Today dose-controlled insulin injection is widely applied to treat insulin-dependent diabetes. An alternative therapy for T1D is islet transplantation-replenishing the body with new and healthy beta cells. Cadaveric islet transplantation has proved to be an effective therapy for T1D [2, 3]. However, due to shortage of cadaveric islets, this efficacious treatment can only be extended to a small number of patients [4]. It is imperative to develop new technologies to generate functional insulin-secreting cells for transplantation therapy to treat severe forms of diabetes in which significant beta cell loss occurs.

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Several promising technologies have been developed in recent years to produce beta cells in vitro. In 1998, Thomson et al. first established the in vitro culture of human embryonic stem cells (hESCs) and differentiated hESCs into all three germ layers [5]. As pluripotent stem cell-related studies advanced in recent years, increasingly refined differentiation protocols were developed for pancreatic beta cell generation in vitro [6–14]. The differentiated beta-like cells express key beta cell markers and possess glucose-stimulated insulin secretion (GSIS) after transplantation in vivo [12, 15, 16]. However, derivation of beta-like cells from hESCs remains technically challenging, often producing multi-hormonal cells or cells with limited GSIS in vitro [17–20]. Nevertheless, several companies (ViaCyte Inc. and SEMMA Therapeutics) are moving this technology towards clinics.

Lineage-determined cells can be reprogrammed, or converted, from one specialized cell type to another, producing stem cells or other types of lineage-determined cells [21]. Although rare in nature, cellular reprogramming has attracted much attention in the past decade due to its enormous potential in generating functional cells for cell therapies [22]. Perhaps the most prominent example of reprogramming is the generation of induced pluripotent stem cells (iPSCs) from murine and human skin fibroblasts with defined factors, which opened up the possibility of deriving different tissues by iPSC re-differentiation [23–25]. Alternatively, lineage-determined cells may also be reprogrammed directly into other types of lineage-determined cells. These types of reprogramming are sometimes referred to as lineage reprogramming, direct reprogramming, or transdifferentiation. The lineage reprogramming approach has been used to generate beta-like cells from other adult cell types, including pancreatic alpha cells [26–31], delta cells [32], acinar cells [33] and ductal cells [34, 35]. Other endodermal cell types, including hepatic cells [36–41], biliary cells [42] and gastrointestinal cells [43] have also been induced to become insulin$^+$ cells with cocktails of reprogramming factors.

The in vivo reprogramming approach for regenerating insulin-secreting cells was pioneered by the study of acinar-to-beta reprogramming [44]. This study and all other reprogramming studies are generally guided by our knowledge of pancreatic beta cell development during embryogenesis. In this chapter we will first summary key developmental steps of pancreas in mouse and in human before discussing different in vivo beta cell reprogramming models.

2.2 Development of the Pancreas

The pancreas is both an exocrine and an endocrine organ derived from endoderm. The exocrine part of the pancreas secrets digestive enzymes into the duodenum, and the endocrine pancreas (islets) secrets hormones into the blood to regulate blood glucose level. Past studies are mostly focused on mouse pancreas development. Comparatively less is known about human pancreas development due to limited accessibility to early human embryos [45]. There are many excellent reviews on pancreas development [45–47]. We will herein briefly summarize key developmental steps and several well-studied developmental regulators.

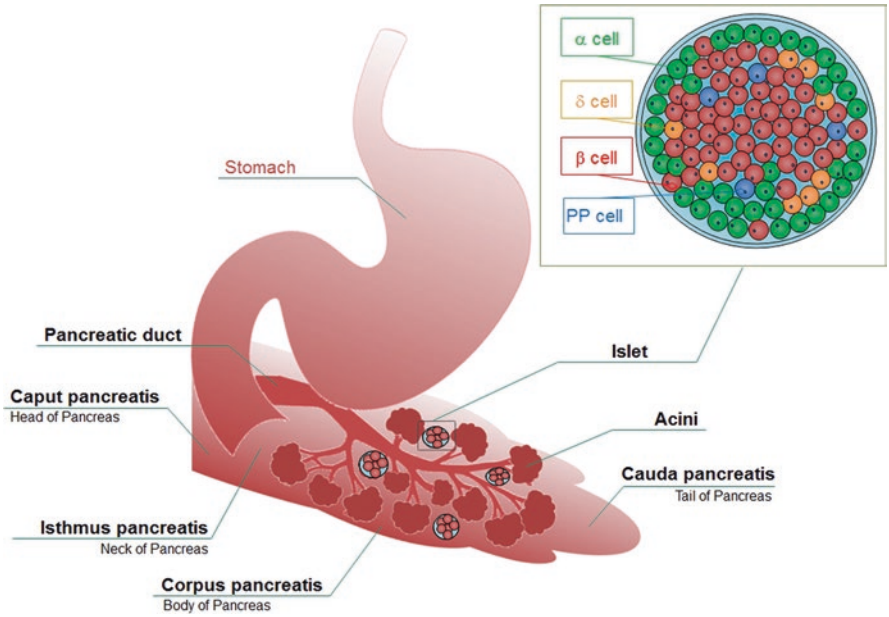

Fig. 2.1 The pancreas and the main pancreatic cell types. Schematic diagram of the stomach and pancreas, the main anatomical components of human pancreas, and the major islet endocrine cell types (*inset*). The anatomical components include caput pancreatis, isthmus pancreatis, corpus pancreatis and cauda pancreatis. Pancreatic alpha cells, beta cells, delta cells and PP cells in the islet are shown in the enlarged islet

2.2.1 Mouse Pancreas Development

All pancreatic cells arise from two buds originating in the posterior foregut endoderm in mouse (Fig. 2.1). The dorsal bud in mouse begins to form at E8.75 and the ventral bud forms several hours later. Studies have identified two waves of development in rodent pancreatic formation: the "primary" and "secondary" transitions. During the "primary transition" stage between E9.5 and E12.5, the first wave of endocrine cells, mainly glucagon$^+$ cells appear in mouse pancreas. The functions of these early endocrine cells remain enigmatic [49]. At E11.5, the gut tube begins to rotate, which brings the dorsal bud close to the ventral bud for their future fusion [48]. At this stage, microlumens begin to appear in the buds and local spreading of these microlumens occurs. The buds are dramatically transformed into a tubular epithelium that grows into the pancreatic mesenchymal layer [50]. The "secondary transition" begins at E13.5 when the epithelium begins to differentiate into three main pancreatic lineages. During the early branching morphogenesis of the embryonic epithelium, a tip and a trunk domain can be discerned. Genetic lineage tracing studies suggest that the tip domain contains a transient population of multipotent progenitor cells (MPCs), which further differentiate into acinar, duct and endocrine cells [51].

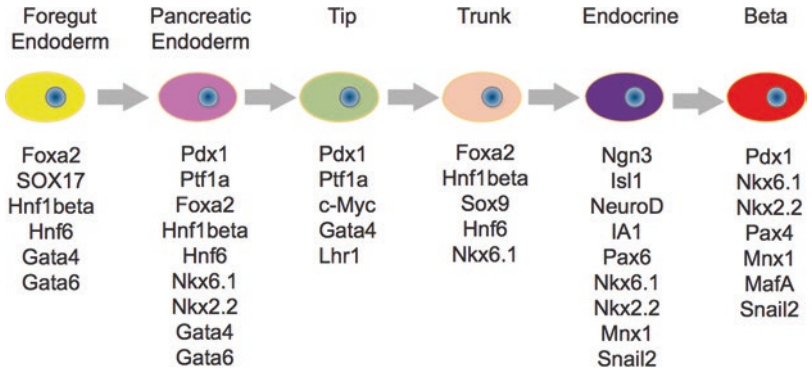

Fig. 2.2 Transcription factor profiles in mouse pancreatic beta cell differentiation. Each tissue type is marked on top of the corresponding cell symbol. Representative transcription factors of each cell type in the developmental stage are listed

Endocrine differentiation in mouse pancreas is programmed by specific expression of a constellation of transcription factors (Fig. 2.2). Pancreas and duodenum homeobox 1 (Pdx1) begins to be expressed as early as E8.5 in the endoderm around the junction of stomach and intestinal epithelium [48]. Ptf1a expression initiates shortly thereafter and likely represents the first gene that specifically marks the developing pancreatic domain [52]. Paired box 4 (Pax4) is one target of Ngn3 that plays important roles in inducing beta- and delta-cell lineages by inhibiting alpha-cell fate [53, 54]. Paired box 6 (Pax6) is expressed in all endocrine cells and is required for the development of four endocrine cell subtypes in islet cell differentiation [55]. The homeobox factor Nkx2.2 functions in beta-cell specification and can be detected in alpha-, beta- and PP-cells in mature islets [56]. Nkx6.1 is important for both endocrine precursor formation and later, proper insulin secretion [57]. Maf gene family acts in the terminal differentiation of beta-cells. MafA is initially expressed in E13.5 and it interacts with insulin promoter directly and trans-activates insulin expression [48]. Finally, Ngn3 is the key determinant for pancreatic endocrine development; its function is both necessary and sufficient for formation of all islet cell types [58, 59].

2.2.2 Human Pancreas Development

The human pancreas is made up of the head (caput pancreatis), the isthmus (isthmus pancreatis), the body (corpus pancreatis) and the tail of the pancreas (cauda pancreatis) [60, 61]. Similar to mouse development, the human pancreas originates from two buds, each arises from either side of the distal foregut endoderm. The dorsal bud forms first at gestational day 26 (G26d) and then two ventral buds appear at approximately G30d [47, 62] (Table 2.1). There is no “primary transition” in human pancreas development [45]. At G25–27d, the human dorsal pre-pancreatic

Table 2.1 Key events in human and mouse pancreatic development

Carnegie stage	Gestational day	Key events in human embryonic pancreas development	Key events in mouse embryonic pancreas development	Approximate equivalent stage of mouse development
CS9	G22–26d	Dorsal bud formation at G26d		E7.5–E8
CS10	G25–27d	Anterior intestinal portal formation; transient notochord-foregut endoderm contact		E8–E8.5
CS11	G27–29d		The dorsal pancreatic bud begins as a local thickening of endoderm	E8.5–E9
CS12	G29–31d	Dorsal aortae fusion; Pdx1 expression in pancreatic buds	Evagination of the early gut endoderm	E9–E9.5
CS13	G30–33d	Clear dorsal and ventral pancreatic buds formation	Glucagon$^+$ and insulin$^+$ cells can be detected	E9.5–E10
CS14	G33–35d		The buds grow and initiate branching; Pioneer microlumens begin to appear in the buds	E10–E11.5
CS15	G35–37d	Separation of fused dorsal aorta from endoderm; dorsal bud outgrowth		E11.5–E12.25
CS16	G37–40d	Growth of organ and proliferation of multipotent pancreatic progenitors	Pancreatic branching morphogenesis has just begun; primary brunches formed	E12.25–E12.75
CS17	G39–42d			E12.75–E13.25
CS18	G42–45d		Amylase expression appearing in scattered tip cells	E13.25–E14
CS19	G45–47d	Tip-trunk compartmentalization process	The tip and the trunk of the pancreas branch	E14–E14.5
CS20	G47–50d		The tip MPC begins to switch to the pro-acinar condition	E14.5–E15
CS21	G49–52d	First detection of insulin$^+$ cells		E15–E15.5

(continued)

Table 2.1 (continued)

Carnegie stage	Gestational day	Key events in human embryonic pancreas development	Key events in mouse embryonic pancreas development	Approximate equivalent stage of mouse development
CS22	G52–55d			E15.5–E16
CS23	G53–58d G8w	Ngn3 expression and Ngn3$^+$ endocrine progenitors found; Appearance of glucagon$^+$ and somatostatin$^+$ cells	The epithelium expands further	E16–E16.5
N/A	G9w	Appearance of pancreatic polypeptide$^+$ cells		E16.5–E17
N/A	G11w	Initiation of acinar differentiation		E17–E17.5
N/A	G11–14w	Initiation of islet vascularization	Endocrine cells begin to form the islets of Langerhans	E17.5–E18
N/A	G12w	Initiation of active endocrine cell birth, delamination and islet clustering	Ventral and dorsal parts of the pancreas fuse in a single organ structure	E18–E18.5
N/A	G14w	Tip-trunk compartmentalization process completed		

CS refers to Carnegie stages adapted from the UNSW Human Embryo Resource (https://embryology.med.unsw.edu.au/embryology/index.php/Embryonic_Development), *N/A* not available

endoderm is transiently adjacent to the notochord, which excludes sonic hedgehog (SHH) expression from this endodermal region. At G29–31d, Pdx1 expression can be detected in pancreatic buds when the transient notochord-endoderm contact is disrupted by the dorsal aortae fusion with the mesoderm [63].

At G30–33d, several transcription factors such as Pdx1, sex-determining region Y-box 9 (Sox9), GATA transcription factor 4 (GATA4) and Nkx6.1 are expressed in the epithelium of human pancreatic buds [47, 64]. This transcription profile closely resembles that of multipotent pancreatic progenitors cells (MPCs) in the mouse (Fig. 2.2), indicating the high similarity of molecular markers in human and mouse early pancreatic differentiation. During this stage (G30–33d), the primary central lumen and microlumens appear in the stratified epithelium derived from the dorsal bud in human [47].

The right ventral bud migrates posteriorly at G35d along with the gut rotation and fuses with the dorsal pancreatic bud at G6–7w in human, and further differentiates into the inferior part of the head [60, 61]. The left ventral bud regresses gradually [60]. From G45 to G47, the pancreatic epithelium grows and branches actively by the extensions of the pancreatic buds into the surrounding mesenchyme [65]. Later at G7–8w, the epithelium begins to ramify and forms a lobular pattern. Future

studies are required to clarify whether human fetal pancreatic epithelium goes through similar steps of morphogenesis as that of mouse.

As we mentioned above, in the early mouse pancreatic bud, glucagon-expressing cells can be detected during the primary transition, but they cannot be detected in early human embryonic pancreas. At G7w, the human pancreatic epithelium initiates the tip-trunk compartmentalization. At this stage GATA4 expression is detected in the pancreatic epithelium, indicating the presence of pro-acinar cells and tip cells (Fig. 2.2). MPC markers Sox9 and Nkx6.1 are also expressed in the GATA4$^+$ tip cells, suggesting that some MPCs localize in the tip domain [66]. Tip-trunk segregation is completed at G14w when GATA4$^+$ tip cells stop expressing Sox9 and Nkx6.1. In human pancreas at G7.5w, insulin-expressing cells are the first to appear and they are the major endocrine cell type during the first trimester [67]. At G8w, glucagon- and somatostatin-expressing cells appear and 1 week later, pancreatic polypeptide and ghrelin-expressing cells can be detected [67]. In mouse the endocrine cells are only located in the trunk region of the pancreatic epithelium. It is unknown whether this is the same for human endocrine cells [66].

At G9–21w several endocrine progenitor markers can be detected in human fetal pancreas, including Ngn3, Pax6, Pax4, Nkx2.2, Nkx6.1, Islet1, Neurogenic differentiation 1 (NeuroD1) [68]. The transcription factor expressing patterns of human fetal pancreas closely resemble that of mouse at this stage (Fig. 2.2). Ngn3$^+$ cells increase at subsequent developmental stages between G8.5w and G10w. Ngn3$^+$Pdx1$^+$, Ngn3$^+$Insulin$^+$, and Ngn3$^+$Glucagon$^+$cells can be found at this stage without co-expression of the trunk marker Sox9 [47, 64]. In mouse pancreas, the endocrine cells cannot develop further without Ngn3 expression. Similarly, in human the endocrine differentiation from PSCs is also dependent on Ngn3 [45, 69].

Islet formation was proposed to occur with delamination of differentiated hormone-expressing cells, which undergo epithelial-mesenchymal transition (EMT) [70]. Mesenchymal marker vimentin and EMT regulators Snail1 and Snail2 are transiently expressed in the beta-cells delaminating from pancreatic epithelium [66]. In mouse, endocrine precursors first delaminate in clusters and then differentiate [71]. It is not known whether such "cluster delamination" occurs in human. It is also possible that a single cell delaminates first followed by endocrine cell clustering. Scharfmann et al. found that endocrine cells in one human islet came from more than one progenitor, supporting the idea that delamination precedes endocrine differentiation in human [72].

In human the clustering of insulin and glucagon-expressing cells begins in the central region of the pancreas [73]. At G10w endothelial cells start to connect with small endocrine cell clusters, and vascular structures appear at G14w [74]. In mouse pancreas the "endocrine cords" can be detected. But the organization of human endocrine cells and the integration of vascular and neuronal components still remain obscure. Beta-cell is the most abundant cell type in the developing mouse pancreas in the secondary transition. In human, the number of beta-cells are 3–4 times that of alpha-cells in the initial islet clusters (G9–13w) but the ratio of alpha-cells to beta-cells decreases to 1:1 by G14–16w [67, 73]. Moreover, the proportion of beta-cells

is equal to delta-cells in human in the last trimester, indicating the slower proliferation rate of beta-cells in the later stages [75].

At G8w, carboxypeptidase A1 (CPA1)-positive pyramidal cells grow from pancreatic epithelium, indicating generation of early pro-acinar population [76]. Meanwhile the pro-acinar markers such as GATA4, MIST1 and trypsin inhibitors are also expressed. At G11–15w, human secondary transition begins with terminal differentiation program [66]. Digestive enzymes and acinar-specific markers such as carboxylester lipase, chymotrypsinogen, trpsinogen, elastase1, and trypsin1 dramatically increase from G11w and stabilize at G15–19w [66]. The acinar cell marker amylase can be detected from G23w. The acinar cell-specific gene in mice, Ptf1a, has not been detected in the human acinar. The ductal differentiation program in human has not been clearly defined. Several ductal cell markers have been detected, including cytokeratin 19, carbonic anhydase 1, mucin 1, cystic fibrosis trans-membrane conductance regulator, but the differentiation status and the heterogeneity of ducts in the epithelium remain unclear [66].

In summary, compared with mouse beta cell development, the molecular programs and pathways that govern human beta cell generation are less well understood. But the key developmental steps and transcriptional regulators appear to share a high degree of similarity between human and mouse beta cells.

2.3 In Vivo Reprogramming of Alpha and Delta Cells to Beta-Like Cells

All pancreatic islet cell types share a common precursor, namely the Ngn3$^+$ endocrine progenitors. Interconverting islet endocrine cells has long been thought of as a possibility [58, 59]. This was demonstrated for pancreatic alpha and delta cell conversion to insulin$^+$ cells after near-total beta-cell ablation in mice [32, 77].

After extreme beta-cell loss in juvenile mice, Chera et al. lineage-traced delta cells, and proposed that delta cells could dedifferentiate, proliferate and become insulin-producing cells [32].

Mouse pancreatic alpha cells have been shown to harbor bivalent chromatin marks at beta cell-specific genes [31]. When most beta cells are destroyed (diphtheria toxin) in mice, a large number of glucagon$^+$ cells appear to become insulin$^+$, indicating alpha-to-beta-like cell conversion [31]. However, the mechanism of this conversion and whether it occurs under more physiological conditions remain unclear. In addition, several studies have demonstrated conversion of alpha cells to beta-like cells by forced expression of transcription factors. For instance, ectopic expression of Pax4 was shown to convert embryonic glucagon-producing cells into beta-like cells [28]. The mis-expression of Pax4 in adult alpha cells also induces their conversion into beta-like cells in mice, which functionally reversed repeated cycles of toxin-induced hyperglycemia [34]. Courtney et al. further discovered that the inactivation of Arx triggered the alpha-to-beta-like cell conversion [29]. Efforts

have also been made to enhance efficiency of alpha-to-beta-like cell conversion. Yang et al. found that forced expression of Pdx1 in the $Ngn3^+$ endocrine progenitor facilitated conversion of alpha cells to beta-like cells [78]. It was also observed that Activin enhanced alpha to beta cell reprogramming [27], indicating that signaling pathway contributed to this cell type conversion. Nevertheless, further studies are necessary to fully ascertain how closely these new beta-like cells resemble endogenous beta cells in terms of their molecular characteristics and function.

The regenerative capacity of alpha cells and the feasibility of alpha-to-beta-like cell conversion potentially open a new avenue to develop regenerative therapies for T1D. In 2017, Nouha et al. identified γ-aminobutyric acid (GABA) as an inducer of alpha-to-beta-like cell conversion in vivo [79]. Long-term GABA exposure was found to act on $GABA_A$ receptor and induce the conversion of alpha cells into functional beta-like cells through the downregulation of Arx expression, which successfully reversed chemical-induced diabetes in vivo. Meanwhile, GABA administration on transplanted human islets could also give rise to alpha-to-beta-like cell reprogramming. This discovery provides a promising approach to induce beta-like cells in pancreas without gene delivery.

2.4 In Vivo Reprogramming of Acinar Cells to Beta-Like Cells

One of the earliest examples of converting non-beta cells to beta-like cells was carried out with pancreatic acinar cells using a cocktail of beta-cell transcription regulators. Prior to this study, Zhou et al. exhaustively mapped expression of transcription factors in developing mouse pancreas and described distinct domains of gene expression using in situ hybridization of more than 1100 transcription factors [51] (Table 2.2). More than 20 transcription factors were found to be expressed in beta-cells and endocrine progenitors, including Pdx1, NeuroD, MafA, Nkx2.2, Nkx6.1, Pax6, Isl1, Foxo1, Hnf1a, Hnf3a, Hnf4a [44, 51] (Table 2.3).

