Dissertation

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Mathias Bernhardt, Master of Science in Molecular Medicine

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Reprogramming of human cancer cells towards induced pluripotency as a tool to study the influence of epigenetic modifications on the tumor phenotype

Referees: Prof. Dr. Viktor Umansky
Prof. Dr. Jochen Utikal

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Mathias Bernhardt

This thesis is dedicated to my parents for
their endless support.

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III. List of abbreviations

°C	Degree Celsius
µ	Micro
2-Me-5HT	2-Methyl-5-hydroxytryptamine
ABCB5	ATP-binding cassette transporter B5
AC	Adenylat cyclase
AIRD2	AT rich interactive domain 2
Akt	Protein kinase B
ALCAM	Activated leukocyte adhesion molecule
ALDH	Aldehyde dehydrogenase
AP	Alkaline phosphatase
APS	Ammoniumpersulfat
AS	Alternative state
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
bFGF	Basic fibroblast growth factor
BRAFi	BRAF inhibitor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CDH1	E-Cadherin
CDK4	Cyclin-dependent kinase 4
CDKN1C	Cyclin-dependent kinase 1C
CiPSCs	Chemically induced pluripotent stem cells
CML	Chronic myeloid leukemia
cMVPs	Cancer-specific methylation variable positions
CREB	cAMP response element-binding protein
D	Aspartic acid
DAPI	4’,6-diamidino-2-phenylindole
DCT	Dopachrome tautomerase
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DNMT3L	DNA methyltransferase 3-like
Dox	Doxycyline
DZNep	3-deazaneplanocin A
E	Glutamic acid
ECL	Enhanced chemoluminescence
ECT	Ectoderm
EGF	Epidermal growth factor receptor
EGFR	Epidermal growth factor
eIF4F	Eukaryotic initiation factor 4F
EMT	Epithelial-to-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
EpiSC	Epiblast stem cell

ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
ETS	E-twenty-six transformation specific
FCS	Fetal bovine serum
FSK	Forskolin
FSPα	Fibroblast-specific protein α
G	Gram
GAPDH	Glyceraldehyde 3-phosophate dehydrogenase
GNS cells	Glioblastoma-derived neural stem cells
gp100	Glycoprotein 100
GSK3ß	Glycogen synthase kinase 3
GSMs	γ-secretase
h	Hours
H&E	Haematoxyline and eosin
HGF	Hepatocyte growth factor
HH-GLI	HEDGEHOG-GLI
HMGS	Human melanocyte growth supplement
HT-144-dFLCs	HT-144-derived fibroblast-like cells
HT-144-dNLCs	HT-144-derived neuronal-like cells
hu	Human
iASPP	Inhibitor of apoptosis-stimulating protein of p53
ICAM1	Intercellular adhesion molecule I
ICM	Inner cell mass
IGF-1R	Insulin-like growth factor receptor 1
IL	Interleukin
iPCCs	Induced pluripotent cancer cells
iPSCs	Induced pluripotent stem cells
JAK	Janus kinase
JARID1B	Jumonji AT-rich interactive domain 1B
JNK	c-Jun N-terminal kinase
K	Lysine
KOSR	Knockout serum replacement
KSR	Kinase suppressor of RAS
l	Liter
LIF	Leukemia inhibitory factor
LNGFR	Low-affinity nerve growth factor receptor
LSCC	Lung squamous cell carcinoma
LTR	Long terminal repeat
M	Methionine
m	Milli
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
Mbd3	Methyl-binding-protein 3
MC1R	Melanocortin 1 receptor
McSCs	Melanocyte stem cells
ME	Mesoderm

MEK	Mitogen activated protein kinase 1 (MAP2K1)
MEKi	MEK inhibitor
MET	Mesenchymal-to-epithelial transition
MHC	Major histocompatibility complex
min	Minutes
MITF	Microphthalima-associated transcription factor
mM	Millimolar
MMP-3	Matrix metallo-protease-3
mRNA	Messenger RNA
MSCs	Mesenchymal stem cells
mTORC2	Mammalian target of rapamycine complex 2
n	Nano
NC	Neural crest
NCCs	Neural crest cells
NEAA	Non-essential amino acids
NF-κB	Nuclear factor kappa B
NHF	Normal human fibroblasts
NHiPSCs II	Normal human melanocyte-derived induced pluripotent stem cells
NRAS	Neuroblastoma rat sarcoma oncogene
NRAS	Neuroblastoma RAS viral oncogene homologe
N-region	Negative-charge regulatory domain
NRG1	Neuregulin 1
NSADs	Non-steroidal anti-inflammatory drugs
NSCLCs	Non-small cell lung cancer
NuRD	Nucleosome remodeling complex
ORF	Open reading frame
p16ink4A	P16 inhibitor of cell cycle kinase 4A
PanINs	Pancreatic intraepithelial neoplasia
PBS	Phosphate-buffered saline
PDACs	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PDK1	3'-phosphoinositide-dependent kinase-1
pERK	Phosphorylated ERK
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PRC	Polycomb repressive complex
PSC	Pluripotent stem cells
PTEN	Phosphatase and tensin homologe deleted on chromosome 10
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real-time polymerase chain reaction
R	Arginine
RAC1	Ras-related C3 botulinum toxin substrate 1
RAS	Rat sarcoma oncogene
RB	Retinoblastoma protein

RBD	RAS-binding-domain
RNA	Ribonucleic acid
Rock	Rho-associated coiled coil kinase
RPCs	Retinal pigmented cells
rPuro	Puromycin resistance
RTK	Receptor tyrosine kinase
SCCs	Squamous cell carcinoma
SCF	Stem cell factor
SCNT	Somatic nuclear transfer
shRNA	Short-hairpin RNA
siRNA	Small-interfering RNA
sm-mRNA-FISH	Single-molecule-mRNA-fluorescence-*in-situ*-hybridization
SNP	Single-nucleotide polymorphism
STAT3	Signal transducer and activator of transcription
STEMCCA	Stem cell cassette
SWI/SNF	SWIth/Sucrose NonFermentable chromatin-remodelling complex
TBS	Tris-buffered saline
TEMED	Tetramethylenediamine
TERT	Telomerase reverse transcriptase
TetO	Tetracycline operator
TNF	Tumor necrosis factor
Tp53	Tumor protein p53
TRP1	Tyrosine-related protein 1
TRP2	Tyrosine-related protein 2
Tuj-1	Neuron-specific class III β-tubulin
UV	Ultraviolet
V	Valine
v/v	Volume/volume
VC6T	VPA, CHIR99021, 616452, Tranylcypromine
VEE	Venezuelan equine encephalitis
VEGF	Vascular-endothelial growth factor
w/v	Mass/volumne
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
wt	Wild-type
αMSH	α-Melanocyte-stimulating hormone
kDa	Kilodalton
k	Kilo

IV. Abstract

Melanoma is the deadliest form of skin cancer. Traditional therapeutic options include surgery, radiation, chemotherapy and immunotherapeutic options. Typically, around 20% of patients develop metastases as the disease progresses, which reduces therapeutic options to provide only palliative benefit in the majority of cases. The genomes of a large number of primary and secondary melanomas were recently sequenced, leading to the identification of melanoma driver mutations. Targeting pathways, activated by cancer-specific genetic alterations, enabled researchers to develop novel therapeutic drugs. Although these drugs effectively fight melanoma cells, cancer cells develop various resistance mechanisms regulated by epigenetic changes, which leads to cancer recurrence in most patients.

Nuclear factor-based reprogramming was implemented in melanoma cells to test whether malignant cancer cells can reacquire developmental pluripotency, and moreover to analyze reprogramming-associated epigenetic changes on the tumor cell phenotype.

The results showed that the constitutive overexpression of Oct4, Sox2, and Klf4 reprograms melanoma cells into a murine embryonic stem cell-like state. In contrast to fibroblasts, melanoma cells do not require exogenous c-Myc for the induction of a pluripotent stem cell state, characterized by the reactivation of endogenous pluripotency markers and loss of the transcriptional profile of melanoma cells. However, continuous transgene expression is required to maintain an undifferentiated state. When injected into immunocompromised mice, melanoma-derived reprogrammed cells formed teratoma-like tumors containing cell types of all three germ layers, and despite their oncogenic mutations, rarely contained melanoma-like structures. *In vitro* directed differentiation into neuronal-like and fibroblast-like cells demonstrated that reprogrammed tumor cells acquired the potential to execute terminal differentiation pathways. Although most melanoma cell lines are highly depended on MAPK signaling, reprogrammed tumor cells and their differentiated daughter cells became resistant against BRAF- or MEK-targeting inhibitors, suggesting that epigenetic remodeling processes facilitated therapy resistance against targeted melanoma therapy. Furthermore, global gene expression profiling demonstrated that nuclear reprogramming and subsequent differentiation induced deregulation of tumor suppressors and oncogenes.

In conclusion, reprogramming cancer cells allows the investigation of a cancer genome in the context of a specific epigenetic cell state and might help study how alterations in the epigenetic signature control the biological behavior of tumor cells and their response to therapy.