Table 2.2 Pancreatic transcription factors expressed in developing mouse pancreas can be grouped into five general patterns

Localization	Pan-epithelium	Tip	Trunk	Mesenchyme	Vasculature
Pancreatic genes	Pdx1 Sox9 Hnf1b Hnf3a Hnf3b Hnf4a Nkx6.1 Hex Nkx6.2 Nkx2.2 Hnf6 Prox1 FoxP4 Hex TGIF2 HMGb4 RorC	Ptf1a Nr5a2 Etv5 Mist1 c-Myc Id1 Id2 Xbp1 HMG1 Pse HMGb2	Ngn3 Pax4 Pax6 NeuroD Myt1 Brn4 Isl1 Arx MafB Wbscr14	Osr1 Osr2 Prrx1 Tbx3 Islet1	Sox17 Sox18 Epas1 Hif3 Elk3 HeyL Crip2 Ptrf HMG ERG

From Reference Zhou et al. [51]

Table 2.3 A list of pancreatic transcription factors expressed in pancreatic progenitors, endocrine progenitors, and beta cells

Pancreatic progenitor	Endocrine progenitor	Adult beta cell
Pdx1 Ptf1a Sox9 Hnf6 Hnf1b Hnf3b Hnf4a Hex Prox1 Hb9 Nr5a2	**Ngn3 NeuroD Nkx2.2 Nkx6.1 Pax4 Pax6 Isl1 MafA** MafB Brn4 Arx Myt1 Wbscr14 VDR IA1	**Pdx1 NeuroD MafA Nkx2.2 Nkx6.1 Pax6 Isl1** Foxo1 Hnf1a Hnf3a Hnf4a

Factors in bold were tested in reprogramming studies

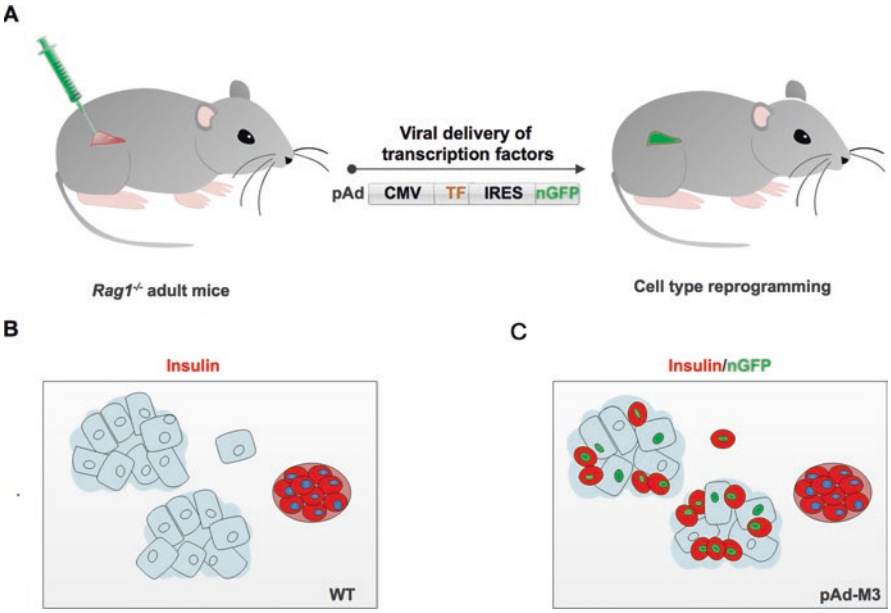

Fig. 2.3 In vivo reprogramming of pancreatic acinar cells into beta-like cells. (**a**) Schematic diagram illustrating the reprogramming strategy to induce beta-like cells from mouse pancreas by injecting adenoviruses encoding transcription factors and nGFP linked by IRES element. (**b**) Wild type pancreas showing the exocrine tissue and insulin-expressing beta-cells in islets (outlined). (**c**) Numerous insulin$^+$ cells appeared outside of islets 1 month after infection with a combination of Ngn3, Pdx1 and MafA adenoviruses

Among pancreatic transcription factors, genetic knock-out studies show that at least nine genes are directly involved in endocrine and/or beta-cell development. As a result, these nine genes were picked as candidate reprogramming factors (in bold font in Table 2.3). Exocrine cells were chosen as the cells to be engineered because their close lineage relationship. Adenoviruses carrying each of the nine genes together with nuclear GFP (nGFP) were made. A mixture of the nine adenoviruses was injected into the pancreata of 2-month-old adult mice Rag$^{-/-}$ (Fig. 2.3a). One month after viral delivery, insulin$^+$ cells could be detected among viral infected

GFP$^+$ extra-islet cells (Fig. 2.3c). Subsequent tests showed that only three of the nigh factors, Ngn3, Pdx1, and MafA (referred to as M3 or NPM factors) were essential (Fig. 2.3). Twenty percent of the infected cells were reprogrammed into insulin$^+$ cells by M3 (Fig. 2.3). Most infected cells were found to be amylase$^+$ exocrine cells in the in vivo M3-induction experiments. It was demonstrated that the reprogramming effects were specific for exocrine cells, given that other cell types, including skeletal muscle in vivo or fibroblasts in vitro didn't turn on insulin expression when infected with M3 factors.

Morphologically, the induced insulin-secreting cells are indistinguishable from endogenous islet beta-cells. The induced beta-like cells possess small dense insulin granules and lose large zymogen granules that exocrine cells have. Induced beta-like cells express essential beta-cell genes including glucose transporter 2 (Glut2), glucokinase (GCK), prohormone convertase 1/3 (PC1/3), and key beta-cell transcription factors such as NeuroD, Nkx2.2 and Nkx6.1. Further, the induced insulin-secreting cells lose amylase and Ptf1a expression and CK19 expression, suggesting loss of exocrine identity. Interestingly, the induced beta-like cells synthesize vascular endothelial growth factor (VEGF), which induces angiogenesis. Injection of Adeno-M3 induced formation of insulin$^+$ cells and ameliorated hyperglycemia of STZ-induced diabetic mice. This study reveals one possible reprogramming pathway to convert terminally differentiated pancreatic exocrine cells into beta-like cells by expression of developmental transcription factors.

In a follow-up study, Li et al. further increased the induction efficiency of insulin$^+$ cells from acinar cells and assessed their therapeutic potential [80]. By using polycistronic version of M3 factors, insulin$^+$ cell induction increased to 40–50%, which led to formation of islet-like clusters that persisted in the pancreas for up to 13 months. This is the first study to investigate the long-term fate of the induced beta-like cells. The new beta-like cells induced in this way responded to high glucose at 2 months after induction, and the glucose-responsiveness of the induced beta-like cells improved over time and eventually approached that of endogenous beta cells.

To investigate mechanisms of acinar reprogramming, global DNA methylation patterns of acinar cells, induced beta-like cells and endogenous islet beta-cells were analyzed [80]. The results show that DNA methylations increase significantly in the first 10 days of the conversion from acinar cells to induced beta-like cells. Transcriptome analysis showed that the majority of transcriptional remodeling was completed by 2 months after viral transduction. The induced beta-like cells expressed moderate to high levels of beta-cell genes and had no expression of acinar genes, which was verified by single cell qPCR. Therefore the transcriptional network of the induced beta-like cells resembles that of endogenous beta cells, and the induced and the endogenous beta cells are similar at the single-cell level.

Besides acinar to beta-like reprogramming, it has been reported that acinar cells can be transdifferentiated to delta-like and alpha-like cells in vivo by Ngn3 and Ngn3+MafA, respectively [81]. In conclusion, the acinar cells can be reprogrammed into three major islet endocrine subtypes with different combinations of NPM factors in vivo [81].

2.5 Reprogramming of Gastrointestinal Tissues

It is reasonable to postulate that cells in the endoderm-derived organs could potentially be reprogrammed to beta cells due to their shared lineage history. In recent years in vivo reprogramming toward beta-like cells has been successfully achieved from gastrointestinal cells and liver cells (In vitro reprogramming was successful in a wider array of cells including pancreatic ductal cells and gall bladder cells) [35, 82].

The gastrointestinal (GI) epithelium is a highly regenerative tissue due to large numbers of adult stem cells and progenitors where the hormone-secreting enteroendocrine cells originate from [83, 84]. Part of the stomach (antrum) and intestine (duodenum) share particular developmental similarity with pancreas [85, 86]. Moreover, both the gut enteroendocrine cells and pancreatic endocrine cells develop from Ngn3$^+$ progenitor cells [59, 87].

Insulin-secreting cells were successfully generated in vivo by ablating Foxo1 in Ngn3$^+$ endocrine progenitors in mouse gut [88, 89]. Intestinal crypts were also found to generate "neo-beta cell islets" by expression of NPM factors [90]. However, the induced beta-like cells from intestine appear to possess limited function and lack key beta cell genes Nkx6.1 and Prohormone Convertase 2 (PC2).

In 2016, Ariyachet et al. reported that enteroendocrine cells from the antral stomach could be reprogrammed into functional beta-like cells more efficiently than that of intestine with NPM factors [91]. The antral enteroendocrine cells were demonstrated to share a high level of transcriptional similarity with pancreatic beta cells (Fig. 2.4). These insulin$^+$ cells were discovered to arise from multiple endocrine subtypes. The Dox-induced insulin$^+$ cells in GI tract could reverse diabetes long-term (up to 6 months).

Ariyachet et al. further created bioengineered "mini-stomach" using gastric gland units extracted from the antrum of CAGrtTA::TetONPMcherry (CAG-NPM) animals. After transplanting into the omentum of immunodeficient NSG mice and Dox treatment, insulin$^+$ cells were induced, and ameliorated hyperglycemia in a subset of the transplanted mice. The existence of Sox9$^+$ progenitor cells and Ki67$^+$ proliferating epithelial cells indicates the persistence of a stem compartment [91]. Therefore the induced antral insulin$^+$ cells are considered to be a renewable insulin-secreting cell source to treat diabetes.

2.6 Reprogramming of Liver Cells

The pancreas and the liver arise from adjacent regions of foregut and share close lineage relationship (Fig. 2.1). Insulin$^+$ cells and other endocrine cells have been found to arise from hepatobiliary duct cells in mouse. Thus, the hepatic lineage has long been evaluated for their ability to give rise to insulin$^+$ cells using the reprogramming approach.

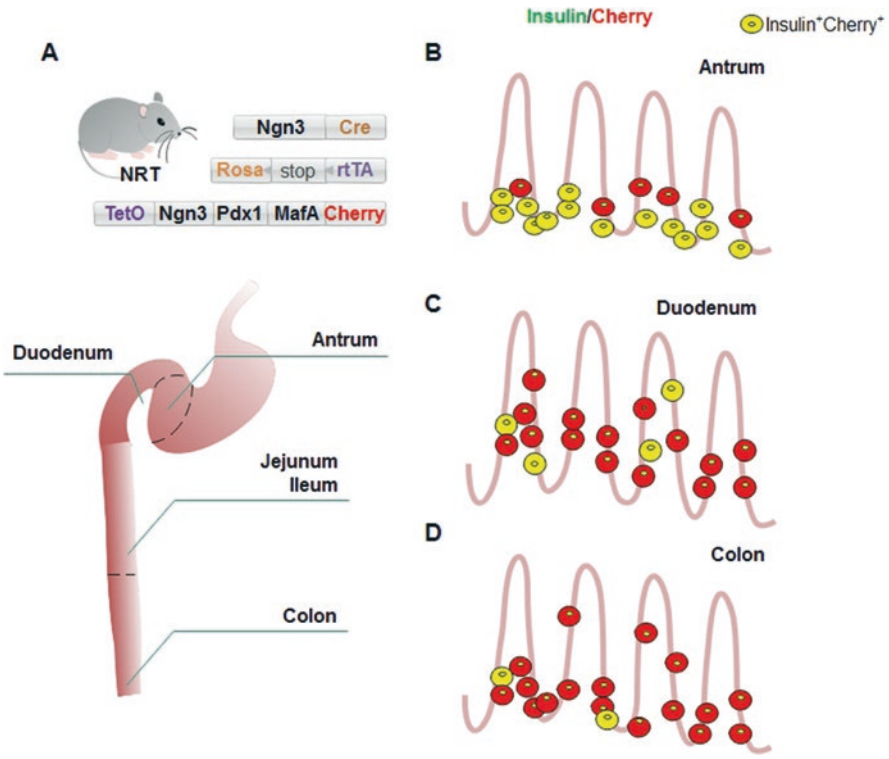

Fig. 2.4 In vivo reprogramming of gastrointestinal epithelial cells into insulin-secreting cells with NPM factors. (**a**) Generation of the NRT transgenic mouse by crossing TetO-NPMcherry mouse line with BAC-transgenic Ngn3-Cre line and the knock-in Rosa-floxed-rtTA line. (**b**–**d**) Generation of insulin$^+$ cells in the antrum (**b**) duodenum (**c**) and colon (**d**) of NRT animals. *Yellow balls* represent the induced insulin$^+$Cherry$^+$ beta-like cells, and *red balls* represent Cherry$^+$ cells

Many studies have reported generation of beta-like cells from liver cells in vivo. In *Xenopus*, ectopic expression of an active form of Pdx1 gives rise to pancreatic differentiation [92]. In 2000, adenoviral delivery of Pdx1 in mouse liver was found to successfully activate insulin expression in situ and ameliorated hyperglycemia in diabetic mice [93–95]. Gefen-Halev et al. discovered that Nkx6.1 could promote the Pdx1-induced liver to insulin-secreting cell reprogramming [96]. In 2009, Ngn3 with betacellulin was delivered by AAV into the liver, leading to insulin$^+$ cells in oval cells in the periportal region [97]. Pdx1VP16 and NeuroD fusion protein delivery was also reported to activate beta-cell genes in the liver [98].

In 2012, Anannya et al. injected the adenoviruses carrying Pdx1-Ngn3-MafA polycistronic constructs into the liver of NOD-SCID mice, which resulted in the significant rescue of the STZ-induced diabetes a few days after viral delivery [98]. The normal blood glucose level was maintained over a period of at least 4 months. The induced insulin$^+$ cells in this way are visible as small clusters initially and later (3–16 weeks after viral delivery) become duct-like. Moreover, the dense core insulin

granules in the induced insulin$^+$ cells can be seen from electron microscopy, albeit fewer than normal beta cells. Morphologically, these induced insulin-secreting cells look like ducts and express CK19 and E-cadherin. But these insulin$^+$ ductal cells are glucose responsive and positive for C-peptide, Pdx1, MafA, Isl1, Rfx6 and Sox17. In addition, somatostatin$^+$ glucagon$^+$ cells also exist in the insulin$^+$ duct. Therefore although these unique cells possess some properties of pancreatic beta-cell, they are quite different from native pancreatic beta cells.

Recently, AAV-mediated expression of TGIF2 expression was shown to turn on pancreatic markers in the liver of adult mice [99].TGIF2 is expressed at E8.0 of mouse embryos, particularly in the caudo-lateral region of the ventral foregut where the bipotent hepatic and pancreatic progenitors reside. TGIF2 targets GATA5 and changes its expression in opposite directions when cells commit to pancreatic or hepatic lineages by modulating BMP/TGFbeta signals within the endoderm. After E8, TGIF2 is persistently expressed in pancreas whereas it becomes undetectable in the liver. Nuria et al. demonstrated that definitive endoderm could be induced toward pancreatic fate by transduction of lentivirus carrying TGIF2. In parallel, the expression of liver-specific genes was decreased by TGIF2 expression in hepatic endoderm [100]. Thus TGIF2 was selected as a pertinent candidate to test its capability of reprogramming liver to pancreas ex vivo and in vivo. It was shown that lentiviral delivery of TGIF2 into hepatic cell lines induced a series of pancreatic genes and strongly reduced hepatic genes, demonstrating that TGIF2 is a remarkable ex vivo reprogramming factor to induce liver to pancreas fate [99]. Recombinant AAV2/8 delivery of TGIF2 into the liver effectively induced Sox9 expression in the liver parenchyma. It was observed that Pdx1 as well as other pancreatic transcription factors like NeuroD and Nkx6.1 were induced 1 month after AAV injection. Furthermore, AAV_TGIF2-injected diabetic Akita animals displayed sustainable decrease in blood glucose together with higher levels of circulating insulin.

2.7 Challenges and Future Directions for In Vivo Direct Reprogramming

As discussed above, it has been reported that a number of endodermal tissues can be reprogrammed into beta-like cells in vivo, sometimes accompanied by amelioration of diabetes in mice. Overall, this is a promising strategy to regenerate therapeutic insulin-secreting cells. However, all of the published studies were carried out in mice and it is unknown whether these reprogramming methods could be applied to human. For further clinical development, several significant challenges need to be addressed.

Firstly, the safety of the in vivo reprogramming approach needs to be evaluated. Foxo1 ablation in intestine tissues generated insulin$^+$ cells, but this process was accompanied by beta cell failure due to the protective effect of Foxo1 [101]. With the present acinar-to-beta strategy, immature beta-like cells are generated in the

initial transition, which poses a potential risk of hypoglycemia [80]. To reduce such risk, acinar reprogramming should be approached by reprogramming a relatively small number of cells at a time. How to deliver the reprogramming factors into the pancreas without eliciting inflammation is another challenge. To evaluate the safety of reprogramming human tissues, we will likely need relevant humanized mouse models, primate animal models, or human mini-organ models [102–104].

Secondly, we need to further optimize our reprogramming cocktails and timing of reprogramming factor expression. For instance, expression of the factors may need to be controlled in a precise temporal manner for better reprogramming efficiency. To more precisely manipulate the reprogramming process, Pratik et al. designed a synthetic lineage-control network to regulate Ngn3 (OFF–ON–OFF) and Pdx1 (ON–OFF–ON) expression with concomitant induction of MafA (OFF–ON) [105]. The insulin-release dynamics of the induced beta-like cells are comparable to endogenous human pancreatic islets, highlighting the importance of timely control of transcription factor expressions.

Thirdly, we need to optimize the gene delivery method. The direct reprogramming efficiency is highly dependent on viral delivery vehicles. In most studies, adenoviruses were used. However, adenoviruses possess high immunogenicity, which lead to tissue inflammation when applied. Adeno-associated viruses (AAVs) have relative low immunogenicity, high transduction efficiency, and are non-integrative. Thus, AAV is considered a far better clinically applicable vector. To further enhance the safety of the reprogramming method, chemical- and protein-mediated reprogramming methods are certainly worthwhile to explore.

In summary, the in vivo reprogramming technology has advanced rapidly in the recent decade. The next major challenge is to develop this promising technology towards clinical applications. We believe in vitro reprogramming using chemicals and protein factors is an important direction to make further progress. With further optimization on safety, efficacy, and delivery method, these technologies could be developed into a powerful clinical approach to treat diabetes.

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Chapter 3
Direct Reprogramming to Beta Cells

Jonathan M.W. Slack

3.1 Introduction

We are currently living in a world-wide epidemic of diabetes. In recent years the incidence of the disease has been increasing rapidly, especially for type 2 diabetes. It is estimated that there were 382 million sufferers in 2013 and this number is projected to rise to 600 million by 2035 [1]. About 10% of all patients are type 1 diabetics who suffer a loss of insulin-secreting beta cells due to autoimmunity. Most of the remainder are type 2 diabetics. Although type 2 diabetes is a complex and heterogeneous condition, it has become clear in recent years that there is usually an element of beta cell insufficiency involved in addition to insulin resistance of peripheral tissues [2]. This is why insulin therapy has an important part to play for both types of the disease. Nearly all type 1 diabetics and about 40% of type 2 diabetics take insulin daily by injection and this is a key part of the management of the disease.

Insulin is normally produced by the beta cells which lie in the Islets of Langerhans in the pancreas. Beta cells release insulin when the glucose level in their environment increases. The circulating insulin provokes glucose uptake by tissues, particularly the adipose tissue and muscles, and the system normally keeps the blood glucose stable at about 5.5 mM (100 mg/dl). Insulin therapy and the monitoring of diabetes have become very sophisticated and are successful in managing the disease. However there are inevitably frequent excursions of blood glucose outside the normal range and these gradually damage blood vessels leading to the complications of diabetes which generate considerable morbidity. These include heart failure, stroke, peripheral circulatory failure leading to amputations, and blindness arising

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from damage to the retina. These complications generate considerable suffering to the patients affected, and are also very costly to treat and manage. It is estimated that about 12% of health care budgets in all developed countries is spent on diabetes care in one form or another. As the prevalence of diabetes increases, these costs will rise proportionately.

Because of the high prevalence of diabetes and the suffering and costs that it causes, it has long been a potential target for new techniques of regenerative medicine. The key objective is to replace or supplement the beta cell complement of the body. Beta cells have the capacity to respond rapidly to increases in glucose concentration and to release insulin accordingly. The precise characteristics and time course of the insulin secretion is very important for minimizing blood glucose excursions. The exquisite sensitivity of the beta cell response cannot be matched by insulin injections and it is even difficult to replicate using the "closed loop" insulin pumps which respond to continuous glucose monitoring of subcutaneous interstitial fluid and use very advanced algorithms to calculate insulin doses [3].

It is important to note that certain solutions that may appear attractive at first sight are not necessarily effective. For example, inserting the *insulin* gene controlled by a glucose-sensitive promoter into the liver does not mimic beta cell function very well because of the time delay for a transcription-controlled process between glucose sensing and insulin secretion. So the goal must be to produce actual beta cells, or cells with very close physiological similarity to beta cells, and use them to augment the endogenous capacity.

The clinical technique of islet transplantation has been practiced with some success since the introduction of the Edmonton protocol for immunosuppression in 2000 [4, 5]. Islets from the pancreases of organ donors are infused into the portal vein of the patient's liver. They lodge within the liver and, although the position is ectopic, they can continue to function for some time. Islet transplantation is usually conducted for type 1 diabetes patients with unawareness of hypoglycemia. This is a very dangerous condition because the patients are liable to die during sleep or to suffer accidents as a result of passing out without warning. In recent experience virtually all islet transplant recipients show improvement of this condition and up to 50% can remain insulin-independent for 3 years. Islet transplants are a considerable success for diabetes treatment but, as with other types of transplantation, the number that can be performed is severely restricted by the shortage of organ donors. Also, being allografts, islet transplants require lifelong immunosuppression. The immunosuppressive drugs can be unpleasant and have various side effects, and they also tend to damage the grafted islets themselves, thus reducing the effective survival time of the graft. Fortunately the degree of immunosuppression required to secure tolerance to an allograft is also sufficient to suppress the autoimmunity characteristic of type 1 diabetes.

There are several potential ways of producing more beta cells using the techniques of regenerative medicine [6, 7]. One possibility is to expand the available beta cells in vitro to increase the cell numbers for transplantation. Unfortunately this has proved very difficult. Beta cells divide rather rarely in vivo, and in vitro they tend to de-differentiate. So the front-running method at present is to make beta cells

from pluripotent stem cells: either embryonic stem (ES) cells or induced pluripotent stem (iPS) cells. The protocols for achieving this have become quite good [8, 7, 9], although there are still some problems of reproducibility between labs. Since it is preferred to avoid immunosuppression, the main task for effective clinical implementation is to find an effective method of encapsulation of the grafted cells that protects them from immune attack by the host but allows sufficient interchange of materials for glucose sensing and insulin secretion.

This article will deal with experiments aimed at generating beta cells, or beta-like cells, by reprogramming in situ. This has the potential advantage of avoiding alloimmunity of cells grafted from another individual, and could also avoid the cost and safety issues of culturing cells in vitro. On the other hand, less resource has been put into this approach and it is not yet ready for clinical trials.

3.2 Reprogramming in *Xenopus* and In Vitro

If the aim is to generate beta cells by reprogramming, attention must be given both to the target tissue type and to the genes to be used for reprogramming. We started from the presumption that potential target cells more like to respond appropriately if they were developmentally related to beta cells in some way. In the normal embryo there are about six distinct stages of developmental commitment through which cell populations pass between being part of the early embryo and finally becoming beta cells. Each of these stages involves changes to the repertoire of transcription factors controlling gene expression and changes in the constitution of the chromatin determining which regions of the genome are accessible to transcription factors.

In normal embryonic development the pancreas arises as two buds from the epithelium of the foregut. The ventral bud is anatomically closely related to the rudiment for the liver and the gall bladder. In response to fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) from the adjacent cardiac mesenchyme, the future liver upregulates various transcription factor genes including *Prox1* and *C/EBPα* while the pancreatic bud upregulates *Pdx1* and *PTH1* [10, 11]. The distinction between the gall bladder region and the ventral pancreatic bud depends on upregulation of *Sox17* and consequent repression of *Pdx1* [12]. So the liver is developmentally quite similar to the pancreas, and the gall bladder, with associated extrahepatic biliary ducts, perhaps even closer. The latter similarity is underlined by the fact that the loss of a single gene, encoding the transcription factor HES1, provokes the conversion of extrahepatic bile ducts to pancreatic tissue [13]. This, of course, is a germline gene loss so has effects throughout the whole of development and, as we shall see, it is much easier to provoke changes of cell type during development than in adult life. It may also be noted however that even in normal mice there are a few pancreatic endocrine cells that arise naturally in the extrahepatic biliary tract [14, 15].

Our first experience of generating ectopic pancreas, including beta cells, was in the frog *Xenopus*, which is an important model organism for studies of embryonic

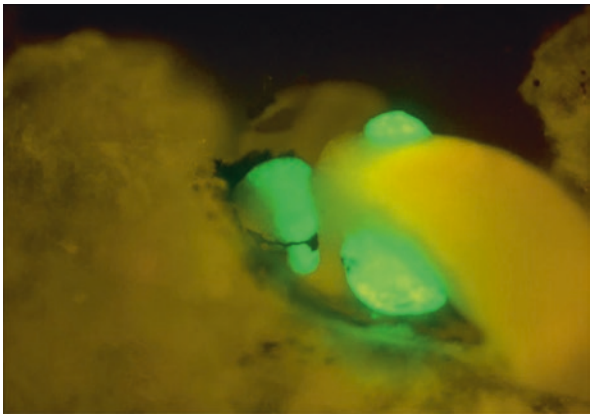

Fig. 3.1 Reprogramming of the *Xenopus* tadpole liver to pancreas. The tadpole (about 1 week old) is transgenic for *TTR-Xlhbox8VP16;elastase-GFP*. Pancreas is *green* because of the presence of the GFP marker. The two right hand zones are the normal dorsal and ventral buds. The left hand zone is part of the liver, now converted to pancreas

development. A transgenic construct was prepared containing the gene for an activated form of the transcription factor PDX1 (*XlHbox8VP16*) driven by a liver specific promoter (*TTR*). In the resulting tadpoles, the activated PDX1 was expressed shortly after the first formation of the liver and it caused the formation of ectopic patches of pancreas (Fig. 3.1 and [16]). This was an exciting result although later work showed that the conversion only worked when the transgene was expressed during the few days following liver bud formation and would not work in more mature liver.

We were encouraged to introduce the same reagent (*TTR-XlHbox8VP16*) into the HepG2 cell line, which is an established line derived from a human hepatoma [17]. Although the delivery method used at the time (transfection) was very inefficient, we did see the production of some insulin-positive cells, with some evidence for induction of endogenous *Pdx1* expression and glucose-stimulated insulin secretion.

We attempted to introduce a similar construct into adult mouse liver using both hydrodynamic gene delivery [18] and adenovirus [19]. However these experiments were unsuccessful. Although other labs had reported pancreatic gene expression in the liver following delivery of *Pdx1*, sometimes in combination with other beta cell genes [20–24], their results were hard to reproduce and our efforts eventually convinced us of two things. First, more than one gene was needed. Secondly, the process is very inefficient in adult cells because of the degree of condensation of the chromatin. In order to reprogram cells in the liver we felt it would be necessary to make the chromatin more active. Several of the published studies had used procedures causing some degree of liver damage which would have provoked this effect through induction of cell division.

We then carried out a screen of pancreatic transcription factors by transfecting groups of factors into cultures of primary rat hepatocytes and looking for expression of insulin. The most active combination turned out to be *Pdx1, Ngn3* and *NeuroD*. Unfortunately our screen was commenced before the important role of *MafA* in beta cell maturation had been recognised [25], so we had not included this gene. A simultaneous screen conducted by the Melton lab came up with the superior combination

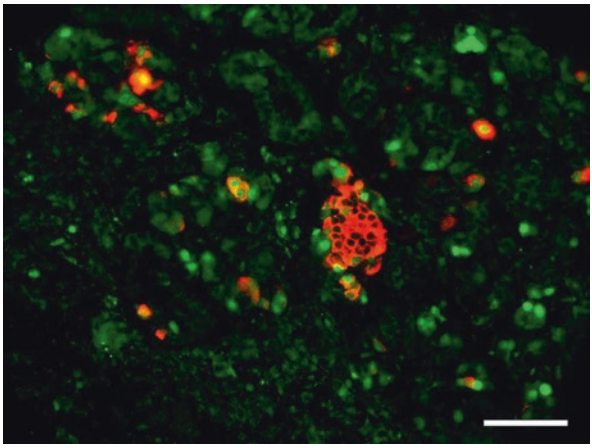

Fig. 3.2 Pancreatic exocrine cells reprogrammed to a beta-like phenotype. A mouse pancreas was transduced with adeno-PNM in vivo. The *green* fluorescence shows NGN3, one of the virus-encoded proteins, and the *red* fluorescence shows insulin. One normal islet is visible and the scattered fluorescent cells are the result of reprogramming. Scale bar 100 m

of *Pdx1, Ngn3* and *MafA* [26]. This combination is very logical: *Pdx1* (earlier called *IPF1*) is needed for growth and development of the early pancreatic buds [27]. *Ngn3* is needed for generation of endocrine precursor cells [28]. *MafA* is needed for beta cell maturation [25]. In our subsequent work we adopted this three gene cocktail introduced by Zhou et al.

We commenced investigation of the *Pdx1-Ngn3-MafA* gene cocktail (hereinafter called "PNM") by making a single adenoviral vector expressing all three. In this construct the coding regions are connected by 2A sequences which enable self-splicing of the nascent polypeptide into the three individual proteins. Transcription is driven by the strong *CAGS* promoter. This vector proved much more effective than the three separate single gene adenoviral vectors.

Zhou et al. [26] had introduced the three genes into exocrine pancreas of the mouse and claimed to generate ectopic beta cells. We confirmed that our adeno-PNM construct could indeed generate cells producing insulin from the exocrine pancreas (Fig. 3.2), but these did not have the appearance of real beta cells. A detailed characterization was made in vitro using the rat pancreatic exocrine cell line AR42j-B13 as the target cell type. This study showed that expression of PNM could generate a high proportion of insulin-positive cells, which ceased dividing and adopted a stellate appearance (Fig. 3.3). They expressed a range of typical beta cell genes, but did lack some, such as *Slc2a2* (*Glut2*) and *Kcnj11* (*Kir6.2*), both encoding essential components of the mechanism for glucose-stimulated insulin secretion. Consistent with this, the insulin-positive cells did not show an enhanced secretion of insulin following glucose challenge. Although the transformed cells ceased to divide they could survive for at least 4 weeks. But because the cells are not dividing the adenoviral DNA may well persist this long so it is not possible to say that this system exhibits genuine reprogramming.

In addition to the pancreatic exocrine AR42j-B13 cells, we also examined the effects of adeno-PNM on a variety of other cells in vitro [29]. Partial transformations were observed for rat (but not mouse) primary hepatocytes and for a line of mouse

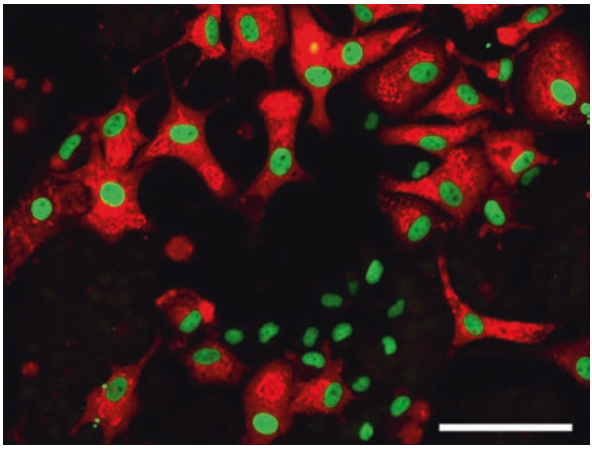

Fig. 3.3 Pancreatic exocrine cells reprogrammed to a beta-like phenotype. AR42j-B13 cells were transduced with adeno-PNM and consequently acquire a stellate shape and cease dividing. The *green* fluorescence shows PDX1, one of the virus-encoded proteins. The *red* fluorescence shows insulin. Scale bar 100 m

hepatocyte-derived cells. These both showed immunopositive insulin and upregulation of a variety of typical beta cell genes. However little or no effect was seen for a variety of fibroblast lines. These results are consistent with the presumption that developmentally related cell types are more easily reprogrammed. We also found that the proportion of insulin-positive cells could be increased by treatment with small molecules. The optimum combination consisted of three substances: BIX-01294 (a diazepinquinazolinamine derivative, which has a chromatin-opening activity) N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester (DAPT) (an inhibitor of Notch signaling), and 5′-N-ethylcarboxamidoadenosine (NECA) (an agonist of adenosine). These substances increased the proportion of cells responding but did not affect the spectrum of beta cell genes expressed, or the small degree of glucose-stimulated insulin release which was seen with adeno-PNM alone.

A consistent theme in direct reprogramming studies has been the greater lability of committed but immature cell types compared to fully differentiated adult cells. We examined the effect of adeno-PNM on cells from fetal mouse liver and found that the peak production of insulin-positive cells occurred at E12–14, declining to zero by birth [30]. The responding cells were judged to be hepatoblasts (progenitors of both hepatocytes and biliary epithelial cells [31]), based on their co-expression of *a*-fetoprotein and other markers. The fetal insulin-positive cells differed from those derived from cell lines in two respects: firstly they expressed all of the beta cell markers that were examined, including the glucose-sensing components. Secondly they co-expressed hormones characteristic of other pancreatic endocrine types, namely glucagon, somatostatin and peptide YY. The cells did not show consistent glucose-stimulated insulin secretion, and this, combined with the presence of multiple hormones, suggests that they resemble the multihormone endocrine cells which arise early in pancreatic development, rather than mature beta cells.

Because of the finding that mice lacking *Hes1* have ectopic pancreas in the extrahepatic biliary system [13], we also investigated the effect of inhibiting HES1

activity in biliary epithelial cell cultures [32]. In normal pancreatic development, *Hes1* transcription is stimulated by Notch signaling and the gene encodes a bHLH transcriptional repressor which acts to repress endocrine cell formation. We created a gene for a dominant negative form of HES1 by removal of its DNA binding domain and this was cloned into an adenoviral vector. Introduction into cultures of primary mouse biliary epithelial cells caused some synthesis of insulin RNA and protein, together with a battery of other beta cell genes. Some glucose-stimulated insulin secretion was observed. Effects were greater when the biliary cells were co-transduced with a virus encoding Pdx1.

3.3 Reprogramming in Adult Mice

With our initial in vivo experiments we had considerable difficulty repeating reports by other workers of the induction of pancreatic gene expression in the liver by administration of *Pdx1* or other beta cell genes [20–24]. In the end we concluded that success requires the right gene combination, very good quality high titer virus, and some stimulation of cell division in the target cell population. The last requirement arises from the fact that none of the factors used is a "pioneer factor", namely a transcription factor that can locate its target DNA sequences within inactive chromatin [33]. Cell division is favorable for reprogramming because the chromatin structure has to be opened to enable DNA replication and this provides an opportunity for transcription factors to recognize target sequences in inactive regions of chromatin. In fact the virus doses that can provoke a response on their own are very high and probably cause some modest liver damage with consequent cell division.

The agent we have used for enhancing the in vivo effect of adeno-PNM is WY14643 (pirinixic acid). This is an agonist of peroxisome proliferator activated receptors (PPAR) α and γ. It causes hyperplasia of the liver, stimulating both the hepatocytes and the biliary cells to divide (Fig. 3.4a). It is given orally to the mice and, if administered for a few days around the injection of adeno-PNM, it reduces the virus dose required for a given effect by a factor of about 20, compared with no WY feeding [34]. In these experiments mice are made diabetic by treatment with streptozotocin, a toxin which destroys mouse beta cells. A high dose (2×10^{10} pfu) of adeno-PNM, or a lower dose (1×10^{9} pfu) of adeno-PNM plus WY14643, will cure the diabetes and bring the blood glucose down into the normal range. However the effect of the high dose adeno-PNM alone is lost after a few weeks while that of the lower dose adeno-PNM + WY14643 persists long term.

In these experiments two distinct types of insulin-positive cell arise in the liver. First there are scattered cells within the liver parenchyma. These closely resemble the hepatocytes around them except for the presence of immunopositive insulin, for example they continue to express albumin (Fig. 3.4b). The number of these cells declines over several weeks, coincident with the decline of expression of GFP from viral vectors containing the *GFP* gene, and with the loss of blood glucose control in the adeno-PNM alone animals. Secondly there are structures resembling hyperplastic

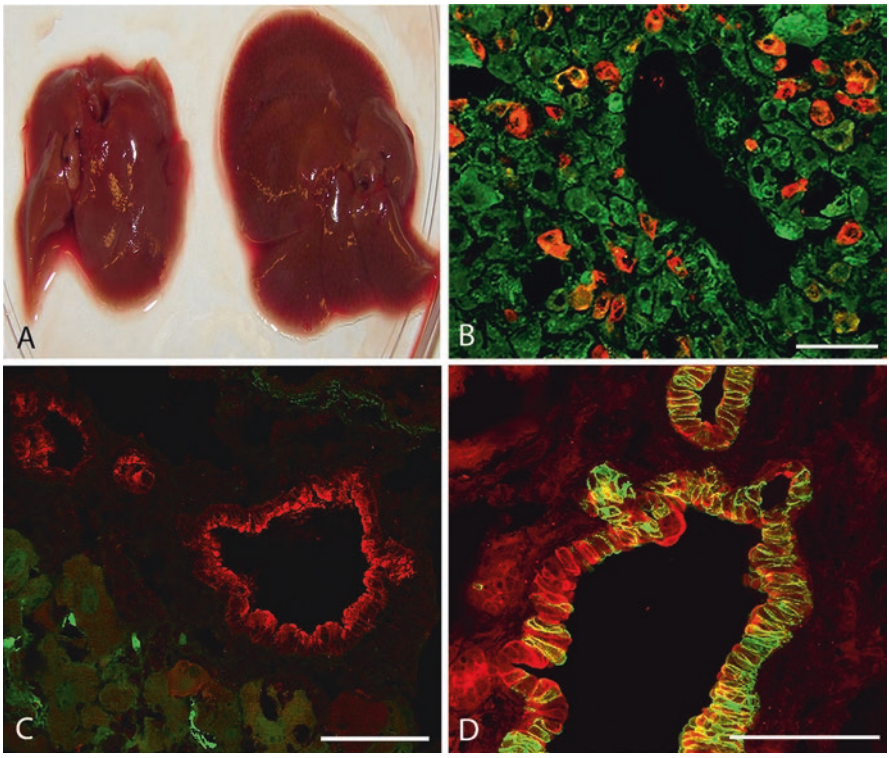

Fig. 3.4 Reprogramming events in the liver. (**a**). Liver hyperplasia caused by feeding with WY14643 for 6 days. (**b**). Scattered insulin-positive hepatocytes seen in a CD1 mouse treated with adeno-PNM and WY14643. Albumin is *green* and insulin *red*. (**c**). An insulin-producing hyperplastic duct seen in a NOD-SCID mouse treated with adeno-PNM. Here the viral vector also encoded GFP but little signal from it remains. Insulin: *red*, GFP: *green*. (**d**). Evidence that insulin-positive hyperplastic ducts arise from *Sox9*-positive progenitors. The mouse was *Sox9-CreER; mT/mG* and was treated with tamoxifen during fetal life to label *Sox9*-positive cells with GFP. In adulthood it was made diabetic and given adeno-PNM. Insulin is *red*, GFP *green*. Scale bars 100 μm

bile ducts which are insulin-positive as well as retaining expression of a number of ductal markers (Fig. 3.4c). These structures are more abundant in the adeno-PNM + WY14643 animals and they remain present as long as the experiments were continued (20 weeks). It is probable that both of these populations secrete insulin, as the glucose handling of the animals was considerably improved over controls both in the short term, when the scattered hepatocyte-like cells were abundant, and in the longer term, when the hyperplastic ducts were the predominant insulin-positive cells remaining. In these experiments no regeneration of beta cells in the pancreas was observed.

Although the use of WY14643 enables effective results to be obtained with CD1 mice, we had commenced these experiments using NOD-SCID mice. This was

because the original study on PNM by Zhou et al. had used immunodeficient mice (in their case a Rag⁻ strain). In fact NOD-SCID mice behave rather like CD1 mice given WY14643, in other words they are much more sensitive to the virus than normal mice. The reason for this is not known. It is probably not simply due to their immunodeficiency as the behavior could not be replicated by administering the immunosuppressive drug tacrolimus (a calcineurin inhibitor) to CD1 mice [34].

Using NOD-SCID hosts we were able to isolate insulin-positive cells from the livers of the treated animals by cell sorting following staining with Newport Green dye, which binds to the zinc associated with insulin crystals and fluoresces green [35]. This enabled more detailed study of the gene expression and physiology of the transformed cells. The cells expressed a wide range of beta cell genes, including endogenous *Pdx1* and those required for glucose sensing, but also genes for non-beta cell pancreatic endocrine hormones. Unlike many of the in vitro models described above, the cells showed good glucose-stimulated insulin release, comparable to that of control islets. The duct-derived cells also show the presence of dense core granules in transmission electron microscopy, a feature characteristic of beta cells.

The morphology and co-expression data made it likely that the hepatocyte-like cells derived from hepatocytes while the hyperplastic ducts derived from small intrahepatic bile ducts. Since it is the insulin-positive ducts that persist long term, and maintain normoglycemia long term in the treated animals, we made a cell lineage study using *Sox9-CreER; mT/mG* mice. SOX9 is a key transcription factor involved in the development of the biliary system [36]. The mice were dosed with tamoxifen during pregnancy to label *Sox9*-positive cells. This generated mice with the intrahepatic biliary system labeled with GFP. Following induction of diabetes with streptozotocin, and administration of adeno-PNM, the livers were examined. The insulin-positive ducts were also GFP-positive indicating that these structures indeed derived from the small intrahepatic bile ducts (Fig. 3.4d). We consider that the hyperplastic ducts are genuinely reprogrammed because in experiments where adeno-PNM-GFP was used the GFP signal is lost from the ducts after a few weeks indicating that the vector DNA is no longer present.

3.4 Other In Vivo Work

This review has focused on the work of my own lab. However there is of course much valuable work going on elsewhere.

Zhou and colleagues initially introduced the PNM cocktail in experiments on the exocrine pancreas in vivo [26]. The resulting insulin-positive cells were not permanent and did not provide complete rectification of diabetes. In subsequent work [37] they used a tricistronic vector, as we had, and found a higher percentage of acinar cells becoming transformed. In these experiments the insulin-positive cells acquired a gene expression profile close to beta cells over 2 months, and acquired glucose sensitivity by 4 months. They also aggregated into islet-like structures. At least

some of the mice showed a complete correction of induced diabetes. Interestingly, experiments using the same reagent on pancreatic duct tissue, rather than acinar cells, gave a less good response, without the acquisition of glucose sensitivity [38].