V. Zusammenfassung

Das Melanom ist die gefährlichste Form aller Hautkrebsarten. Traditionelle Therapieoptionen umfassen operative Entfernung, Bestrahlung, Chemotherapie und immuntherapeutische Ansätze. Etwa 20% aller Patienten entwickeln während des klinischen Verlaufs Metastasen, wodurch sich die Behandlungsmöglichkeiten auf eine lediglich palliative Behandlung der Patienten reduziert. Während der letzten Jahre wurden zahlreiche Genome primärer und sekundärer Melanome entschlüsselt, was die Identifizierung von Melanom-initiierenden Mutationen ermöglichte. Das Wissen um die Aktivierung spezifischer, onkogener Signalwege erlaubte die Entwicklung zielgerichteter innovativer Medikamente. Diese neuartigen Medikamente bekämpfen zwar effektiv Melanomzellen, jedoch kommt es während der Behandlung zur Entstehung von Resistenzen. Bei vielen Patienten sind diese erworbene Resistenzen, die für die Bildung von Rezidiven verantwortlich sind, auf epigenetisch-regulierte Entstehungsmechanismen zurückzuführen.

In der vorliegenden Arbeit wurde zunächst untersucht ob Melanomzellen mit Hilfe von Transkriptionsfaktoren reprogrammiert werden und das Entwicklungspotential einer pluripotenten Stammzelle erwerben können, um dann die Auswirkungen epigenetischer Veränderungen auf den Phänotyp von Melanomzellen zu studieren.

Die vorliegenden Ergebnisse zeigen, dass die konstitutive Expression von Oct4, Sox2, und Klf4 ausreichend ist um Melanomzellen in einen Zustand zu reprogrammieren, der embryonalen Stammzellen der Maus ähnelt. Im Gegensatz zu Fibroblasten benötigen Melanomzellen dafür keine exogene Expression des onkogenen Transkriptionsfaktors c-Myc. Der stammzellähnliche Zustand der reprogrammierten Melanomzellen zeichnet sich durch die endogene Reaktivierung von Pluripotenzfaktoren und durch den Verlust des Transkriptionsprofils der ursprünglichen Melanomzellen aus. Allerdings war eine kontinuierliche Transgenexpression notwendig, um die Zellen in einem pluripotenten Zustand zu halten. In immunkompromittierten Mäusen erzeugten reprogrammierte Melanomzellen Teratom-ähnliche Tumore, die Zelltypen aller drei Keimblätter aufwiesen. Trotz der in den Zellen vorhandenen onkogenen Mutationen, waren in den Tumoren kaum Melanomzellen detektierbar. Die gezielte Differenzierung in neuronale und Fibroblasten-ähnliche Zellen *in vitro* zeigte, dass die reprogrammierten Zellen die Fähigkeit erlangten terminal zu differenzieren.

Obwohl die große Mehrheit der parentalen Melanomzellen von der Aktivierung des MAPK-Signalweges abhängig ist, wiesen sowohl die reprogrammierten als auch die daraus differenzierten Tochterzellen eine vergleichsweise erhöhte Resistenz gegenüber BRAF- und MEK-Inhibitoren auf. Dies führte zur Vermutung, dass epigenetische Veränderungsprozesse die Entwicklung von Resistenzen gegen zielgerichtete Melanom-Medikamente ermöglichen.

Darüber hinaus zeigten globale Genexpressionsanalysen eine durch nukleäre Reprogrammierung und auch durch anschließende Differenzierung induzierte Hoch- und Herunterregulation von Tumorsuppressor- und Onkogen-codierenden Genen.
Zusammenfassend ermöglicht die Reprogrammierung von Tumorzellen die Untersuchung des Genoms einer Krebszelle im Kontext spezifischer epigenetischer Zellprofile. Die vorliegende Arbeit vertieft somit das Wissen darüber, wie epigenetische Modifikationen das biologische Verhalten von Melanomazellen und deren Therapie beeinflussen.

VI. Introduction

VI.1. Pluripotency

The development from the totipotent zygote to the fully evolved organism describes a tightly regulated process of transitions between cellular states. Key events are initialized by transcription factors modifying the cell's epigenome.