The Zhou group have also used a transgenic approach to explore the effect of PNM on other tissues [39]. A transgenic mouse strain (*TetO-PNM-Cherry; Rosa-rtTA*) was created in which PNM is expressed following exposure to doxycycline. Doxycycline activates the Tet activator (rtTA) which drives transcription from the *TetO* promoter. Cherry is a fluorescent protein which is more stable than GFP and is used as a marker. In these mice, following treatment with doxycycline, ectopic insulin production was found from cells in the intestine. The insulin-positive cells showed a transcription profile similar to beta cells, dense core granules visible in the electron microscope, and glucose-stimulated insulin release. Lineage labeling using *Ngn3-CreER* indicated that at least some of these beta-like cells arose from intestinal endocrine cells. NGN3 is an important transcription factor in the formation of both pancreatic endocrine cells and intestinal secretory cells, which include endocrine cells. Interestingly these beta-like cells can regenerate following STZ ablation as they arise from intestinal stem cells, whereas beta cells in the pancreas show very limited regeneration.

The Zhou group have focused on the role of the NGN3-expressing cells in an interesting study using a transgenic mouse with three components: *Ngn3-Cre; Rosa-rtTA; TetO-PNM-Cherry* [40]. In such mice, all cells expressing *Ngn3* will also express *Cre* and undergo DNA splicing to cause continuous subsequent expression of rtTA (the Tet activator). When treated with doxycycline the PNM-Cherry cassette is thereby expressed in *Ngn3*-positive cells. *Ngn3* is normally expressed in many cells of the developing nervous system and gastrointestinal system as it is involved in the differentiation of neurons in the CNS, and of various secretory cells, including endocrine cells, in the gut. Consistent with the non-pioneer status of the PNM factors, no effects are seen in the nervous system. But in the gut many insulin-positive cells appear. In the intestine these are not glucose-responsive, but in the gastric antrum they are glucose responsive and can ameliorate experimental diabetes. This may be associated with the fact that the gastric antrum, like the hepatopancreatic buds, arises from the region of the foregut expressing *Pdx1* in early development.

In addition to these results it has been found that ablation of the *FoxO* gene in cells expressing *Ngn3* (*Ngn3-Cre; floxed FoxO* mouse) can generate insulin-positive cells in the intestine that show many properties of beta cells including glucose-stimulated insulin secretion [41].

3.5 Conclusions

In conclusion, our own results suggest that the biliary system is a favorable target for reprogramming to beta cells. Cells exhibiting glucose-sensitive insulin secretion could be obtained both from the gall bladder cultures in vitro and from the duct

system of the mouse livers in vivo. The occasional presence of pancreatic endocrine cells, including beta cells, in the extrahepatic bile ducts of normal mice [14, 15] indicates a developmental propensity towards a beta cell fate. Work elsewhere has shown that pancreatic exocrine cells and endocrine cells of the GI tract may also be favorable targets [40].

For any work on reprogramming it is important to establish that genuine reprogramming has occurred, not just the upregulation of genes directly regulated by the transgene products themselves. The criteria for genuine reprogramming are generally considered to be as follows. Firstly the gene expression pattern should shift in its entirety from one phenotype to the other. Secondly the morphology and physiological behavior of the cells should match the new gene expression phenotype. Thirdly the change should be permanent and should not require the continued presence of the initiating transgenes. Although many claims have been made for cell type reprogramming provoked by selected transcription factors, detailed characterization has tended to show that few if any cases are really fully reprogrammed [42].

In our own studies none really correspond to complete reprogramming to beta cells except for the initial *Xenopus* experiment. However this involved very immature liver, corresponding to the hepatoblast stage of the mouse fetus. For the adult tissues, the best results arose from the reprogramming of small bile ducts. These did not generate beta cells, but did generate hyperplastic ducts expressing a repertoire of beta cell genes and showing glucose-stimulated insulin release. In terms of the third criterion, the permanence of the transformation in the absence of the initiating transgenes, this system also meets it. The fate of the administered adenovirus can be tracked using a construct expressing GFP in addition to the PNM factors. The GFP signal disappears after a few weeks and this time also corresponds to the loss of the scattered hepatocyte-like insulin-positive cells. We believe that these insulin-positive hepatocytes do require the continued presence of the transgenes whereas the hyperplastic ducts do not. Although the phenotype of the hyperplastic ducts differs considerably from that of beta cells, it should also be borne in mind that an incomplete transformation may still be of clinical value, so long as it is safe, permanent, and meets the clinical requirements of the situation.

In terms of future work, we consider that the PNM combination, useful though it is, is probably not optimal for reprogramming to beta cells. Some transcription factors are known as “pioneer” factors because they are capable of finding their target sites in the DNA even when they are buried in inactive(=“low signal”) chromatin [33]. The gene combinations used for other types of transformation, to cardiomyocytes, neurons or hepatocytes, include pioneer factors [43–45]. By contrast, the PNM combination does not include a pioneer factor. This is very obvious in experiments where adeno-PNM is administered to fibroblasts, or PNM expression is induced in Ngn3-positive cells of the nervous system, and virtually no response at all is seen. These tissues are far enough away from beta cells in developmental history for the key target genes to be in low signal chromatin and inaccessible to non-pioneer factors. We consider that inclusion of a pioneer factor in the cocktail would be desirable, and also probably some modification of doses and relative times of the factors.

In work of this type, reviewers generally insist on seeing animal rescue experiments in which diabetic animals have their blood glucose restored toward the normal range by the treatment. While important, this is not a very discriminating criterion. Any source of insulin, including injections of the protein or slow release pellets, will relieve experimental diabetes. This is why we have placed a greater weight on the ability of the transformed cells to exhibit glucose-stimulated insulin secretion, which is the behavior required for any potential clinical application.

In terms of clinical applications for diabetes, methods involving the reprogramming of cells have yet to be tested. At present the front running regenerative medicine solution for diabetes is the transplantation of beta cells made from pluripotent stem cells. A clinical trial was commenced in 2014 by the Viacyte company of implants containing pancreatic progenitor cells made from human ES cells. But so far no results have been released and it may be that different approaches, including in vivo reprogramming will eventually be tried.

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Chapter 4
In Vivo Lineage Reprogramming of Fibroblasts to Cardiomyocytes for Heart Regeneration

Li Wang, Jiandong Liu, and Li Qian

4.1 Introduction

Heart disease is the leading cause of adult mortality in the developed world and continues to be a heavy burden to health care systems [1]. Resulting from the limited regenerative capacity of adult cardiomyocytes, it's difficult for heart to functionally recover after lesions such as myocardium infarction (MI). The lost cardiomyocytes in the injured area are replaced by activated cardiac fibroblasts (CFs) that proliferate and secrete excessive extracellular matrix to form scar tissues and pathologically remodel the myocardium. Although recently studies showed that mammalian hearts possess modest self-renewal and turnover under certain scenarios [2–5], it is still insufficient to regenerate a damaged heart.

Recent development of direct reprogramming, which directly converts cells from one differentiated phenotype to another without transitioning through the intermediate pluripotent state, offers a promising alternative approach for regenerative medicine. A single or multiple transcription factors have been shown to drive cell fata conversion from fibroblast into neuron like cells, hepatocyte like cells and many other somatic cell types [6]. As for heart, the activated residential CFs upon injury could serve as an endogenous source of new CMs for regenerative purpose if they could be directly reprogrammed into functional CMs. Several groups have successfully converted fibroblasts in to induced CM-like cells (iCMs) using a cocktail of transcription factors that reside at top of developmental regulatory hierarchy for cardiogenesis, both in vitro and in vivo. Alternatively, combinations of small molecules and microRNAs have been developed to either directly reprogram or enhance

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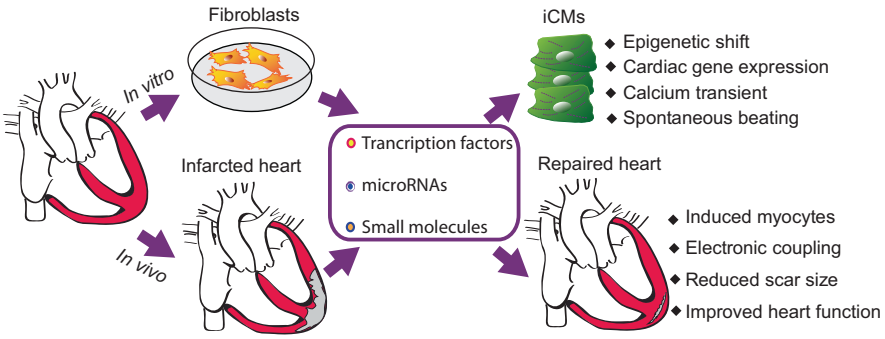

Fig. 4.1 Schematic of direct cardiac reprogramming in vitro and in vivo. Reprogramming factors including transcription factors, microRNAs and small molecules can be to delivered to cardiac or dermal fibroblasts in vitro, and can be transferred into infarcted heart to induce cell fate conversion

reprogramming of iCMs (Fig. 4.1). Now more effort has been taken into studying the mechanisms underlying this process. Here we will summarize current advances in direct iCM reprogramming and discuss its challenges and further applications for regenerative medicine.

4.2 Direct Reprogramming of Mouse Fibroblasts into iCMs

4.2.1 Transcription Factors

Starting from 14 transcription factors, Ieda et al. discovered that a specific combination of three factors, Gata 4 (G), Mef2c (M) and Tbx5 (T) (collectively referred to as GMT) was sufficient to transform mouse Thy1+ dermal or cardiac fibroblasts into iCMs [7]. The iCMs exhibit similar global gene expression and epigenetic imprinting as endogenous CMs, whereas the fibroblasts program is significantly repressed. Functionally, iCMs show calcium oscillation and spontaneous beating. Importantly, iCMs do not pass through a cardiac progenitor stage (in particular Mesp1+ and Isl1+ lineages), suggesting iCM generation is a direct conversion from one somatic cell type to another. In accordance with this observation, the fully reprogrammed iCMs morphologically and functionally resemble neonatal cardiomyocytes.

The following in vivo studies using genetic linage tracing strategies demonstrated the regenerative capacity of iCM reprogramming. It has been demonstrated that retroviral delivery of GMT after coronary ligation produced iCMs characterized with mature CM features including bi-nucleation, well-organized sarcomere structures as well as similar gene expression and electrophysiological properties [8]. Importantly, the in vivo reprogramming efficiency is much higher than the in vitro one, suggesting the environmental factors may contribute to the enhancement

of this conversion process. In vivo delivery of GMT also improved heart function, decreased infarction size and reduced fibrosis in mice with myocardial infarction [8]. Similarly, injection of GMT retroviruses into immunosuppressed mouse heart suffering MI resulted in newly emerged iCMs 2 weeks after surgery [9]. To overcome the disadvantages of the retro- and lenti-viral infection (integration and chronic expression), Mathison et al. generated replication-deficient adenovirus expressing GMT (Ad-GMT) [10]. These Ad-GMTs appeared to be as efficient as lentiviral GMT for rat iCM reprogramming both in vitro and in vivo [10].

In addition, other combinations of transcription factors have been reported to succeed in converting fibroblasts into iCMs. It has been shown that another transcription factor, Hand2, can function together with GMT (referred to as HGMT) to improve iCM reprogramming efficiency of adult fibroblasts in vitro and produce iCMs in vivo to attenuate heart dysfunction after myocardial injury [11]. In comparison with GMT, HGMT appears to generate diverse cell types including atrial, ventricular and pacemaker cardiomyocytes in vitro [12]. Protze et al. screened a pool of 10 transcription factors in MEFs and found another three factor combination (Mef2c, Tbx5 and Myocd) could induce iCMs with cardiac program and functionally these cells are more mature than GMT derived iCMs [13].

4.2.2 MicroRNAs

MicroRNAs (miRNAs) are a class of small noncoding RNAs of 21–25 nt in length that in general repress gene expression at the posttranscriptional level by degrading their target mRNAs and/or inhibiting their translation [14, 15]. MiRNAs play pivotal roles in governing gene expression during cardiovascular development and disease [16, 17]. For example, miR-1 was the first reported miRNA to be involved in regulation of heart development through targeting Hand2 [18, 19]. Recent studies imply additional import roles of miRNA in controlling cell fate conversion. Using combination of miRNAs, both mouse and human fibroblasts could be directly converted into induced pluripotent cells and neurons [20–22]. Based on the potential roles of miRNAs, Jayawardena et al. identified a combination of miRNAs 1, 133, 208, and 499 (referred to as miR combo) that are capable of inducing iCMs both in vitro and in vivo [23–25]. iCMs generated by miR combo are characterized with similar gene expression as endogenous CMs, spontaneous calcium flux and contraction. Mice harboring miRNA combo after MI showed newly derived iCMs originated from fibroblasts, and resulted improvement in cardiac function. JAK inhibitor I treatment further increased miR combo mediated iCM reprogramming efficiency [23]. Mechanistically, removal of tri-methylation of the lysine 27 of histone H3 (H3K27me3) is essential for miR combo to initiate the reprogramming [26]. Most recently, with the development of three-dimensional (3D) tissue-engineered cardiac hydrogel patches, miR combo directed iCM reprogramming was further enhanced with observation of strong environmental matrix metalloproteinases expression [27].

4.2.3 Small Molecules

Canonical reprogramming utilizes retroviral or lentiviral based strategies to deliver transcription factors in vitro and in vivo. Application of virus inevitably brings up the challenge of viral integration into the host genome and thus limits the clinical translation. An alternative approach is to use small chemical compounds, which are cell permeable, nonimmunogenic and could be easily handled for the delivery procedure to be standardized. Proof-of-concept studies have demonstrated that combinations of small molecules can replace master transcription genes to initiate iPS production [28]. With this concept, Want et al. demonstrated the transdifferentiation of mouse fibroblasts into cardiomyocytes with a single transcription factor Oct4 and a defined small molecule pool consisting SB431542 (ALK4/5/7 inhibitor), CHIR99021 (GSK3 inhibitor), parnate (LSD1/KDM1 inhibitor), and forskolin (adenylyl cyclase activator) [29]. Fu et al. developed a full chemical approach to generate chemical induced cardiomyocyte-like cells (CiCMs). Using compounds (CRFVPTZ (C, CHIR99021; R, RepSox; F, Forskolin; V, VPA; P, Parnate; and T, TTNPB) together with optimized culture medium, MEFs were amenable to become contractile cardiomyocytes [30]. Of note, different from GMT induced direct reprogramming, CiCMs pass through cardiac progenitor stage with high expression of progenitor markers Msp1 and Isl1 [29, 30].

4.3 Enhancement of Mouse iCM Generation

4.3.1 Optimization of Transcription Factors

Three transcription factors GMT are sufficient to induce cell fate conversion from fibroblasts to iCMs. Suffering from the relatively low efficiency and incomplete reprogramming, several studies aimed at improving reprogramming efficiency through harnessing the transcription factor pool. Addition of MYOCD and SRF alone or in combination with Mesp1 and SMARCD3 enhanced GMT activated basal cardiac gene expression, though no significant difference was observed in terms of myocyte functionality [31]. Taking advantage of a transgenic calcium florescent reporter system driven by cardiac specific Troponin T promoter, Addis et al. evaluated several transcription factor combinations for their capacity to produce functional iCMs [32]. Interestingly, they found that addition of Nkx2-5 to HGMT cocktail (referred to as HNGMT) resulted in highest reprogramming efficiency [32]. There are also studies attempting at modifying activity of one reprogramming factor Mef2c. MyoD was one of the skeletal muscle master genes which has been identified to transform fibroblasts into myoblasts [33]. Hirai et al. fused MyoD transactivation domain to Mef2c and demonstrated that chimeric Mef2c together with Gata4, Tbx5 and Hand2 (referred to as MM3-GHT) yields larger contractile

iCM clusters with shortened time window in comparison with traditional HGMT [34]. Alternatively, Abad et al. enhanced binding of Mef2c to the promoter region of cardiac genes, which also resulted in higher reprogramming efficiency [35].

4.3.2 Stoichiometry of Transcription Factors

The use of transcription factor cocktails raised a critical question on relationship between expression level of each exogenous factor and outcome of iCM conversion. During heart development, delicate regulation and dose-spatial-temporal balance of these transcription factors are required to initiate and maintain cardiac specification and differentiation properly [36–39]. To address this question, Wang et al. manipulated expression level of GMT using polycistronic constructs and showed distinctive protein expression based on splicing orders among identical self-cleaving 2A sequences. They further demonstrated that relative ratio of G, M, T protein was crucial for efficient iCM reprogramming. An optimal expression of GMT with relative high level of M and low levels of G and T achieved by using polycistronic MGT vector (hereafter refer to as MGT) significantly increased reprogramming efficiency and improved iCM quantity and quality in vitro [40, 41]. Moreover, in vivo MGT delivery generated more iCMs and further improved heart functions than traditional delivery of GMT separate viruses [42, 43]. Another two polycistronic constructs encoding GMT were also reported to improve in vivo reprogramming [9, 44]. These reports emphasized the importance of stoichiometric expression of transcription factors and established a single vector platform to facilitate consistent and reproducible iCM reprogramming and further moved the basic and translational research on iCMs.

4.3.3 Addition of Small Molecules

Enhancement of iCM reprogramming could be achieved through addition of small molecules and microRNAs to base transcription factor cocktails (Table 4.1). In brief, there are three main groups of the supplements. First group consists of proteins and peptides like Thymosin β4, Akt and growth factors such as VEGF and FGFs. Thymosin β4 is a natural peptide that has been implied to be critical for cardiac development and play cardio-protective roles upon heart injury [45–48]. Co-administration of thymosin β4 resulted in a better delivery of GMT to reactivated fibroblasts, hence increased iCM numbers and resulted in further functional improvement in ejection fraction and cardiac output in vivo [8, 49]. Another in vivo research demonstrated that preconditioning rats with VEGF promoted iCM generation and improved cardiac function after MI [50], suggesting the possible important role of angiogenesis for heart repair. In vitro, treatment of serum free

Table 4.1 Small molecules enhance transcription factors induced murine iCM reprogramming

Reprogramming factors	Supplements	Targets/function		Reference
GMT	Thymosin β4	Pro-angiogenic and fibroblast-activating peptide	In vivo	[8, 49]
GMT	VEGF	Increased angiogenesis	In vivo	[50]
GMT/MT	FGF2, FGF10 and VEGF	Activation of p38MAPK and PI3K/AKT pathways	In vitro	[51]
GHMT	Akt/protein kinase B		In vitro	[52]
MM3-GHT	GSK126	Ezh2 inhibitor	In vitro	[56]
MM3-GHT	UNC0638	G9a and GLP inhibitor	In vitro	[56]
HNGMT	SB431542	TGFβ inhibitor	In vitro	[57]
MGT	MM408, MI503	Mll1 complex specific inhibitor	In vitro	[58]
GHMT	DAPT	Notch inhibitor	In vitro	[35]
GMT	SB431542; XAV939	TGFβ inhibitor; WNT inhibitor	In vitro and In vivo	[53]
GMT	miR-133	Repress snail1	In vitro	[54]
GHMT	miR-1 and miR-133		In vitro	[55]
GHMT2m	Y-27632/Thiazovivin/ SR-3677; A8301	ROCK inhibitors; TGFβ inhibitor	In vitro	[55]

medium containing VEGF with FGFs leads to faster maturation of iCMs induced by GMT, possibly through activation of Akt [51]. Overexpression of Akt also led to more efficient generation of contractile iCMs [52]. Moreover, VEGF and FGFs can substitute Gata4 and contribute to direct iCM generation with M and T [51].

The second group consists of chemical compounds targeting epigenetic modifiers and signaling pathways that will be discussed in later section. Noticeably, almost all the drugs were tested in dish, only a very recent study from Srivastava group showed the applicability of small molecules to enhance in vivo reprogramming [53]. They first depicted the reinforcement of in vitro reprogramming by using TGFβ inhibitor and WNT inhibitor with GMT (referred to as GMTc) characterized with shortened duration and enhanced iCM quantity and quality. Mice exposed to GMTc developed smaller scar size, thicker re-muscularized myocardium and further improvement in heart function than GMT alone. At cellular level, the number of ex vivo isolated iCMs from GMTc group was five-fold higher than that from GMT group, and GMTc-iCMs are functionally closer to adult cardiomyocytes in terms of their electrophysiological properties [53].

The third group includes microRNAs with or without chemical compounds. Although miRNA itself could generate iCMs in vitro and in vivo [23, 24], addition of miRNAs to cardiac transcription factors enables higher iCM reprogramming

efficiency and better cellular quality. Ectopic expression of miR-133 alone with GMT increased beating iCMs by sevenfold and noticeably enhanced the speed (from 30 to 10 days) for iCM maturation [54]. Overexpression of miR-1 and miR-133 together with GHMT (referred to as GHMT2m) induced more matured iCMs that started to beat by day 8 [55]. In combination with these two miRNAs, ROCK inhibitor and/or TGFβ inhibitor converted fibroblasts into functional iCMs with the efficiency over 60% when quantifying the percentage of beating cells [55].

4.4 Molecular Mechanisms Underlying iCM Generation

4.4.1 Epigenetic Regulation of iCM

Epigenetic regulation plays fundamental roles in cellular specification and lineage commitment during development. Emerging evidence indicates that dysregulated epigenetic landscape contributes to cardiomyopathy and heart failure [59, 60]. Recent studies on cellular reprogramming also demonstrated the dynamic alternation of epigenetic modifications [61–64]. In the first iCM paper, Ieda et al. discovered that trimethylation of histone H3 at lysine 27 (H3K27me3), a commonly used marker to mark transcriptionally inactive chromatin, was significantly reduced at the promoter region of several cardiac specific genes in iCMs 4 weeks after GMT induction [7]. Whereas, trimethylation of histone H3 at lysine 4 (H3K4me3), which labels an open chromatin, was increased at the same promoter region in iCMs compared to fibroblasts. Liu et al. further analyzed the repatterning of H3K27me3, H3K4me3 at cardiac and fibroblast loci at the MGT mediated reprogramming day3 and day10 [65]. Loss of H3K27me3 at cardiac gene loci appeared as early as day3, suggesting the rapid suppression of fibroblast signatures and early activation of cardiac program. Furthermore, data from ChIP-Seq revealed that upon transduction of GHMT, H3K4 dimethylation (H3K4me2, a general marker of both promoter and enhancer regions [66, 67]) peak shifted from fibroblast toward myocyte status at reprogramming day 7, indicating the existence of epigenetic orchestration at gene regulatory regions during early phase of iCM reprogramming [55].

To explore the underlying mechanism and identify potential epigenetic barriers to iCM reprogramming, our lab performed the first loss of function screen with a shRNA pool consisting 35 components that were involved in chromatin remodeling and modification and identified several factors that could either facilitate or blunt iCM reprogramming [68]. In particular, Bmi1, an important component of the Polycomb repressive complex 1 (PRC1) [69, 70], functioned as a major epigenetic barrier at the early stage of iCM reprogramming. Bmi1 suppressed iCM reprogramming through direct binding to a battery of cardiogenic loci including Gata4, Nkx2.5, Isl1, Pitx2, Tbx20, and Hand2. Furthermore, we demonstrated that Bmi1 depletion could replace Gata4 and convert fibroblasts into iCMs together with a single vector encoding Mef2c and Tbx5.

Liu et al. adopted a gain-of-function approach and identified Men1 and Suv39h1 as epigenetic inhibitors of iCM reprogramming [58]. Men1 is an essential component of a MLL/SET1 histone methyltransferase (HMT) complex responsible for H3K4 methylation and H3K9 methylation [71–73]. Suv39h1 also mediates H3K9 methylation [71, 74]. Chemical inhibitors targeting MLL1 complex to repress H3K4 methyltransferase activity significantly enhanced reprogramming efficiency, indicating that Men1 regulate iCM generation through modifying H3K4m3 instead of H3K9m3.

Enhancer of Zeste Homolog 2 (Ezh2), a catalytic subunit of PRC2 complex for H3K27me2 and H3K27me3, behaved as one of the epigenetic barriers for MM3-GHT mediated reprogramming [56]. Exposure to Ezh2 inhibitor GSK126 resulted in a decrease of H3K27me3 and an increase of beating iCM clusters. Similarly, UNC0638, an inhibitor to G9a and GLP that mainly controls H3K9me and H3K9me2, led to a higher iCM reprogramming efficiency in association with lower level of H3K9me2 in iCMs [56].

Taken together, iCM reprogramming is largely guided by specific cardiac transcription factors and the associated chromatin modifiers to establish authentic myocyte cell fate in another distinct cell type.

4.4.2 Suppression of Fibroblast Program

During reprograming, transcription factors drive fibroblast toward a differentiated cardiomyocyte lineage. Genome wide transcriptome research demonstrated that iCM reprogramming requires depletion of the original fibroblast signatures and de novo establishment of myocyte programs such as the contractile machinery, sarcomere structures, high mass of mitochondria and the metabolic switches [35, 51–55]. Suppression of fibroblast program has been shown to fundamentally affect iCM reprogramming. Overexpression of miR133 with GMT repressed Snail1 to silence fibroblast signatures and activates cardiac programs [54]. Snail1 is one of the major mediators of epithelial-mesenchymal transition (EMT) that contributes to cardiac fibrosis [75, 76]. MiR133 directly targeted Snail1 for degradation and overexpression of Snail1 inhibited iCM reprogramming.

Accumulating studies revealed the pivotal role of TGFβ signaling pathway during iCM conversion [53, 55, 57]. TGFβ signals activate cardiac microvascular endothelial cells to undergo endothelial-to-mesenchymal transformation and contributes to cardiac fibrosis [77, 78]. TGFβ also behaves as a repressor for embryonic cells differentiation toward cardiomyocytes [79]. Generally, TGFβ superfamily members bind to TGFβ type II receptor, which subsequently recruits and triggers phosphorylation of TGFβ type I receptor. Phosphorylated type I receptor activates SMAD molecules and leads to formation of SMAD complex. Activated SMAD complex translocates into nucleus and interacts with other DNA binding factors, transcription factors, thus regulates the transcription of target gene [80]. One of the most commonly used TGFβ inhibitors is SB-431542 that selectively blocks the TGF-β

type I receptor including ALK4 and ALK5, as well as ALK7 [81]. Both Srivastava group [53] and Gearhart [57] group screened out this inhibitor for its application in iCM reprogramming, while Song group [55] identified another TGF-β inhibitor termed as A8301 that inactivates similar receptors for its use in enhancing iCM induction. Through disturbing TGFβ signaling with chemical inhibitors, all three researches achieved much greater reprogramming quality. Most iCMs generated with the help of TGFβ inhibitors were relatively more reprogrammed beating cells with transcriptome more similar to adult cardiomyocytes [53, 55, 57].

4.5 Direct Cardiac Reprograming in Human Cells

Compared to the rapid advances of murine iCM programming, generation of human iCMs in vitro is more complicated thus much delayed. Neither GMT nor GHMT was sufficient to induce human iCMs [82–84]. Screening additional transcription factors finally led to successful induction of cardiomyocyte-like cells from human fibroblasts. Fu et al. discovered that the combination of GMT with ESRRG, MESP1 was sufficient to turn on cardiac specific markers in transduced human fibroblasts. Addition of myocardin and ZFPM2 further enhanced the reprogramming and resulted in iCMs exhibiting calcium flux and action potential [82]. More recently, with the help of two chemical inhibitors (TGFβ inhibitor and WMT inhibitor), the seven transcription factors (7c) induced reprogramming was further accelerated [53]. In addition, the 7c cocktail could be cut down to a four-factor recipe (GMT plus myocardin) with the two inhibitors, indicating the critical role of TGFβ and WNT signaling for human iCM reprogramming. Wada et al. showed that addition of Mesp1 and Myocd to GMT (referred to as GMTMM) cocktail transformed HCF (human cardiac fibroblasts) and HDF (human dermal fibroblasts) to iCMs that expressed a broad panel of cardiac markers, exhibited calcium oscillation and contracted synchronously when co-cultured murine primary CMs [84]. Later they demonstrated that miR-133 mediated snail1 inhibition in human fibroblasts is as important as that for mouse iCM reprogramming. Inclusion of miR-133 or snail1 depletion promoted GMTMM induced human iCM reprogramming [54]. Nam et al. showed that combination of GHMT with myocardin generates few beating cells after 11 weeks in culture [83]. Addition of miR590 to GHMT with myocardin upregulated cardiac gene expression and further suppressed fibroblast marker genes by directly inhibition of Sp1 (specificity protein 1) expression [85].

Generation of expandable cardiac progenitor cells (CPC) from fibroblasts shed lights on the production of CMs through de-differentiation. CPCs can differentiate into three major cell types of heart- endothelial cells, CMs and smooth muscle cells [86, 87]. Transcription factors ETS2 and MESP1 have been reported to transdifferentiate HDF into cardiac progenitors [88]. Lalit et al. reprogrammed adult mouse fibroblasts into induced CPCs (iCPCs) with a cocktail of five transcription factors (Mesp1, Tbx5, Gata4, Nkx2.5, and Baf60c) with two compounds (BIO and LIF) [89]. iCPCs were capable of proliferation and differentiation into all three cell types

and generated new myocardium post MI [89]. In comparison, Zhang et al. transiently expressed four Yamanaka factors (Oct4, Sox2, Klf4 and c-myc) in fibroblasts, cultured these primed cells in conditioned medium and ended with acquisition of iCPCs [90]. However, the precise outcome of the final differentiation is difficult to be controlled, with the likelihood of contamination of cells from non-cardiac lineages. Independent of transcription factors, Cao et al. demonstrated that 9 chemical compounds, called 9c, successfully transformed human foreskin fibroblasts to cardiomyocyte like cells [91]. The chemically induced iCMs (ciCM) sequentially expressed mesoderm, CPC and CM genes and eventually became spontaneously beating cells. It appears that ciCMs acquire similar transcriptional and epigenetic signatures, as well as functional properties that are similar to human CMs.

In summary, current studies (summarized in Table 4.2) paved a great foundation for future translational applications that require generation of mature human cardiomyocytes from different resources. Induction of human iCMs takes longer time and requires more factors; in addition human iCMs are far less mature, all of which suggest the need of further refinement from laboratory work. Research using cells from large animals in particular non-human primates could serve as an alternative to study the combination of factors with small molecules on the outcomes of iCM reprogramming, the outcome of which may facilitate the ultimate goal of effectively generating human iCMs.

4.6 Conclusions and Perspectives

Direct cardiac reprogramming holds great potential for regenerative medicine by offering an alternative strategy for treatment of heart disease and disease modeling. Recent studies indicated that the reprogramming efficiency is steadily increased by utilizing multiple strategies and through understanding the molecular mechanisms. Although it has been progressed rapidly, there are still challenges for this field.

First, the reprogramming efficiency is still low and varies between labs. The majority of reprogramed cells are not functionally fully matured cardiomyocytes. It's undoubtedly required to further optimize the platform and remove the molecular barriers so as to obtain sufficient highly matured iCMs for drug screening and disease modeling. One of the intriguing areas is to identify the contributing factors by evolving "omics" technologies, such as genomics, transcriptomics, proteomics and metabolics, which would reveal the genetic and epigenetic regulation, protein interaction network and metabolite profiles. Interrogating data from these techniques can help to understand more thoroughly about the events and mechanisms underlying the iCM reprogramming. Recent single cell RNA sequencing analysis (scRNA-seq) offers another opportunity to dissect the reprogramming trajectory, profile dynamic gene expression and identify cell fate determinants. Researches from Treutlein et al. set an example as how to use scRNA-seq to gain mechanistic understanding for induced neuron reprogramming [93].

Table 4.2 Direct programming of human fibroblasts into iCMs

Transcription factors	microRNAs	Supplements	Cell source	Phenotypes	Reference
GMT, ESRRG, MESP1, Myocardin, ZFPM2			Human fibroblasts derived from ESCs, HDFs and HCFs	Around 10% cTnT+ for ESC derived fibroblasts; around 4% with HDF, calcium transients in 4 weeks and action potentials in 10 weeks	[82]
GHMT, myocardin			Neonatal HFF	Around 20% cTnT+, beating in 11 weeks	[83]
GHMT, myocardin	miR-1 and miR-133		HFF, adult HCFs and HDFs	Around 10% cTnT+ for HCFs and around 4.4% cTnT+ for HDFs(2 week), calcium transients in 8 weeks	[83]
GHT, myocardin	miR-1, miR-133		HFF, adult HCFs and HDF	Around 13% cTnT+ for HCFs and around 9.5% cTnT+ for HDFs(2 week); around 35% cTnT (4w) for HFF	[83]
GMT, Mesp1, Myocd			HCF and HDFs	Around 5% cTnT+; calcium transients in 4 weeks; contact with co-cultured CMs	[84]
GMT, Mesp1, Myocd	miR-133		HCFs	23–27% cTnT+	[54]
GHMT, Myocardin	miR-590		HCFs	Around 6% cTnT+	[85]
GMT, Myocd, NKX2-5	miR-1, miR-133	JAK1i, GSK3i; IGF1 and NRG	HDFs	Calcium transients in 1 week	[92]
GMT, ESRRG, MESP1, Myocardin, ZFPM2		TGFbi (SB431542) and WNTi (AXV939)	Immortalized HCFs	Around 12% cTnT+, calcium transients in 10 days	[53]

(continued)

Table 4.2 (continued)

Transcription factors	microRNAs	Supplements	Cell source	Phenotypes	Reference
GMT, Myocardin		TGFbi (SB431542) and WNTi (AXV939),	Immortalized HCFs	Around 12% cTnT+, calcium transients in 10 days	[53]
		CHIR99021, A83-01, BIX01294, AS8351, SC1, Y27632, OAC2, SU16F and JNJ10198409	HFFs	Around 6.6% cTnT+ at day30, form beating clusters	[91]

Second, most studies used virus-based strategies to deliver reprogramming factors, which inevitably raises up the safety issues such as genomic integration and subsequent tumorigenesis [6]. To address this concern, safer delivery vectors, such as bio-safe Adeno-associated virus (AAV) based vectors [94], could be developed and optimized of reprogramming. Moreover, the use of small molecules would be another promising way for the clinical application of iCM reprogramming.

Last but not least, direct in vivo reprogramming yields iCMs at higher efficiency, better quantity and quality than iCMs generated from in vitro [8], clearly suggesting the fundamental effective amelioration of environmental niches. Mechanical forces, inflammatory responses, angiogenesis and extracellular matrix could be potential contributors for iCM maturation. Studies using growth factors and small peptide to mimic the environmental changes showed the enhancement of iCM reprogramming, however, the detailed mechanism remains unclear [8, 49, 51]. Identification and understanding the role of these environmental factors could further benefit iCM production and harnessing this approach for regenerative purpose. An alternative approach to identify microenvironmental clues is the application of recently advanced bioengineered materials. Biomaterials have been shown to influence cell fate and behavior through mutual interaction between cells and their environment [95]. Manipulation of the biophysical and biochemical properties of certain biomaterials leads to an improvement of iCM reprogramming [27, 96–98], indicating that it can be used to engineer the niches and realize the controllable release of reprogramming factors and small molecules in situ for in vivo reprogramming. One more consideration for experimental biologists is the utilization of large animal species for in vivo iCM reprogramming, such as the porcine model that raises exciting prospect for future iCM based therapies. Compared to murine models, pigs are anatomically and physiologically more similar to humans in cardiovascular, skeletal muscle, immune, and metabolic systems. It can not only solve the aforementioned biosafety issues and explore environmental niches, but also allows researchers to overcome the possible adverse effects of arrhythmia caused by newly formed iCMs in the scar region of myocardium. In particular, efficient and improved genetic engineering

approaches for pigs are now available, facilitating the establishment of tailored animal models for mimicking human diseases.

In summary, direct cardiac reprogramming converts injury-activated fibroblast into terminally differentiated cardiomyocytes in situ, holding tremendous potential for healing the injured heart. After better understanding the molecular mechanisms and overcoming the obstacles discussed, we anticipate that we can ultimately harness the iCM reprogramming and translate it to mend the broken heart.

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Chapter 5
In Vivo Cell Reprogramming to Pluripotency

Irene de Lázaro

5.1 Introduction

Pluripotency is defined as the capacity of an undifferentiated cell to differentiate into cell types representative of all three germ layers (endoderm, mesoderm and ectoderm) that form embryonic and, eventually, adult tissues. In the mammalian organism and under normal circumstances, pluripotent stem cells are only found at early developmental stages, primarily in the inner cell mass (ICM) of the blastocyst, from where the so-called embryonic stem cells (ESCs) can be isolated and established in culture. As development continues, differentiation potential is progressively lost, and the majority of cells in the adult organism are terminally differentiated to perform specific functions within the tissue hierarchy. Although some tissues count with specific populations of stem cells to maintain tissue homeostasis, those are multi- or oligopotent (i.e. can only differentiate into a few cell types, most commonly within the same lineage), and no pluripotent cells remain naturally in the adult [1].

It is precisely their ample differentiation potential that has attracted great interest in pluripotent stem cells. Since they can be re-differentiated into specific progenitor and mature cell types in the laboratory, they are seen as invaluable research tools in developmental biology and to investigate cellular responses to drugs and other substances. More importantly, they are considered by many the "holy grail" in regenerative medicine, since they could potentially serve as starting source to generate new cells that replace those lost due to tissue injury or degeneration in a variety of conditions [2]. For all such reasons, the generation of pluripotent stem cells has been largely pursued and explored in the cell culture laboratory. By contrast, the

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generation of pluripotent stem cells directly in vivo has only recently seen the first proof-of-principle studies although, partly thanks to the knowledge generated in the culture dish, it is rapidly advancing to unveil its potential applications.

5.1.1 Sources of Pluripotent Stem Cells: From ESCs to Induced Reprogramming

Pluripotent stem cells, in particular ESCs, can be isolated from the ICM of mouse [3, 4] and human [5] blastocysts, and maintained as such in culture [6] (Fig. 5.1a). However, ethical implications brought by the destruction of embryonic material, as well as tight regulations that restrict their use and the capacity to fund and patent research in which ESCs are involved [7, 8], have prompted the search for less controversial sources of pluripotent stem cells (Fig. 5.1). Thanks to John Gurdon's pioneering work on nuclear transfer [9–11] and Davis' and Weintraub's discoveries on the roles of transcription factors in cell fate decisions [12, 13], among many others, it is now understood that cell differentiation is not driven by irreversible changes to the genetic information of the cell, but by changes to the epigenetic landscape that can be reverted. Therefore, terminally differentiated cells retain all genetic information necessary to recapitulate the development of an entire organism. The differentiated status of adult cells is stable, but not irreversibly fixed, and it can be "reprogrammed" back to pluripotency as long and the appropriate switches are activated. Somatic cell nuclear transfer (SCNT), a process in which the nucleus of a somatic cell is introduced in an enucleated egg [14], has been one of the most popular approaches to achieve the pluripotent conversion (Fig. 5.1b). The oocyte contains molecular cues able to epigenetically reprogram the somatic nucleus back to pluripotency. The reprogrammed nucleus is in turn able to support the development of an entire new adult organism, and this technique is behind popular breakthroughs such as the first cloning of an adult frog [11] and the birth of Dolly the sheep [15]. Pluripotent stem cells have also been obtained in the laboratory by fusion events between somatic and embryonic carcinoma cells (ECCs), which are pluripotent cells isolated from germ cell tumors (Fig. 5.1c) [16]. However, none of these approaches is devoid of technical hurdles. Indeed, it took more than 20 years to adapt the SCNT protocol from amniotes to mammalian cells (sheep), and even harder efforts have been required to translate it to human cells, a procedure which is still today technically daunting [17, 18]. In addition, the specific molecular mechanisms and factors driving the conversion to pluripotency were never elucidated in the above studies.

Precisely aiming to identify the molecular cues that trigger and orchestrate the induction of pluripotency, Yamanaka and Takahashi embarked on the ambitious challenge to screen a pool of transcription factors, all present in the oocyte and/or in ESCs and many of them with already-known roles in the maintenance of pluripotency. In a groundbreaking study published in 2006, which was awarded the 2012 Nobel Prize of Physiology and Medicine together with John Gurdon's contributions

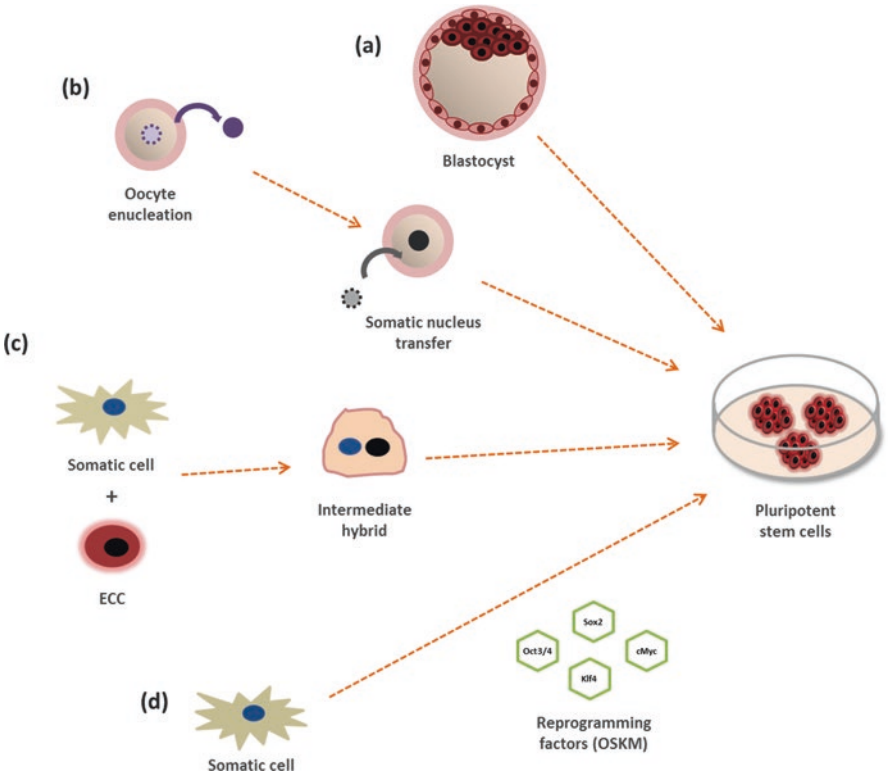

Fig. 5.1 Sources of pluripotent stem cells. Several strategies can be utilized to isolate naturally-occurring pluripotent stem cells or generate them artificially from other cell sources. (**a**) Naturally-occurring ESCs can be isolated from the ICM of the blastocyst and maintained in culture. (**b**) Pluripotent stem cells can be generated via transfer of a somatic cell nucleus into an enucleated oocyte. Factors present in the oocyte reprogram the somatic nucleus to a pluripotent state able to support the development of a whole adult organism. This procedure is known as Somatic Cell Nuclear Transfer (SCNT). (**c**) Fusion of a somatic cell with a pluripotent ECC generates an intermediate hybrid in which the pluripotent phenotype eventually outweighs the differentiated state. (**d**) Somatic cells can be reprogrammed to the pluripotent state via overexpression of defined transcription factors (*Oct3/4, Sox2, Klf4, c-Myc,* OSKM), generating so-called iPS cells

to the field, they identified a cocktail of four transcription factors (*Oct3/4*, *Sox2*, *Klf4* and *cMyc*, also known as OSKM or Yamanaka factors) as the most efficient combination to reprogram mouse embryonic and adult fibroblasts back to a pluripotent-like state (Fig. 5.1d) [19]. Their protocol was later refined to generate *bona fide* pluripotent cells that contributed to all adult tissues in murine chimeras [20], while the same and similar combinations of transcription factors (i.e. substituting KLF4 and cMYC by NANOG and LIN28) were shown to induce pluripotency in human cells [21, 22]. The resulting cells were named induced pluripotent stem (iPS) cells and have completely revolutionized the fields of stem cell research and regenerative medicine.