These epigenetic modifications are reversible, so the differentiation process is not a one-way street. The most feasible technique, discovered in Shinya Yamanaka's ground-breaking research, is the ectopic overexpression of key transcription factors that facilitate pluripotency. The resulting cells are called induced pluripotent stem cells (iPSCs), and have had enormous influence on interdisciplinary research fields such as developmental biology, regenerative medicine, and oncology to name only a few. A hallmark of these cells is their potential to differentiate into all somatic and germline cells of a developing organism. Therefore, hopes are high that pluripotent stem cells can be made to differentiate into specialized cells suitable for cell replacement therapies, drug tests, and disease modelling.

VI.1.1. Pluripotent cells during development

Pluripotent stem cells occur at early stages during embryonic development and can be isolated from different sources. Ethical issues have limited research mainly to mice, but experiments have shown that much of the information gained from studying stem cell development in mice can be applied to human pluripotent stem cells. Nevertheless, certain features are unique to each system. This section will first summarize the current state of the art, and then discuss the differences between murine and human pluripotent stem cells in detail.

As the totipotent zygote undergoes cell divisions, a hollow structure forms in which the inner cell mass (ICM) resides. The explantation of the ICM generates colonies of embryonic stem cells (ESCs). *In vivo*, the ICM develops into the epiblast and the hypoblast, but only epiblast stem cells (EpiSCs) have the potential to give rise to all somatic cells. Besides ICM cells and EpiSCs also primordial germ-line cells can be used as source for pluripotent stem cells (Shamblott et al. 1998; Shim et al. 1997). They develop during gastrulation, migrate through the embryo, and form the gonads later in life. Although pluripotent stem cells can be derived from these three sources, each cell type requires particular culture conditions *in vitro*. Here the features of the most common types, ESCs and EpiSCs, are outlined.

VI.1.2. Embryonic stem cells

It has been demonstrated that cells from the ICM can be isolated and expanded on feeder cells in the presence of leukemia inhibitory factor (LIF) (Smith et al. 1992). This cytokine belongs to

the interleukin (IL)-6 family and has a broad influence on a variety of physiological and developmental processes (reviewed in Hirai et al. 2011), such as the activation of Signal Transducer and Activator of Transcription 3 (STAT3) signaling, which is of crucial importance for ESC maintenance. After the LIF receptor binds its ligand, it heterodimerizes with gp130, followed by the phosphorylation of Janus kinase (JAK) I and II. Activated JAKs phosphorylate gp130, enabling STAT3 to bind the kinases, promoting phosphorylation and homodimerization of STAT3. The dimers translocate into the nucleus, regulating the expression of its target genes by binding their enhancer elements (Chen et al. 2008). In particular, STAT3 induces Nanog expression, a key molecule of the pluripotency network (Chambers et al. 2003). Consequently, the level of STAT3 activation correlates with the potential to keep ESCs from differentiating, indicating that a critical limit of STAT3 activation is required to prevent differentiation (Raz et al. 1999). In 2008, Austin Smith and colleagues discovered that protecting ESCs from differentiation-inducing stimuli provides optimal culture conditions to maintain undifferentiated ESCs. Therefore, ESCs were cultivated in the presence of the mitogen activated protein kinase kinase (MEK) inhibitor PD0325901, the glycogen synthase kinase-3 (GSK3) inhibitor CHIR99021, and LIF (2i+Lif). Under these specific conditions, ESCs homogenously expressed Oct4 and Nanog at strongly correlating levels (Descalzo et al. 2012), and were characterized by lower expression of lineage specifiers, reactivation of both X-chromosomes in female cells, and fewer bivalent chromatin domains, indicating a more naïve state of pluripotency (Marks et al. 2012).

VI.1.3. Epiblast stem cells

EpiSCs are at a later developmental cell stage than ESCs. Upon implantation of the embryo in the uterus, a single layer of epithelial cells derives from the ICM to form the epiblast. These cells can be isolated from embryos on day 5.5-7.5 and cultivated in the presence of basic fibroblast growth factor (bFGF) and Activin A (Brons et al. 2007; Tesar et al. 2007), but they spontaneously revert to ESC-like cells under permissive culture conditions (Bao et al. 2009). Besides the conditions required for isolation and cultivation of ESCs and EpiSCs, the cell types differ in other ways. EpiSCs grow in single layers of colonies and require cell-cell contact for survival (Greber et al. 2010). Their developmental potential is more restricted, as demonstrated by their poor contribution to chimeric mice and by the inactivation of one X-chromosome in female cells (Brons et al. 2007; Tesar et al. 2007). Furthermore, EpiSCs express early lineage-commitment markers and major histocompatibility complex (MHC) class I molecules. Nevertheless, *in vitro* the cells are capable of multilineage differentiation, and *in vivo* they form teratomas. Notably, they are dependent on bFGF, ERK, Activin A, and TGF-ß, but independent of LIF/STAT3, indicating that EpiSCs respond differently to self-renewing and differentiation

stimuli compared to ESCs. These cells represent a population of pluripotent stem cells that is already primed for certain differentiation stimuli.