First, the implications of Yamanaka and Takahashi's work are huge for the efforts in understanding the biological mechanisms behind the process of reprogramming to pluripotency, and have opened the doors to extensive research in this topic [23, 24]. In addition, identification of the OSKM cocktail has also provided a relatively straightforward as well as versatile "recipe" to generate pluripotent stem cells in the laboratory. Indeed, iPS cells have been generated from a wide variety of terminally differentiated cell types, with little or no variation in the combination of transcription factors and experimental protocols utilized, of which the most popular nowadays are skin fibroblasts and peripheral blood cells [25–29]. The use of those as starting cell types avoids the ethical and legal hurdles associated to the use of embryonic material, while it allows derivation of patient-specific iPS cells through minimally invasive procedures, such as a simple skin biopsy or a blood test. Obtaining iPS cells from individuals suffering from a particular disease has opened tremendous opportunities to study the evolution of the specific condition through different developmental stages, as well as to interrogate responses to drugs and treatments [30]. In the context of regenerative medicine, the possibility to generate replacement cells from the very same patient in need for the transplantation is thought to diminish the chances for graft rejection [31].

5.2 iPS Cells Escape the Culture Dish. Proof-of-Principle Studies of In Vivo Reprogramming to Pluripotency

The first years after Yamanaka and Takahashi's groundbreaking study focused primarily on (1) the optimization of the reprogramming protocol to increase its efficiency and ensure reprogramming to ground-state pluripotency, (2) further elucidation of the mechanisms behind reprogramming, (3) the characterization of genomic integrity and stability of iPS cell clones and their direct comparison to ESCs and (4) the establishment of specific protocols to derive a plethora of differentiated cell types from patient-specific iPS cells, to be tested as disease models and in cell therapies. All of such studies were performed in the culture dish. However, the question whether the OSKM cocktail would be able to induce adult cells to pluripotency in vivo remained unanswered, in spite of undeniable interest from the mechanistic point of view and the obvious implications in regenerative medicine that will be discussed in Chap. 6.

In the culture dish, the induction of reprogramming takes place under defined and controlled conditions that are set to promote and maintain the pluripotent state. For example, mouse embryonic fibroblasts (MEFs) are used as feeder cells in many iPS cell generation protocols [19]. Leukaemia inhibitor factor (LIF) [6] is also supplemented in the cell culture medium to avoid differentiation and maintain pluripotency. In certain reprogramming protocols, additional substances are required to reach ground-state pluripotency. GSK3 and Mek1/2 inhibitors—widely known as 2i conditions—have been used to force partially reprogrammed intermediates, unable

to differentiate into all three lineages, to ground-state pluripotency [32]. On a very different picture, the induction of pluripotency directly in vivo cannot benefit from such controlled and pluripotency-favorable conditions, but has to overcome the effect of pro-differentiation signals naturally present in the tissue microenvironment. This was therefore the first uncertainty on the feasibility of in vivo reprogramming to pluripotency.

More importantly, the fact that pluripotent stem cells have both the capacity to proliferate actively and to differentiate towards all developmental lineages and tissue types adds an additional concern and questions whether generating cells with such capabilities within the living organism would be safe. In fact, previous experience in the transplantation of ESCs and iPS cells confirmed that the administration of pluripotent cells in vivo can generate teratomas, tumors composed of cells from all three germ layers [33, 34]. Indeed, the teratoma formation assay is routinely used as a tool to assess pluripotency [35].

In spite of these initial doubts, the field jumped its way onto the in vivo scenario in the early 2010s with two independent studies that used episomal plasmid DNA (pDNA)—in pre-metamorphic tadpoles [36] and mice [37, 38], respectively—to demonstrate that in vivo overexpression of reprograming factors can outweigh the pro-differentiation signals present in the tissue microenvironment and reprogram cells back to pluripotency. Since then, the number of strategies to induce pluripotency in various different tissues via OSKM overexpression has grown, but the outcomes of such studies differ significantly depending on the duration of OSKM expression, which will be discussed in Chap. 6 of this book. For a summary of the studies on in vivo reprogramming via OSKM overexpression see Table 6.2 in Chap. 6 of this book.

5.2.1 *In Vivo Reprogramming to Pluripotency in the Developing Tadpole*

The first proof-of-principle study to demonstrate that cells can be reprogrammed to pluripotency in vivo was performed in pre-metamorphic tadpoles (Fig. 5.2) [36]. Direct intramuscular injection of a reprogramming pDNA encoding three of the Yamanaka factors (*Oct3/4*, *Sox2* and *Klf4*, with the absence of *c-Myc*) in the tail muscle generated clusters of highly proliferative cells that showed several hallmarks of pluripotency. Among them, expression of endogenous pluripotency markers, upregulation of key epigenetic and chromatin remodeling markers and high alkaline phosphatase (AP) activity. Beyond their molecular signature, the capacity of the reprogrammed cells to differentiate into different lineages was tested both in vivo and in vitro. When left to re-differentiate within the tissue, they gave rise to neuron-like cells, representative of the ectoderm lineage. Transplantation of in vivo reprogrammed cells generated in pCar-GFP transgenic tadpoles into wild-type (WT) counterparts confirmed the capacity of such reprogrammed cells to re-differentiate

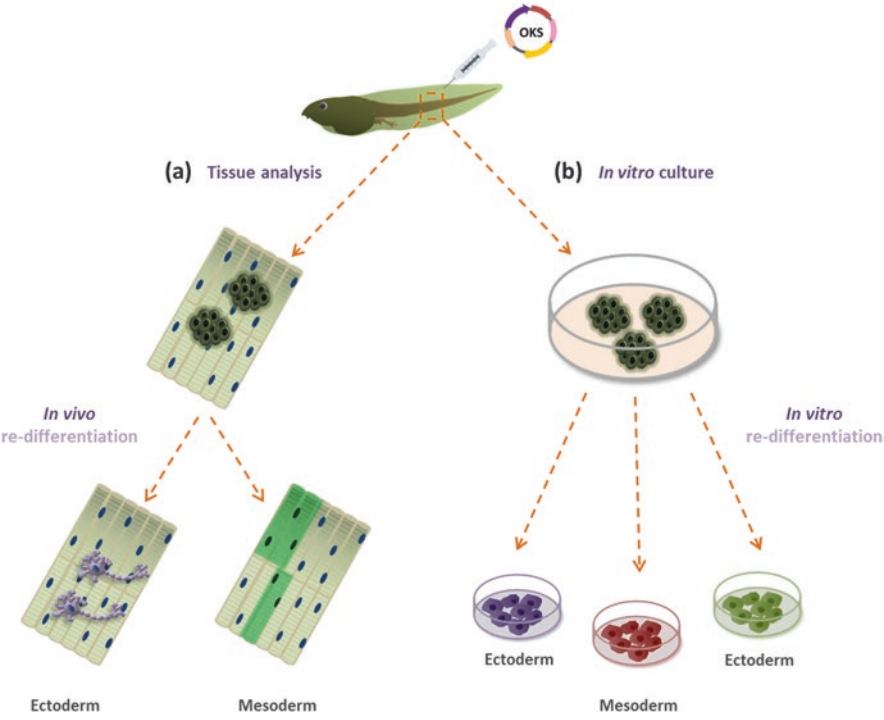

Fig. 5.2 Proof-of-principle of in vivo reprogramming to pluripotency in pre-metamorphic tadpoles. (**a**) Direct intramuscular administration of pDNA encoding OSK reprogramming factors generated highly proliferative cell clusters within the muscle tissue that showed several hallmarks of pluripotency and re-differentiated spontaneously into representatives of ectoderm and mesoderm lineages. (**b**) Reprogrammed muscles cultured in vitro generated cell colonies that morphologically resembled those of iPS and ESCs and whose differentiation could be directed to all three embryonic lineages (ectoderm, mesoderm and endoderm) [36]

towards the mesoderm lineage and generate new muscle fibers. Notably, both events took place even without the administration of growth factors that favor differentiation towards specific lineages. In vitro culture of OSK-injected muscles gave rise to cell colonies with morphology similar to that of ESCs, and that showed high AP activity. The differentiation of such cells could be directed to all three germ layers, which confirmed reprogramming to a pluripotency.

Within reprogrammed tissues, the morphology of the observed cell clusters was also very different from that of the regular muscle fibers. Nuclei were disorganized in such structures and significantly smaller than those in differentiated myofibers. However, proliferation was only observed within the clusters by PH3 expression for 14 days after injection and, in fact, the number and size of cell clusters peaked at day 7 to then decrease progressively, confirming that the reprogrammed cells did not persist as proliferative clusters within the tissues. An increase in the number of apoptotic cells was found in reprogrammed tissues at precisely the same time

when clusters started to disappear. However, based on the results on cellular re-differentiation towards the ectoderm and mesoderm lineages mentioned above, it is likely that at least a percentage of the reprogrammed cells survived but re-differentiated spontaneously in response to the pro-differentiation signals present in the tissue. Importantly, the expression of reprogramming factors (OSK) was only detected for 7 days after pDNA injection. The impact of the duration of OSK expression in the fate of reprogrammed cells will be further discussed in Chap. 6.

Overall, this study demonstrated that pluripotency features can be artificially re-induced in a tissue within a developing living organism. Proof-of-concept in the fully developed adult is presented in Sect. 5.2.2.

5.2.2 *In Vivo Reprogramming to Pluripotency in Adult Mouse Liver*

Similar episomal pDNA cassettes (with the addition of *c-Myc*) were used to demonstrate that the pluripotent conversion can indeed take place in the adult, fully developed, mammalian organism [37, 38]. The mouse liver was the organ selected to direct such investigations, given the relative high efficiency of pDNA transfection that can be achieved via hydrodynamic tail vein (HTV) administration. This technique, which has been extensively exploited in the gene therapy field to specifically transfect hepatocytes, consists in a rapid intravenous injection of a large volume of fluid (10% of the body weight) containing the pDNA of interest [39]. Administration of such a large volume in only 5–7 s generates a transient heart failure that provokes the reversion of all the administered fluid to the hepatic circulation. The mechanisms by which pDNA is taken up by hepatocytes are not fully elucidated. Some studies point at a receptor-based mechanism, while others suggest the opening of pores in the hepatocyte membrane given the sudden influx of fluid. In addition, such a rapid cellular internalization of the nucleic acid avoids degradation driven by circulating nucleases and thus also contributes to the high levels of transgene expression achieved [40, 41].

Interestingly, very similar observations to those reported in pre-metamorphic tadpoles were made in the mouse study (Fig. 5.3), in spite of the significant differences between species and developmental stages involved in both models. In brief, clusters of cells expressing endogenous pluripotency markers appeared promptly in the liver after HTV injection of reprogramming pDNA. Moreover, the expression of some hepatocyte-specific markers was downregulated in reprogrammed tissues, which further indicated transient de-differentiation and loss of the specific cell phenotype [37]. Importantly, cells directly isolated from in vivo reprogrammed livers demonstrated potential to re-differentiate towards all three embryonic lineages when injected in the dorsal flank of immunocompromised mice. In such an environment, they generated teratomas [42, 43]. Since no ex vivo manipulations or exposure to LIF or other molecules that could favor the pluripotent conversion were used

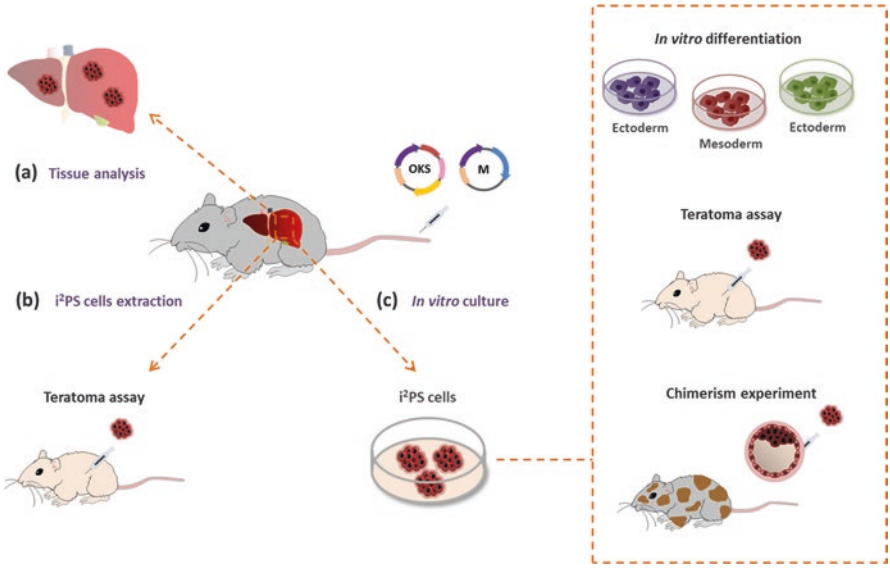

Fig. 5.3 Proof-of-principle of in vivo reprogramming to pluripotency in an adult mammalian tissue (mouse liver). (**a**) HTV injection of reprogramming pDNA encoding OSKM transcription factors generated clusters of reprogrammed cells within the liver tissue, which expressed pluripotency markers and showed signs of hepatocyte de-differentiation, but which did not persist in the tissues for prolonged periods of time [37]. (**b**) Direct injection of cell suspensions extracted from in vivo reprogrammed mouse livers in the dorsal flak of nude mice generated teratomas, which confirmed the presence of cells with pluripotent potential within the reprogrammed tissue [37]. (**c**) In vivo induced pluripotent stem (i²PS) cells were extracted from reprogrammed livers and maintained in culture. Several studies confirmed *bona fide* pluripotency, among them: in vitro spontaneous differentiation towards the three embryonic lineages, teratoma assay and generation of chimeric mice via blastocyst injection [43]

prior to implantation, this study confirmed that the pluripotent conversion took place within the tissue, and that the microenvironment of adult mammalian tissues is indeed permissive to epigenetic reprogramming to pluripotency. On the contrary, no teratomas were observed in the liver or any other organs of reprogrammed mice, monitored for a period of up to 120 days [37]. Similarly to what was observed in the tadpole, the expression of reprogramming factors and pluripotency markers was only detected for a few days, after which their levels reached again those observed in the control non-reprogrammed group, and the distinct clusters of pluripotent-like cells in the tissue were observed only up to day 4 after HTV administration. It is therefore possible that pluripotency is achieved in vivo via OSKM overexpression, but not maintained unless the reprogrammed cells are removed from their native microenvironment. The interactions between reprogramming and different pro-differentiation signals present in different tissues, those which the reprogrammed cells originally belonged to or not, are of great interest to further investigate in vivo reprogramming. However, given the very infant stage of the field at the moment, such interactions are still largely unexplored.

Cells directly reprogrammed in the mouse liver can also be isolated and established in culture. When maintained under standard ESC culture conditions, they form dome-shaped compact colonies with very prominent nuclei that morphologically resemble ESC and in vitro generated iPS cells colonies. They are termed in vivo induced iPS cells (i^2PS cells) in reference to their direct generation in the living organism and to distinguish them from their in vitro generated counterparts [43]. Such colonies express endogenous pluripotency markers and show high AP activity, both considered hallmarks of pluripotency. In addition, they spontaneously differentiate into cell types representative of all three germ layers when pro-pluripotency conditions (i.e. LIF supplement) are withdrawn. Their pluripotent potential has been further confirmed by the teratoma assay and the contribution to all adult tissues in chimeric mice generated via blastocyst injection. Overall, such studies proved that i^2PS cells are *bona fide* pluripotent stem cells.

5.3 The "Reprogrammable Mouse" a Tool for the Study of In Vivo Reprogramming to Pluripotency

Delivery of reprogramming factors to target cells is one of the limiting steps that compromise the efficiency of reprogramming to pluripotency. In vitro, an extensive list of viral vectors including retrovirus [19], lentivirus [44], inducible lentivirus [45], excisable lentivirus [46], adenovirus [47] and Sendai virus [48] has been explored to optimize the induction of pluripotency. Non-viral vectors and vector-free methods such as cell electroporation have also been utilized to deliver not only reprogramming DNA in several forms [49, 50] but also mRNA [51], microRNA [52] and proteins [53].

In vivo, the delivery challenge is even more daunting considering the additional biological barriers that vectors have to overcome to reach the appropriate target, and the presence of a variety of enzymes in biological fluids that can trigger degradation of the cargo. Naked pDNA has been used when the administration method allows relatively high uptake in the target tissue, as it is the case of liver transfection via HTV injection [37, 38]. Direct pDNA injection has also been used to reprogram tissues known to be naturally permissive to the uptake of the nucleic acid, such as skeletal muscle [36, 54]. To target other organs that do not allow such a phenomena—for example, the brain cortex—retroviral vectors have been used [55].

In spite of the examples above, the issue of delivery remains one of the main obstacles for the induction of pluripotency in vivo. In light of such complications, and to be able to separate the impact of inefficient delivery from the intrinsic poor efficiency and stochastic character of the reprogramming process, various proof-of-principle studies have relied on the use of genetically engineered mice that contain OSKM reprogramming factors inserted in the genome to induce pluripotency in vivo [56–62].

Different approaches have been followed to engineer so-called "reprogrammable mice", including secondary systems through generation of iPS cells with integrating

viral vectors [63] and direct insertion of the OSKM cassette in ESCs [64]. Wernig et al. first constructed a secondary system based on the infection of MEFs, which contained the reverse tetracycline-dependent transactivator *M2rtTA* in the ubiquitously expressed *Rosa26* locus (*Rosa26-M2rtTA* MEFs), with four integrating lentiviruses encoding each of the OSKM factors under the control of a doxycycline-inducible promoter (tetOP) [63]. Addition of the drug to the culture medium generated iPS cells that contained OSKM factors inserted in the genome and were subsequently used to generate reprogrammable mice via blastocyst injection. In a different strategy, Stadtfeld et al. utilized gene targeting mediated by Flp recombinase to avoid the complications associated with secondary systems [64]. A doxycycline-inducible polycistronic OSKM cassette was placed in the 3′ untranslated region of the collagen type I, alpha 1 gene (*Col1a1*) in *Rosa26-M2rtTA* ESCs that were, similarly to the previous approach, used to generate chimeric reprogrammable mice. Both strategies are illustrated in Fig. 5.4.

The reprogrammable mouse is an excellent source of "reprogrammable cells" for in vitro studies. Not only MEFs, but also somatic cells obtained from adult reprogrammable mice, can be reprogrammed to pluripotency in the culture dish when doxycycline is added to the culture medium. In addition, the OSKM cassette can also be induced in vivo with the administration of the drug (Fig. 5.4c). Doxycycline administration in drinking water induces widespread OSKM expression, since the transgenes are ubiquitously inserted in the genomes of all cells [56]. Targeted expression of reprogramming factors in specific tissues can also be achieved via localized doxycycline administration, for example by intramuscular administration [61].

The advantages brought by this model are numerous. First, it avoids the need to use viral or other delivery vectors, and with that it circumvents the problems associated with poor and heterogeneous OSKM delivery and ensures efficient reprogramming. In addition, genetic homogeneity of the reprogrammed cells is also higher in terms of number and location of transgene integrations. This allows direct comparison of the reprogramming process, its mechanisms and efficiency in different tissues and cell types of different developmental origin and maturation status; also thanks to the fact that the OSKM transgenes are ubiquitously present in all cells throughout the organism. In fact, induction of OSKM expression in the reprogrammable mouse highlighted that certain tissues are more resilient to reprogramming than others. In the skeletal muscle, OSKM expression was not enough to induce the generation of teratomas at doxycycline doses that efficiently formed such tissue aberrations in stomach, intestine and pancreas [60].

In addition, the doxycycline-inducible promoter (tetO) driving OSKM expression in the reprogrammable mouse model is an excellent tool to customize the pattern of expression of the factors. Their expression can be easily switched on an off at different times via administration or withdrawal of the drug. Thanks to this inducible system, the impact that the duration of OSKM expression has on the extent of reprogramming (partial vs complete reprogramming to pluripotency) and on the overall fate of in vivo reprogrammed cells in the tissues has been elucidated [65]. Full understanding of this relationship is key to ensure the safety of in vivo reprogramming to pluripotency and facilitate the path towards potential clinical applications, and therefore it will be further dissected in Chap. 6 of this book.

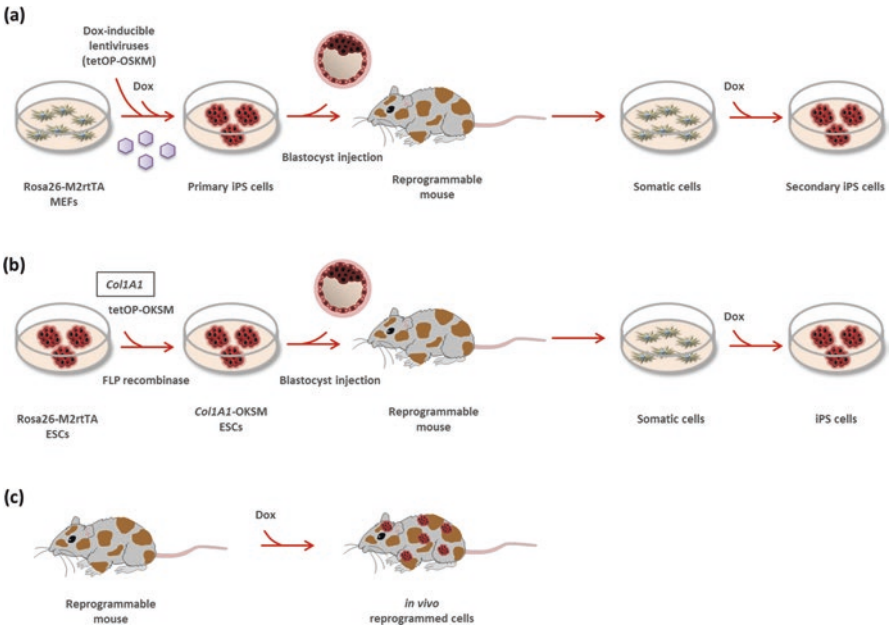

Fig. 5.4 Genetic engineering strategies to generate reprogrammable mice. In vitro and in vivo applications. Different genetic engineering strategies have been used to build reprogrammable mice with OSKM transgenes inserted in their genome. (**a**) MEFs expressing the tetracycline-depended transactivator in *M2rtTA* in the ubiquitously expressed *Rosa26* locus (Rosa26-M2rtTA MEFs) were infected with fours lentivirus vectors expressing each of the OSKM transgenes under the control of a doxycycline-inducible (tetOP) promoter. Primary iPS cells obtained upon doxycycline (Dox) supplementation were used to generate chimeric mice via blastocyst injection, termed "reprogrammable mice" [63]. (**b**) Gene targeting via Flp recombinase was used to insert a polycistronic OSKM cassette under the control of a doxycycline-inducible promoter (tetOP) in the collagen type I, alpha 1 gene (*Col1A1*) in ESCs that expressed an optimized tetracycline-dependent transactivator in the ubiquitously expressed *Rosa26* locus (Rosa26-M2rtTA ESCs)). Reprogrammable mice were generated via the injection of such genetically engineered cells in donor blastocysts [64]. Embryonic and somatic cells extracted from both reprogrammable mouse models generate iPS cells upon the addition of doxycycline in the culture medium. (**c**) Reprogrammable mice can also be used to induce adult cells to pluripotency within the adult organism upon systemic or localized administration of doxycycline [56, 61]

5.4 Downstream Effects of OSKM Expression in the Reprogrammed Tissue Microenvironment

In vivo overexpression of OSKM factors generates cells with pluripotency features within reprogrammed tissues, as demonstrated in proof-of-principle studies described in Sect. 5.2 of this Chapter [36, 37]. However, this is not the only downstream effect that OSKM induction triggers in the cells that express the factors and in those in the surrounding tissue.

5.4.1 *In Vivo Reprogramming to Pluripotency and Tumorigenesis*

The proliferative nature of pluripotent stem cells and their potential to differentiate into cell types from all three embryonic lineages are often regarded as excellent features that offer opportunities in stem cell research and regenerative medicine. However, such capabilities can also compromise the safety of in vivo reprogramming to pluripotency if excessive proliferation and uncontrolled re-differentiation take place in the living organism. Indeed, in vivo OSKM overexpression can lead to the generation of dysplastic lesions and teratomas within reprogrammed tissues [56].

Teratomas are defined as tumors that originate from the uncontrolled expansion and random differentiation of pluripotent cells and that are therefore composed of tissue types representing all three germ layers [66]. Abad et al. reported the occurrence of such tissue abnormalities following ubiquitous induction of OSKM expression in the reprogrammable mouse model [56]. Administration of doxycycline in the drinking water induced reprogramming of a variety of cell types, from hematopoietic and non-hematopoietic lineages, across different tissues. Reprograming and de-differentiation were confirmed by expression of the pluripotency marker NANOG, which was found concomitantly with loss of specific cell type markers. However, OSKM overexpression also led to the appearance of tumor masses, the majority of which were classified as teratomas that contained $NANOG^+$ reprogrammed cells.

Indeed, the process of reprogramming to pluripotency shares various common events with the early stages of tumorigenesis. Those include the acquisition of self-renewal properties, loss of cell differentiation status and re-expression of genes typically enriched in embryonic developmental stages [67], as well as metabolic changes in the cell that involve significant upregulation of glycolytic pathways [68]. Similar telomere changes have also been observed during reprogramming and the onset of tumorigenesis, which indeed support the acquisition of self-renewal properties. Telomeres are short repetitive DNA sequences at the end of the chromosomes that protect them from degradation but are shortened through cell division [69]. Therefore, telomere length determines the lifespan of a cell. Increased activity of telomerase—the enzyme in charge of telomere elongation and normally only active during embryonic development and in adult stem cell compartments—is one of the hallmarks of tumorigenesis, and leads to immortalization of the cancerous cells [70]. During reprogramming, telomere elongation is a mandatory step to achieve *bona fide* pluripotency and it also determines the efficiency of reprogramming [71]. Although this was initially observed in vitro, it is now known that cells within in vivo reprogrammed areas have longer telomeres that those in non-reprogrammed tissues and show high telomerase activity [62].

Similarities between common types of tumors and those generated upon OSKM overexpression in the respective organ have also been reported. Kidney tumors in reprogrammable mice shared common histological, gene expression and DNA methylation features with Wilms tumor, a common pediatric kidney tumor [57].

While such observation adds to the list of shared features between induced reprogramming to pluripotency and tumorigenesis, it should be considered with care since the genetically modified nature of reprogrammable mice prevents any clinical relevance.

Generation of teratomas following OSKM overexpression has been reported in a number of studies [56, 57, 59, 62, 60], not all of which made use of the reprogrammable mouse model [55]. However, other studies have demonstrated that reprogramming can be induced in the absence of tumorigenesis and therefore the causal relationship between in vivo OSKM overexpression and teratoma formation should not be interpreted as general [37, 61, 54]. The main determinants that trigger or prevent teratoma formation are discussed in Chap. 6 of this book.

5.4.2 In Vivo Reprogramming to Pluripotency, Tissue Damage and Cellular Senescence

In addition to telomere shortening, reprogramming via OSKM overexpression is able to reverse various other hallmarks of ageing, including increased expression of aged-related stress response genes, double-stranded DNA breaks, mitochondrial dysfunction and abnormal architecture of the nuclear envelope. In fact, complete reprogramming to pluripotency is not necessary to attain such cell "rejuvenation", which can be achieved with partial reprogramming [61]. However, the relationship between cell reprogramming and cellular senescence is much more intricate, especially at the tissue level.

While reprogrammed cells are "molecularly rejuvenated", in vivo expression of OSKM factors induces senescence in surrounding cells within the tissue, which secrete senescence-related cytokines [59]. Interestingly, the presence of such senescence signals, of which IL-6 is the main player, renders the tissue microenvironment more permissive to the induction of reprogramming [59, 60]. Indeed, the efficiency of in vivo reprogramming has proven to increase in a variety of scenarios, including the administration of pharmacological agents that promote senescence [59], physiological ageing [59], progeria (a condition of extremely premature ageing) [59] and tissue injury [59, 60], all of which share cellular senescence as a common factor.

Therefore, the cross-talk between senescence and reprogramming seems to be beneficial for the latter, which could be of great interest to develop in vivo reprogramming strategies for tissue regeneration.

5.5 Conclusions

Proof-of-principle studies in a developing amniote and an adult, fully developed, mammalian model have demonstrated that specific reprogramming transcription factors are indeed able to induce pluripotency features in vivo, even in the presence

of pro-differentiation signals in the tissue microenvironment. However, it is also clear from these and other studies that forced expression of such genes triggers additional downstream effects in in vivo reprogrammed cells and their surrounding tissue. While confirmation that pluripotent cells can be generated in vivo envisions a plethora of potential applications in regenerative medicine, knowledge on the links between reprogramming, tumorigenesis and cellular senescence, among other events, is still limited and warrants further investigation.

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Chapter 6
In Vivo Reprogramming Towards Pluripotency for Tissue Repair and Regeneration

Irene de Lázaro and Kostas Kostarelos

6.1 Introduction

A series of proof-of-principle studies demonstrated at the beginning of this decade that cells within adult, fully differentiated tissues can be directly reprogrammed in situ to acquire several hallmarks of pluripotency, including the capacity to proliferate. This process is induced by overexpression of defined transcription factors—the combination of *Oct3/4*, *Sox2, Klf4* and *cMyc*, also known as OSKM—that can reset the primitive plasticity of undifferentiated cells in spite of the presence of pro-differentiation signals that naturally govern the adult tissue microenvironment [1–4]. Since then, the induction of pluripotency in vivo via OSKM overexpression has been used to further unravel the mechanisms behind reprogramming, as well as to investigate its connections with other cellular processes, including the onset of tumorigenesis and cellular senescence (see Chap. 5 of this book). However, beyond the invaluable role of in vivo reprogramming models as research tools to answer the questions above, the therapeutic applications that could be developed from this strategy, in particular to induce or enhance tissue regeneration, have also been envisioned. In this Chapter, we discuss the rationale behind the use of in vivo reprogramming towards pluripotency to assist tissue repair. We also analyse opportunities and challenges on the road towards clinical translation and review the studies, although scarce, that have already confirmed the potential of in vivo reprogramming to pluripotency to enhance the regenerative capacity of injured and degenerated tissues.

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6.1.1 Hypothesis: Generation of an In Situ Source of De Novo Cells to Repair Injured or Aged Tissues

A plethora of conditions and insults to the organism trigger the loss of specific cell populations, some of which cannot be efficiently replenished by the adult mammalian organism. Such is for example the case after myocardial infarction, whereby the ischemic accident leads to death of a large number of cardiomyocytes. Current treatments can only help the heart adapt to the new situation by decreasing its workload and thus minimise the risk of future ischemic events, but fail to induce the generation of new cardiomyocytes that restore the intact function of the organ [5]. Similarly, lack of blood supply to certain parts of the brain during ischemic or haemorrhagic stroke results in loss of neuronal cells that cannot be replaced [6].

Direct reprogramming of surviving cells within and injured or degenerated tissue via in vivo OSKM overexpression has been proposed as a novel strategy to induce or enhance its repair and regeneration [7]. Thanks to their capacity to proliferate but also to re-differentiate back into mature phenotypes, in vivo reprogrammed cells could be used as an in situ source of de novo cells to replenish those lost upon injury or degeneration. Such hypothesis is illustrated in Fig. 6.1. While OSKM factors have sufficiently proven their capability to induce de-differentiation in a variety of cell types in vitro and in vivo, it is expected that pro-differentiation cues present in the tissue microenvironment are able to drive re-differentiation of reprogrammed intermediates into fully functional mature cell types [7]. Experimental evidence that supports this hypothesis has been provided in studies that followed the re-differentiation of reprogrammed intermediates, as well as their re-integration in the host tissue and accomplishment of their physiological function [8].

6.1.2 Lessons Learnt from Nature: De-Differentiation for Regeneration

Induction of cell de-differentiation and proliferation is in fact not a new tool to attain tissue regeneration, at least in the context of lower species that are tremendously efficient at regenerating injured tissues, lost appendages and significant portions of vital organs. In zebrafish, heart regeneration is mediated by cardiomyocyte de-differentiation and proliferation [9]. In the newt, proliferating cells that originate from de-differentiated myofibers contribute significantly to form the blastema that precedes limb regeneration [10].

It is not clear whether such regenerative mechanisms have been completely abolished in the mammalian organism as result of evolution, or simply silenced and dormant [11]. A window of efficient heart regeneration via cardiomyocyte proliferation is indeed reported in the neonatal mouse heart, but such capacity vanishes after the first week of life [12]. In the adult, studies have pointed at a very limited degree of cardiomyocyte turnover [13], by far insufficient to provide efficient regeneration,

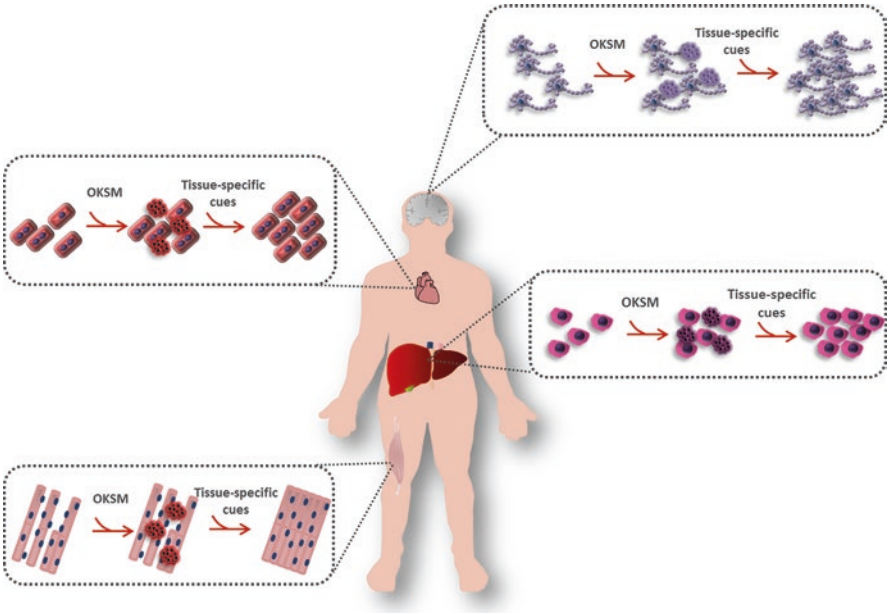

Fig. 6.1 In vivo reprogramming to pluripotency for tissue regeneration. In vivo overexpression of OSKM factors drives reprogramming of a wide variety of starting cell types to a pluripotent-like and proliferative state. It is hypothesised that tissue-specific cues present in the host's microenvironment will be able to orchestrate re-differentiation of the pluripotent intermediates towards appropriate cell phenotypes. Generation of in vivo reprogrammed cells via overexpression of a "universal" cocktail of transcription factors (OSKM) could therefore contribute to enhance regeneration of a variety of injured tissues without the need for ex vivo cell manipulation

and, in addition, the mechanisms by which new cardiomyocytes are generated are yet to be elucidated [14]. Leaving aside rare examples in which spontaneous de-differentiation followed by active division of specific cell populations has been described in vivo [15], the mammalian organism has a lot to learn from lower species.

Overall, regenerative mechanisms in lower species and those still present at the earliest stages of mammalian development have inspired extensive research aiming to recapitulate them in the adult. Many have tried to force or silence the expression of transcription factors and non-coding RNAs known to induce de-differentiation and replenishment of defined cell types in regenerating organisms. Examples of this include exogenous expression of *msx1*, which drives de-differentiation of muscle fibers in urodele amphibians [16] and downregulation of miR99/100 and Let-7a/c, known to induce de-differentiation and proliferation of cardiac myocytes in zebrafish [17]. The role of the Hippo pathway in cardiac regeneration, among other mechanisms, is also under intensive study in the attempts to translate the regenerative capacity of the neonate to the adult mammalian organism [18]. In vivo reprogramming via OSKM overexpression has more recently been proposed as a new alternative in the regenerative medicine portfolio [7, 19].

6.2 In Vivo OSKM Overexpression to Enhance Regeneration After Injury

In spite of the very premature stage at which OSKM-mediated in vivo reprogramming stands today, two independent studies have already provided evidence of enhanced regeneration following administration of the reprogramming cocktail and subsequent generation of pluripotent-like intermediates within injured tissues. Importantly, such evidence has been generated in the context of two distinct injury models that involve different organs, namely traumatic brain injury [20] and skeletal muscle injury [21].

Direct intracranial injection of retroviral vectors encoding OSKM after a controlled cortical impact in mice allowed targeting reactive glia, thanks to the transduction capability of the vectors which is restricted to dividing cells. Indeed, the effect of brain trauma on activating proliferation and migration of glial cells makes them an excellent starting cell source for the generation of in vivo iPS cells via in vivo reprogramming, given their abundance in the injured site. Transduced cells showed hallmarks of pluripotency, including expression of pluripotency marker NANOG and stem cell marker SSEA4, and proliferated actively generating cell clusters that filled the cavity left by the impact. Some reprogrammed cells were found to re-differentiate into neurons and glia, which reassures the potential of this strategy to regenerate the injured brain. However, uncontrolled expansion of reprogrammed cell clusters, caused by the use of integrating gene delivery vectors that sustain long-term expression of reprogramming factors, triggered the generation of teratomas in the brain and therefore compromises any therapeutic application of this strategy as currently designed [20].

In mouse skeletal muscle, forced expression of the same factors encoded in a pDNA cassette via direct intramuscular injection also triggered the appearance of proliferating cell clusters that expressed several pluripotency markers (NANOG, AP, SSEA1) and a marker specific to muscle progenitors (PAX3). However, OSKM expression was not sustained over time, most likely due to the use of an episomal vector that was progressively lost with cell division. As a result, clusters of reprogrammed, pluripotent-like cells were only observed up to 4 days after administration of reprogramming factors and no teratomas were found for the duration of the study (120 days). Morphometric analysis suggested that, on the contrary, in vivo reprogrammed cells could have re-differentiated and fused to existing myofibers, enlarging their calibre. In a clinically-relevant model of severe muscle injury—that involved complete transection of the medial head of the mouse gastrocnemius—OKSM administration accelerated regeneration, as evidenced by the increased numbers of centro-nucleated, small calibre myofibers soon after pDNA administration. Moreover, in vivo reprogramming also showed to prevent excessive collagen deposition, one of the most challenging complications involved in severe muscular injuries that impedes complete recovery of contractile properties [21].

Both studies have opened multiple questions to be answered, not only concerning the safety of the approach but also with regards to the efficiency of reprogramming

achieved and whether this could be sufficient to translate into functional regeneration—not limited to the histological level as in the studies above. Nevertheless, both have offered sound proof of the potential of in vivo reprogramming to enhance regeneration.

6.3 OSKM Overexpression to Rejuvenate Aged Tissues

Induction of tissue regeneration after injury may not be the only therapeutic application provided by the expression of OSKM in vivo. In fact, short but cyclic expression of these factors induces a certain degree of epigenetic remodelling, considered as "partial reprogramming", that does not attain pluripotency but erases several hallmarks of ageing. This event, which may be seen as a strategy for cell "rejuvenation" at the molecular level, could be of interest for the treatment of age-related pathologies. In a mouse model of progeria — a disease in which the onset of ageing is aberrantly premature — cyclic OSKM expression extended the otherwise short life expectancy of the mice and improved the overall condition of various organs and tissues. Even in physiologically aged mice (without the disease), the resilience of aged tissues to injury, impaired compared to that of younger counterparts, increased when OSKM was administered before the insult, following the same cyclic induction protocol. Such improved performance after injury is thought to be achieved through OSKM-driven proliferation of specific cell compartments in charge of tissue turnover and homeostasis, whose numbers normally plummet with age, and was confirmed to occur in two distinct organs. Prior-to-injury expansion of beta cells helped restore glucose tolerance and pancreatic function in a streptozocin model of metabolic disease. In skeletal muscle, satellite cell proliferation prior to intramuscular administration of cardiotoxin, a venom commonly utilised to mimic muscle injury, significantly enhanced tissue regeneration [19].

The observations made in this study confirm that different OSKM induction protocols trigger distinct downstream effects in the tissues, particularly in what concerns the fate of reprogrammed cells. This will be further discussed in Sect. 6.5.1 of this chapter. In addition, it has also become apparent that complete reprogramming to naïve pluripotency may not be a requirement to enhance tissue repair and regeneration in all scenarios [19].

6.4 Opportunities Brought by In Vivo Reprogramming Towards Pluripotency to the Regenerative Medicine Toolbox

In vivo reprogramming via OSKM expression is only one of the numerous strategies currently under preclinical evaluation to achieve efficient cell and tissue regeneration in the adult mammalian organism. However, this particular approach has

attracted increased attention thanks to the promise that it could offer a versatile tool to induce regeneration in virtually any tissue type or organ while avoiding the complications linked to ex vivo cell therapy.

6.4.1 *OSKM: A Universal Recipe to Induce Reprogramming Towards Pluripotency*

Induction of a more plastic, de-differentiated or pluripotent-like status is not the only option in the in vivo reprogramming toolbox that may be used to enhance tissue regeneration. The number of pre-clinical studies that rely on the concept of in vivo transdifferentiation — direct reprogramming between two distinct mature cell types, for example, fibroblasts to cardiomyocytes — is in fact more abundant in the current scientific literature. One of the reasons behind the popularity of this approach is the bypassing of the pluripotent state, which is understood to minimise the risk of tumorigenesis. In addition, use of cell type-specific transcription factors may provide better control over the resulting phenotype, whereas in vivo reprogramming via OSKM overexpression necessarily relies on molecular cues present in the host's tissue to drive re-differentiation towards appropriate cell types [22].

However, in spite of the undeniable advantages of the transdifferentiation approach, versatility of the OSKM cocktail to induce reprogramming in a wide variety of starting cell and tissue types may save significant research efforts and time and should not be underestimated [7]. Indeed, induction of a particular transdifferentiation event requires identification of specific transcription factors that trigger the precise switch between cell types. The many different combinations of transcription factors utilized in in vivo transdifferentiation studies have been a topic of extensive review [22] and are compiled in Table 6.1. On a complete opposite scenario, OSKM has proven able to induce de-differentiation of a large number of different cell types, from different developmental origins and in different maturation stages; even if significant differences in reprogramming efficiencies have been reported. This has not only been illustrated in the culture dish, where iPS cells have been generated from skin fibroblasts [23], peripheral blood cells [24], liver and stomach cells [25] and pancreatic beta cells [26], among others; but also in vivo. Through ubiquitous OSKM expression in reprogrammable mice, it has been confirmed that in vivo iPS cells can be generated from diverse starting cell types, from haematopoietic and non-haematopoietic origin [4], although different tissues may require different OSKM induction levels to undergo efficient reprogramming [27]. Thanks to this versatility, in vivo reprogramming via OSKM overexpression has already been used to enhance regeneration within very distinct tissue types, namely pancreas [19], skeletal muscle [19, 21] and brain [20].