In conclusion, cells representing the *in vitro* counterparts of pre-implantation cells are in the so called “naïve” state of pluripotency, while cells representing post-implantation cells like EpiSCs are in the “primed” state of pluripotency (reviewed in Nichols & Smith 2009).

Features of pluripotent states in rodents	Naive state	Primed state
Cell types (*in vivo/ in vitro*)	Pre-implantation inner cell mass/ ESCs	Post-implantation epiblast/ EpiSCs
Teratoma formation	Yes	Yes
Chimera contribution	Yes	No
Signaling pathways maintaining pluripotency	LIF/STAT3, BMP4	TGF-ß, Activin, bFGF, ERK1/2
Marker signature		
Oct4, Sox2	High	High
Nanog, Klf2, Klf4, Rex1	High	Low
MHC class I	Nearly absent	Expressed
XX status	XaXa	XiXa
Lineage specifiers	Absent	Expressed
Clonogenicity	High	Low
Morphology	Domed-shaped, tightly packed colonies	Flat, large colonies
Response to 2i	Self-renewal	Cell death

Table 1 | Characteristics of naïve state and primed state pluripotent stem cells.

VI.1.4. Human embryonic stem cells

James Thomson was the first to isolate human ESCs. Similar to murine ESCs, a preimplanted blastocyst was explanted and its ICM cells isolated (Thomson et al. 1998). In contrast to mouse ESCs, these cells require bFGF and Activin supplementation, are sensitive to single-cell dissociation, and form flat colonies. Under conventional conditions, isolated hESCs display X-chromosome inactivation, although this also depends on oxygen levels. Isolation under physiological oxygen conditions (5% O_2) results in ESCs with pre-inactivated X-chromosomes (Lengner et al. 2010). Taken together, human ESCs share more features with murine EpiSCs than with mESCs, raising questions about their state of pluripotency. One possible explanation might be that human cells derived from the inner cell mass continue to differentiate into emerging EpiSCs which are stabilized by bFGF. In rodents, the late blastocysts can be blocked

in their development, resulting in the inhibition of the implantation process (Lopes et al. 2004). This stasis is called diapause, and it can be reversed by LIF signaling (Nichols et al. 2001), suggesting that cells derived from explanted mouse blastocysts might remain in a diapause-like state, allowing ESCs to be isolated. Nevertheless, there are hints that a pause in the blastocyst's development is also possible in mammals (Ptak et al. 2012), suggesting that human stem cells in early developmental stages exist in a dynamic pluripotent state based on environmental conditions. According to these results, overexpression of Klf4 or c-Myc in human ESCs, and also iPSCs under 2i+LIF conditions, cause them to revert to a more naïve state, corresponding to murine ESCs in terms of X chromosome inactivation, resistance to single-cell dissociation, and global gene expression profile (Hanna et al. 2010a). In the search for conditions that stabilize human ESCs without the need for transgene expression, researchers identified inhibitors of Jun kinase and p38 MAP kinase that convert human ESCs or iPSCs in the naïve state in combination with 2i+LIF, bFGF, and TGF-ß (Gafni et al. 2013). Morphologically similar to mESCs, these cells show various epigenetic modifications that lead to a global reduction in DNA methylation and bivalent chromatin marks. Interestingly, these cells were able to contribute to murine chimeric embryos at a developmental state comparable to E8.5-E10.5 after microinjection into morulae. Nevertheless, recent discoveries indicate that these cells fail to express crucial epigenetic regulators and remain dependent on bFGF and TGFβ signaling. Instead, short-term expression of Nanog or Klf2 for eight days allows iPSCs to be cultivated in serum and growth factor depleted medium containing GSK3β and PKC inhibitors, supplemented with human LIF (Takashima et al. 2014). Due to their epigenetic resetting, these cells are called "reset human iPSCs" and share the transcription factor circuitry, facilitating naïve pluripotency in murine ESCs. Application of a kinase inhibitor mixture comprising MEK, BRAF, GSK3β, Src, and Rho-associated coiled-coil protein kinase (ROCK) inhibitors, in addition to LIF and Activin A, activates Oct4 distal enhancer elements similar to naïve mouse ESCs (Theunissen et al. 2014). These cells showed more similarity in global gene expression with cells derived after short-term expression of Nanog and subsequent cultivation in 2i+LIF (Takashima et al. 2014) than each cell type compared to other potential naïve human iPSCs. There was an additional screen in human ESCs, designed to identify small molecule inhibitors with the ability to enhance Nanog expression. The combination of three inhibitors, namely PD0325901, the GSK3 inhibitor BIO, and the BMP inhibitor Dorsomorphin, is able to keep ESCs pluripotent, although their self-renewal potential was decreased. Supplementation with LIF rescued this phenotype and generated ESCs with a gene signature of pre-implantation epiblast cells (Chan et al. 2013). Another group discovered that epigenetic marks defining the primed state can be reverted by pretreatment with histone deacetylase inhibitors, followed by culture in 2i with bFGF. Alternatively, naive ESCs can be created by direct isolation from embryos in 2i with bFGF (Ware et al. 2014). This suggests that

human naïve pluripotent stem cells (PSCs) require bFGF for pluripotency, in contrast to murine ESCs, which are destabilized by the growth factor.

VI.1.5. Reprogramming somatic cells towards induced pluripotency

Changing a cell's fate by resetting its epigenetic profile to a pluripotent stem cell can be achieved by somatic cell nuclear transfer (SCNT) and cell fusion. Both techniques are technically challenging and require the use of human embryos produced by *in vitro* fertilization or human oocytes, raising ethical issues about the protection of unborn life. Therefore, Shinya Yamanaka's 2006 report on reprogramming somatic cells into pluripotent stem cells similar to ESCs revolutionized not only the field of regenerative medicine, but also our notion of development. He proved that ectopic overexpression of four transcription factors, namely Oct4, Sox2, Klf4, and c-Myc, is enough to reset the epigenetic profile of murine or human somatic cells, generating a pluripotent stem cell similar to ESCs (Takahashi & Yamanaka 2006; Takahashi et al. 2007). These transcription factors are now called Yamanaka factors, and are either introduced by retroviral (Takahashi & Yamanaka 2006; Takahashi et al. 2007) or lentiviral (Stadtfeld et al. 2008a; Yu et al. 2007) vectors, as well as by non-integrating methods like plasmid transfection (Narsinh et al. 2011; Okita et al. 2008; Piao et al. 2014; Si-Tayeb et al. 2010) or adenoviral vectors (Stadtfeld et al. 2008c; Zhou & Freed 2009). Today, there are DNA-free techniques that are more suitable for the generation of clinically applicable iPSCs, such as factor expression by Sendai virus (Fusaki et al. 2009; Seki et al. 2010), direct protein delivery (Kim, D. et al. 2009; Zhou et al. 2009), and mRNA transfection (Warren et al. 2012). Lately, Venezuelan equine encephalitis (VEE) virus-based RNA vectors have been particularly useful in simplifying the generation of iPSCs. The synthetic, polycistronic, self-replicative RNA facilitates transgene-free reprogramming by a single transfection (Yoshioka et al. 2013). MicroRNA genes also hold the potential to promote iPSC generation. Human and mouse somatic cells that overexpress the miR-302/367 (Anokye-Danso et al. 2011) or miR-302/372 cluster (Subramanyam et al. 2011) and a combination of miR-200c, miR-302s and miR-369s (Miyoshi et al. 2011) convert into iPSCs.

Today, we know that induction of pluripotency is hierarchically organized by a network of transcription factors, which means that transcription factors can be substituted by one or a combination of other factors (Buganim et al. 2012). Single-cell expression analysis of 48 genes during reprogramming allowed researchers to model a Bayesian network (figure 1), which is turned on by endogenous activation of Sox2. According to the model, Sox2 switches on Sall4, which itself activates Oct4 and three other factors. Only Nanog and Dppa2 function independently of the key regulators, Sox2 and Oct4. The model was verified by the generation

of fully reprogrammed cells with a combination of Sall4, Esrrb, and Lin28 in combination with either Nanog or Dppa2.