Table 6.1 Transcription factors (TF) that mediate in vivo transdifferentiation

Starting cell type	Resulting cell type	TF cocktail	Reference
Cardiac fibroblast	Skeletal myofiber	*MyoD*	[28]
	Cardiac myocyte	*Gata4, Mef2c, Tbx5*	[29, 30]
		Gata4, Hand2, Mef2c, Tbx5	[31]
		miRNA1, 133, 208, 499	[32, 33]
Ventricular cardiomyocyte	Pacemaker cell	*Tbx18*	[34, 35]
Exocrine pancreatic cell	Insulin-secreting β cell	*Pdx1, Ngn3, MafA*	[36]
Liver cell	Insulin-secreting cell	*Pdx1*	[37–39]
		neuroD, β-cellulin	[40]
		Pdx1/VP16, NeuroD, Ngn3	[41]
		Ngn3	[42]
		Pdx1, Ngn3, MafA	[1, 43]
Astrocyte	Neuroblast	*Sox2*	[44–46]
	Neuron	*Ascl1, Brn2a, Myt1l*	[47]
		NeuroD1, Ascl1, Lmx1A, miR218	[48]
Fibroblast		*Ascl1, Brn2a, Myt1l*	[47]
Glial cell		*NeuroD1*	[49]
Oligodendrocyte		miRNA 4	[50]
Post-mitotic callosal neuron	Corticofugal neuron	*Fezf2*	[51]
L4 post-mitotic neuron	L5 neuron	*Fezf2*	[52]

In vivo transdifferentiation studies published to date are compiled in this table (updated May 2017), including the specific transcription factors required to trigger each particular switch in cell fate

6.4.2 Direct In Vivo Reprogramming to Avoid the Challenges of Ex Vivo Cell Therapy

In the event of cell loss upon injury or degeneration that cannot be addressed by physiological tissue homeostasis, cells grown and/or manipulated in the laboratory can be transplanted to repopulate the injured site. Strategies of this sort have been explored for a number of years, long before in vivo reprogramming and transdifferentiation were considered in the regenerative medicine portfolio, and rely on several sources of replacement cells including embryonic stem cells (ESCs), mesenchymal stem cells (MSCs) and iPS cells, within a very extensive list [53, 54]. However, all such cell therapies involve a series of common hurdles related to ex vivo cell manipulation, which complicate their establishment in routine clinical practice, and that could be bypassed by directly inducing cell reprogramming in vivo.

Firstly, donor cell isolation encompasses complications of different magnitudes based on specific cell sources. Use of ESCs involves ethical and regulatory constraints

linked to the destruction of embryonic material [55, 56]. Invasive biopsy techniques are required to access certain population of progenitor and adult stem cells [57]. The least problematic to this respect are iPS cells, that can be generated from easily accessible sources through minimally invasive biopsies (i.e. skin fibroblasts) or a simple blood test (i.e. peripheral blood cells) [54].

Several complications also arise during the process that turns the starting cell source into the final product, ready for transplantation into the tissue of need. Genomic aberrations may appear due to extensive in vitro culture [58]. Indeed, the length of in vitro protocols required to achieve sufficient numbers of ready-to-use cells is also a cause of concern when therapeutic efficacy depends on their prompt administration after the insult [59]. In addition, such protocols are frequently complicated recipes that require finely tuned exposure to growth factors, xenobiotics and other substances in order to achieve the desired cell phenotype. Designing such recipes and optimising timing and dosage of exposure to specific cues is a daunting task, and substitutions are commonly needed when the presence of specific molecules in the culture is not considered safe for later human transplantation. Finally, even if the optimal cell product can be obtained in the laboratory, poor engraftment is often to blame in the discrete therapeutic efficacy achieved to date by cell replacement therapies [58, 60].

Direct generation of pluripotent or pluripotent-like intermediates in situ could bypass all limitations listed above, since no donor cell isolation, nor in vitro culture and manipulation, are required. Reprogramming is also reported to occur promptly in vivo after the administration of OSKM factors, without the need to co-administer other substances or adjuvants [2, 3]. In vivo, re-differentiation is also thought to take advantage of pro-differentiation signals naturally present in the host's tissue microenvironment, without the need to optimise complicated protocols to obtain specific cell types. In fact, cells differentiated within living tissues have been reported to achieve a more mature phenotype than those differentiated in the culture dish, which mainly attain a phenotype closer to embryonic or progenitor stages [29, 36]. Finally, chances of graft rejection are believed to be diminished since in vivo reprogrammed cells originate from the host's own organism. Indeed, reprogrammed cells have been seen to successfully re-integrate in the tissue and accomplish their physiological function upon re-differentiation in various studies [8, 20].

6.5 Needs on the Road Towards Clinical Translation of In Vivo Reprogramming Towards Pluripotency

While the first pre-clinical studies support the potential of in vivo reprogramming via OSKM overexpression to enhance tissue regeneration, various obstacles that this technology will need to overcome before it may turn into a clinical reality have also been made apparent. Key issues among them are those related to the fate of in vivo reprogrammed cells and to the efficient, yet safe, in vivo delivery of reprograming factors.

6.5.1 Transient OSKM Expression for Teratoma-Free In Vivo Reprogramming

Fear to the generation of teratomas due to uncontrolled proliferation and disorganised re-differentiation of in vivo reprogrammed cells has slowed down the pace of research on the therapeutic applications of in vivo OSKM induction.

Indeed some, but not all, of the studies in which OSKM factors were overexpressed in the living organism reported the generation of tumours within reprogrammed tissues, of which a vast majority were classified as teratomas based on the presence of tissue structures representative of all three germ layers [4, 8, 20, 27, 61, 62]. Others have however demonstrated complete absence of tumorigenesis even for extended periods of time after reprogramming [2, 3, 19, 21]. Table 6.2 compiles all studies on in vivo OSKM overexpression published to date, indicating the induction protocol of choice and the appearance or not or teratomas.

While such studies share similarities and differences in the way that OSKM factors are induced, the duration of their expression has been identified as the main determinant in the fate of in vivo reprogrammed cells and consequently in the appearance or not of teratomas [63]. The first study to report the development of OSKM-triggered teratomas relied on systemic (i.e. oral) doxycycline administration to induce pluripotency in reprogrammable mice — with OSKM transgenes inserted in the genome under the control of a doxycycline-inducible promoter — and already suggested the relevance of the temporal extent of OSKM expression in such an aberrant outcome. Administration of 0.2 mg/ml of the drug in the drinking water for a period of 2.5 weeks caused higher incidence of teratoma formation than a 5-times higher dose (1 mg/ml) that was withdrawn after 1 week. Teratomas were also found to develop faster with the longer induction scheme, and the survival of the animals was shortened compared to the higher — but shorter — dose [4].

Use of integrating viral vectors that sustain transgene expression for prolonged periods of time (i.e. retroviral vectors) to deliver OSKM also led to the development of teratomas within reprogrammed tissues [20]. Therefore, teratoma formation upon in vivo reprogramming is not limited to the use of genetically engineered reprogrammable mice.

Further studies have covered a wider range of induction intervals, always thanks to doxycycline-inducible OSKM expression, and confirmed the direct relationship between time of OSKM expression and incidence of teratomas. Remarkably, many of the animals fed with the drug for less than 5 days did not develop permanent dysplastic growth lesions or teratomas. Even when OSKM expression was maintained for up to 7 days, some of the cells reprogrammed to a de-differentiated and proliferative state where able to re-differentiate into a mature phenotype that successfully integrated in the tissue recapitulating its physiological function. An example of such event was reported in the pancreas of reprogrammable mice, where transiently reprogrammed cells expressed insulin after re-differentiation [8].

Nevertheless, strategies that achieve teratoma-free reprogramming rely on even more transient induction schemes. For example, delivery of OSKM factors in

Table 6.2 Studies on in vivo OSKM overexpression (updated May 2017)

Species	OSKM overexpression	Administration scheme	Target tissue	Teratoma formation	Reference
Tadpole	pDNA (OSK)	Single i.m. administration	Tail muscle	No	[2]
Mouse	pDNA (OSKM)	Single HTV administration	Liver	No	[3]
	Reprogrammable mouse	0.2 mg/ml Dox, 2.5 weeks or 1 mg/ml Dox, 1 week	Ubiquitous	Yes	[4]
	Dox in drinking water				
	Reprogrammable mouse	2 mg/ml Dox, 3-9 days	Ubiquitous	Yes[a]	[8]
	Dox in drinking water				
	Retroviral vectors (OSKM)	Single intracranial injection	Brain cortex	Yes	[20]
	Reprogrammable mouse	0.2 mg/ml Dox, 8 days	Ubiquitous	Yes	[61]
	Dox in drinking water				
	Reprogrammable mouse	1 mg/ml Dox, (2 days + 5 day withdrawal) 35 cycles.	Ubiquitous	No	[19]
	Dox in drinking water				
	pDNA (OSKM)	Single i.m. administration	Gastrocnemius muscle	No	[21]
	Reprogrammable mouse	0.2 mg/ml Dox, 7 days	Ubiquitous	Yes	[27]
	Dox in drinking water				
	Reprogrammable mouse	0.2 mg/ml Dox, 2.5 weeks	Ubiquitous	Yes	[62]
	Dox in drinking water				

This table compiles all studies on in vivo OSKM overexpression published before May 2017, including the species, tissue target, delivery method and appearance or not of teratomas

[a]Not all mice in the study developed teratomas, as highlighted in the text, which was strongly influenced by the duration of OSKM expression

plasmid DNA (pDNA) backbones that remain as episomes. As in vivo reprogrammed cells proliferate actively during the earliest phases of reprogramming, the episome is diluted with cell division and OSKM expression decays rapidly over time [2, 3, 21]. A different strategy established a short but cyclic OSKM induction protocol, again based on the doxycycline-inducible system, whereby the drug was administered for 2 days followed by 5-day withdrawal. Interestingly, this approach has proved to escape tumorigenesis for at least 35 repeats of the cycle [19].

Indeed, different OSKM induction protocols seem to have distinct effects on the extent of de-differentiation acquired by in vivo reprogrammed cells. In Abad et al.'s study, whereby OSKM expression was sustained over extended periods of time,

in vivo reprogrammed cells acquired totipotency features—a more primitive and plastic status than that of ESCs—and proved able to contribute to extraembryonic tissues [4]. On the opposite scenario, the very transient but cyclic induction protocol designed by Ocampo et al. induced sufficient epigenetic remodelling to erase several hallmarks of ageing and "rejuvenate" aged cells but did not lead to their complete de-differentiation, nor to the acquisition of pluripotency features [19].

While the extent of reprogramming and de-differentiation required to induce efficient regeneration (and whether that would be the same in different tissues and injury scenarios) is still not entirely understood, it is clear that transient OSKM expression is an absolute requirement to ensure safe, teratoma-free in vivo reprogramming that holds potential for clinical translation [63].

6.5.2 Efficient, Targeted and Safe Vectors for OSKM Delivery

Many of the studies exploring the concept of in vivo reprogramming to pluripotency to date have relied on the use of "reprogrammable" mice that include OSKM reprogramming factors integrated in their genome (Table 6.2). Such model bypasses complications linked to in vivo gene delivery, ensures high reprogramming efficiency and is unquestionably useful in mechanistic and proof-of-principle studies [64, 65]. However, it is unable to provide clinical relevance given the nature of its genetic modification. The search for appropriate gene delivery vectors that allow clinical translation of in vivo reprogramming via OSKM overexpression is therefore a priority. While it is difficult to make general assumptions (i.e. the design of the vectors may be greatly influenced by the specific requirements of the disease to be tackled through in vivo reprogramming), some common features will need to be considered to ensure efficient, yet safe, reprogramming.

Based on the reported direct relationship between the duration of OSKM expression and the development of teratomas, the main priority should be to identify a vector able to provide transient expression of such factors that is yet sufficient to translate into functional regeneration. Integrating vectors should therefore be ruled out from the list, unless they are accompanied by excisable or silencing mechanisms [63]. Episomal non-viral vectors have so far provided the most encouraging results to this respect [2, 3, 21]. However, experience gathered from the gene therapy field reminds us that the promise of such systems at the pre-clinical level should not be assumed at the clinical setup [66–68].

Targeting specific cell populations may also be a requirement to control the effects of in vivo reprogramming. To date, retroviral vectors have been used to limit OSKM expression to dividing cells, but transgene integration and sustained reprogramming preclude clinical translation [20]. Use of non-integrating vectors with cell specific promoters may offer an alternative to ensure targeting without compromising the safety of the approach.

Overall, the emphasis in vector design for in vivo reprogramming in tissue regeneration should be placed in finding the appropriate balance between safety,

avoiding prolonged and/or ubiquitous expression of reprogramming factors, and efficacy, through the generation of sufficient reprogrammed cells to replenish the lost tissue.

6.6 Conclusions and Future Challenges

Although studies on in vivo cell reprogramming via direct OSKM overexpression are still scarce, the potential of this strategy to contribute to tissue rejuvenation and regeneration has already been confirmed by preliminary but sound studies that involve different tissues and injury models. The extent of reprogramming required for efficient regeneration, either to full pluripotency or via partial reprogramming accompanied by proliferation, remains to be determined and will likely depend on the nature of the specific condition to be tackled. However, some requirements needed to translate in vivo reprogramming towards pluripotency into a viable clinical approach have already been established. Transient OSKM expression is key to avoid tumorigenesis. Therefore, strategies involving sustained expression of reprogramming factors (i.e. sustained pluripotency) will not develop into clinically relevant approaches and should solely be considered as research tools to investigate the mechanisms behind the pluripotent conversion. Special efforts should instead be placed in designing appropriate delivery vectors that ensure efficient yet transient OSKM expression.

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Chapter 7
Challenges and Future Perspectives for In Vivo Reprogramming Technology

Hadiseh Taheri, Cansu Gurcan, and Açelya Yilmazer

7.1 Introduction

In vivo reprogramming is an emerging technology which consists of converting resident tissue-specific cells into the cell types that are lost due to disease or damage. Especially since 2008, the field has accelerated a lot and proved that this technology can be applied for various diseased conditions in preclinical models. Here we discuss the challenges that are encountered during in vivo reprogramming.

7.2 Challenges

7.2.1 Efficiency

In one of the early studies, PDX-1 induced the expression of various pancreas specific genes in the liver. According to the results, cells were able to initiate but not complete the differentiation of liver cells into pancreatic cells [1]. This report suggested that monitoring the final stage of reprogrammed cells and evaluating the reprogramming efficiency are crucial steps to achieve successful reprogramming

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and consequently therapy. Later, Inagawa et al. showed that Gata4/Mef2c/Tbx5 delivery by retroviral vectors have been shown to induce cardiomyocyte-like cells in infarcted hearts [2]. These 3 candidate genes were not sufficient to reprogram all cells, due to the low infection efficiency in vivo. Furthermore, it has been suggested that high doses of MyoD are required to efficiently induce skeletal muscle differentiation in cardiac granulation tissue.

In a study by Su et al., resident astrocytes in the injured adult spinal cord were shown to be manipulated to produce neurons by defined factors, raising the possibility of using these cells as a source for in situ repair of SCI. However, the reported reprogramming efficiency and the number of converted neurons were low [3]. In another murine model which is studying in vivo reprogramming of pancreatic exocrine cells to beta cells, reprogramming success depends on not only the delivery of transcription factors but also a normoglycaemic environment [4]. Hyperglycaemia significantly impaired reprogramming of exocrine to insulin-producing cells in their quantity, differentiation status and function. With hyperglycaemia, the reprogramming of acinar cells towards beta cells was also incomplete.

7.2.2 Safety

Achieving a safe protocol that will not result in tumors or mutagenic effects in cellular therapy is a must of regenerative medicine. In reprogramming experiments directed towards pluripotency, induction of reprogramming factors such as Oct3/4, Klf4, Sox2 or c-Myc, results in the production of induced pluripotent stem cells in situ. As discussed in detail in Chap. 6, transient induction of pluripotency can be followed by differentiation towards cell types according to the tissue. However, studies that included persistant and high levels of reprogramming factors (as discussed in Chap. 5), have shown the development of tumors or teratomas at the target tissue following induction or reprogramming.

Gao et al. reported a strategy to reprogram glia into neurons and convert a non-neurogenic cortex into a neurogenic region [5]. Reprogramming reactive glia into iPSCs in vivo to produce new neurons in their natural environment of the cortex may suggest a strategy for brain repair following traumatic brain injury and other neurodegenerative diseases. The disadvantage of this approach was that the induced stem cells or precursor cells overgrow and may develop into a tumor.

7.2.3 Mechanism

Delineating the mechanism of different in vivo reprogramming approaches will allow further optimization of this technology and could fasten the transition to clinical settings. Therefore, various in vivo reprogramming studies are trying to understand the biological mechanism of these systems. Oct4 induced oligodendrocyte

progenitor cells (iOPCs) induction reprograms adult somatic cells into self-renewing and bipotent iOPCs, thereby allowing large-scale expansion of oligodendrocytes and astrocytes. However the mechanism of Oct4- triggered lineage conversion have not been elucidated yet [6].

In another study, the effect of the p53-p21 pathway regulation on in vivo reprogramming of resident astrocytes to mature neurons was investigated in the adult mouse spinal cord. Their findings have uncovered critical molecular and cellular checkpoints that may be manipulated to boost neuron regeneration after spinal cord injury (SCI) [7]. However, their biological function after SCI have not been elucidated.

In another study, the forced expression of a single transcription factor, Pdx1, in pancreatic acinar cells was sufficient to induce their reprogramming into endocrine cells, including beta cells [8]. These newly generated beta cells increased the serum insulin levels and ameliorated diabetes. However, in this in vivo reprogramming, both EGFP positive somatostatin- and PP-producing cells which were probably derived from acinar cells, but not EGFP-positive glucagon-producing cells. The mechanism of this kind of reprogramming should be studied carefully.

7.2.4 Incorporation to Target Tissue

In one of the pioneering in vivo reprogramming approaches, Zhou et al. reprogrammed adult pancreatic exocrine cells to beta-cells. The induced beta-cells were indistinguishable from endogenous islet b-cells in size, shape and ultrastructure. They expressed genes essential for beta-cell function and can ameliorate hyperglycaemia by remodeling local vasculature and secreting insulin [9]. The reprogramming of exocrine cells to beta-cells did not involve multiple rounds of cell proliferation and furthermore the induced beta-cells-cells did not organize into islet structures and remain as single cells or small clusters.

In another study, Heinrich et al. revealed the unexpected capacity of Sox2 for converting reactive glial cells into induced doublecortin positive neurons in the injured cerebral cortex [10]. Even though results indicated some degree of functional integration of these transduced cells, at the current state, it is unclear whether this reflects de novo acquisition of synaptic contacts following neuronal conversion or maintenance of synapses established onto these cells.

7.2.5 Survival of Reprogrammed Cells

In 2013, Grande et al. published a study in which non-neuronal cells were reprogrammed to generate new neurons. The in vivo environment affects many aspects of neuronal reprogramming in vivo [11]. In the striatum, GFs or Neurog2 alone induced a small but significant number of new neurons after stab wound, and their

combination further stimulated local neurogenesis. In the neocortex, however, Neurog2 alone induced a large number of immature neurons, but only a small number of mature neurons remained at later stages.

As shown by Guo et al., reactive glial cells in the cortex of stab-injured or Alzheimer' Disease model mice can be directly reprogrammed into functional neurons in vivo using retroviral expression of a single neural transcription factor, NeuroD1 [12]. However there are many questions waiting to be answered: whether it is possible to use a virus-free or small-molecule strategy to affect the reprogramming in vivo and whether the in vivo reprogramming can ultimately rescue behavioral deficits, such as cognitive impairment.

7.3 Future Perspectives

In vivo reprogramming is a very young field and much of the initial pioneering work described above needs further confirmation [13–16]. Heinrich et al. have proposed a total of 5 milestones to be accomplished for in vivio reprogramming. According to these milestones, the target cell type for reprogramming should be identified. The best strategy for converting the target cell type into the desired cell type should be defined carefully. The molecular identity and overall phenotype of the reprogrammed should be matched with the desired cell types. The constraints imposed by the host tissue should be defined and optimized. Finally, the functional integration of reprogrammed cells should restore the lost functions [13].

We believe that optimization of in vivo delivery protocols for the reprogramming factors or cues is a key step to achieve safe and efficient reprogramming. A better incorporation of the knowledge accumulated in the gene delivery field will fasten the in vivo reprogramming technology. Development of next-generation vectors that are rationally designed to meet the needs of in situ cell conversion protocols is required.

Considering the physiological functions, the similarity between large mammals and humans may favor testing the safety and efficacy of in vivo reprogramming protocol in at least one large animal model. This crucial step has been also advised in the Guidelines for Stem Cell Research and Clinical Translation, published by the International Society for Stem Cell Research in 2016. Large animals may better represent human physiology as they are often genetically outbred, anatomically similar, and immunocompetent. Therefore, large animals could be better models to improve the tested in vivo reprogramming approach and evaluate the possible clinical complications before starting any clinical trials.

Regulatory approval represents a key pivot point when a novel therapeutic strategy is translated into clinic. The demonstration of an acceptable balance of risk and clinical benefit should be demonstrated before this translation. Therefore, the challenges described above should be carefully considered in order to fasten the transition of this promising technology of in vivo reprogramming technology from pre-clinical models to clinical settings.

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