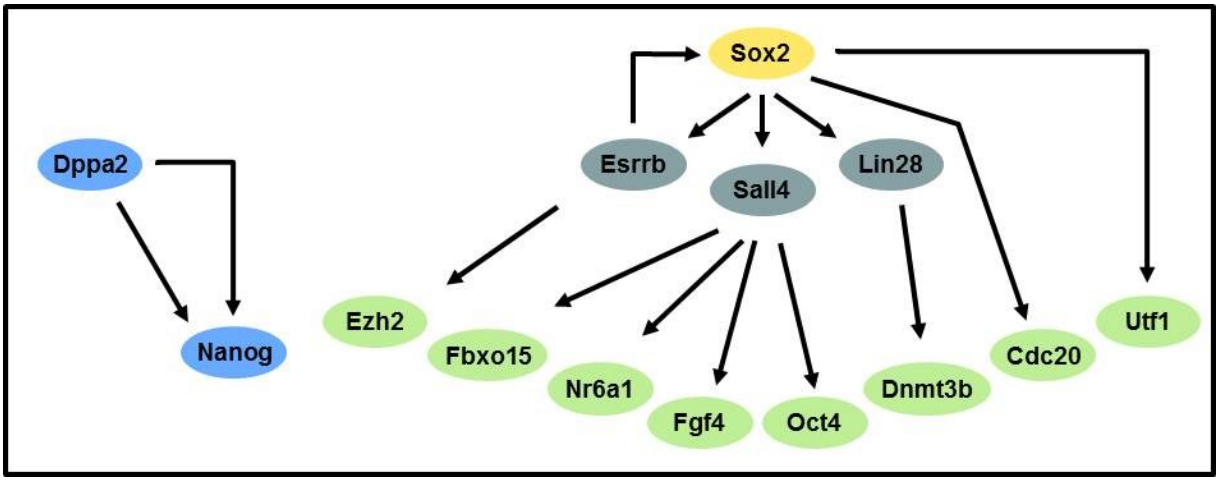


Figure 1 | Hierarchical organization of the transcriptional events in reprogramming. Adapted with permission from Buganim et al. (2012).

In addition, factors unlisted in this model can replace the transcription factors of the Yamanaka cocktail. For example, the orphan receptors Nr5a2, Nr5a1, and Glis1 can replace Oct4 and c-Myc. Furthermore, members of distinct transcription factor families can often take over some of the functions of original Yamanaka factors. Accordingly, Sox1 and Sox3 can replace Sox2; Klf2 and Klf5 can compensate for Klf4, and L- and N-Myc for c-Myc. iPSCs containing exogenous Myc genes harbor a potential tumorigenic risk. Therefore, many studies focus on generating oncogene-free iPSCs more suitable for transplantation studies. Although researchers have discovered that exogenous c-Myc is dispensable for the induction and maintenance of pluripotency (Nakagawa et al. 2008; Wernig et al. 2008b), there is evidence that it facilitates chromatin engagement of the residual Yamanaka factors, therefore enhancing the efficacy of pluripotency induction (Soufi et al. 2012). In mice, more than 16 distinct combinations of transcription factors were identified to produce MEF-derived iPSCs (reviewed in Theunissen & Jaenisch 2014). Nevertheless, in addition to the activity of specific transcription factors, completion of other requirements is a prerequisite for reprogramming. Somatic cells have to complete the epithelial to mesenchymal transition (EMT) (Li, R. et al. 2010) to be able to acquire pluripotency. This event takes place early during the reprogramming process (Samavarchi-Tehrani et al. 2010), and is characterized by the up-regulation of the cell adhesion molecules E-cadherin (CDH1) (Chen et al. 2010; Li, R. et al. 2010), epithelial cell adhesion molecule (EpCAM) (Huang et al. 2011), and intercellular adhesion molecule (ICAM1) (O'Malley et al. 2013). Overexpression of these epithelial regulators prevents nuclear localization of β-catenin, which can replace Oct4 in the Yamanaka cocktail (Redmer et al. 2011).

Far less is known about the substitution of factors in human cells. It has been shown that Nanog and Lin28 can replace c-Myc and Klf4 (Yu et al. 2007), that Glis1 can substitute for c-Myc (Maekawa et al. 2011), and the epigenetic inducer Rcor2 can restore the function of Sox2 (Yang et al. 2011). The surprising discovery that the pluripotency inducers Oct4, Sox2, and Nanog possess specific lineage-inducing functions gave rise to an alternative approach to establish iPSCs. In ESCs, Oct4 induces mesendoderm (ME) and primitive endoderm differentiation, but inhibits the differentiation into ectoderm (ECT) cells, while Sox2 promotes neural ECT differentiation but suppresses ME differentiation (Thomson et al. 2011; Wang et al. 2012). Apparently, maintaining the pluripotent state depends on the balanced activity of lineage-inducing factors. These were initially considered as antagonists of pluripotency factors, but Shu *et al.* and Montserrat *et al.* discovered their potential to restore the pluripotent state by counteracting the up-regulation of ME or ECT genes, enabling them to replace Oct4 or Sox2. More specifically, a larger scale analysis identified Gata6, Sox7, Pax1, Gata4, C/EBPα, HNF4a, and Grb2 as potential Oct4 substitutes, all of them acting as ME lineage specifiers. Applying the same method, they were able to replace Sox2 with Sox1, Sox3, and Gmn, and finally replace Oct4 and Sox2 simultaneously (Shu et al. 2013). In parallel, Montserrat *et al.* demonstrated that human fibroblasts can be reprogrammed in the presence of Klf4 and c-Myc, using Gata3 as a replacement for Oct4, and the ectodermal specifiers ZNF521, OTX2, and PAX6 as a replacement for Sox2. Based on these results, the hypothesis of the so-called “seesaw” model arose, according to which, somatic cells are more likely to acquire pluripotency when opposing differentiation potentials counteract each other. Researchers are now working to generate transgene-free iPSCs that are more suitable for therapeutic purposes. Large-scale analyses of small molecule compounds identified signaling enhancers or inhibitors with the potential to substitute for individual Yamanaka factors. MEFs from transgenic mice carrying GFP in their endogenous Oct4 loci were screened with more than 10.000 molecules revealing a function of Forskolin (FSK), 2-Methyl-5-hydroxytryptamine (2-Me-5HT), and D4476 as Oct4 replacements. Nevertheless, in combination with chemical Sox2 substitution (VC6T (VPA, CHIR99021, 616452, Tranylcypromine)) the cells did not fully reprogram. In order to achieve complete chemical reprogramming, the screen was repeated with cells expressing Oct4 selectively in the early phase of reprogramming. Agonists of cAMP and epigenetic modulators like 3-deazaneplanocin A (DZNep) facilitated late reprogramming. Nevertheless, VC6T treatment, in combination with FSK, followed by the addition of DZNep, resulted in ESC-like cells displaying reduced pluripotency marker expression. After switching to 2i medium, germline-competent chemically induced pluripotent stem cells (CiPSCs) appeared (Hou et al. 2013). Due to the lack of oncogenic transgene integrations, chimeric mice derived from CiPSCs remained healthy for more than 6 months. These results on the chemical replacement of Oct4 and the detailed investigation of different reprogramming phases allow us to speculate

about the mechanism behind the generation of human iPSCs. Sheng Ding's group discovered that combined inhibition of TGF-β, histon-deacetylases, and MEK in combination with the small molecule activator 3'-phospoinositide-dependent kinase-1 (PDK1) in Oct4-expressing human cells successfully produced iPSCs (Zhu et al. 2010). However, their intended aim, to generate human iPSCs only by chemical compounds, has still not been achieved, potentially due to the distinct prerequisites for the induction and maintenance of pluripotency in human cells.
Similar to ESCs, iPSCs can switch between distinct pluripotent states. Reprogramming of fibroblasts by ectopic expression of Oct4, Sox2, Klf4, c-Myc and Nanog in the presence of LIF results in tightly packed colonies expressing surface marker profiles similar to murine ESCs. These so called hLR5 cells shared features of naïve pluripotent stem cells like the response to LIF stimulation. However, the cells did not stabilize the pluripotent state or reactivate endogenous pluripotency genes (Buecker et al. 2010). This might indicate that either the culture conditions were not sufficient to support maintenance of pluripotency or hLR5 were not fully reprogrammed. Besides the composition also the stoichiometry of the exogenous reprogramming factors influence the properties of the resulting iPSCs (Carey et al. 2011; Papapetrou et al. 2009). Taken together, biological properties of iPSCs are dependent on the interplay of the transcription factors, their stoichiometry and the culture conditions used for reprogramming.

VI.1.6. Molecular characteristics in different phases of reprogramming

Reprogramming is a very inefficient process with a technique-dependent range from 0.00001% (transgene-free and viral-free) up to ≤1% (mRNA-based) cells generating iPSC colonies. In need to study the reprogramming process in genetically identical cells, mice entirely derived of iPSCs (all-iPSC mice) were generated carrying inducible stem cell factors. Fibroblasts from all-iPSCs mice displayed higher reprogramming efficiencies than other primary somatic cells (Carey et al. 2010; Wernig et al. 2008a) and enabled researchers to uncover distinct phases during the reprogramming process. Three consecutive events were identified by transcriptome profiling time course experiments: an initiation phase, a maturation and finally a stabilization phase (Samavarchi-Tehrani et al. 2010). The first period is characterized by initial transcriptional and epigenetic changes due to overexpression of the reprogramming factors, resulting in mesenchymal-to-epithelial transition (MET) (Li, R. et al. 2010; Samavarchi-Tehrani et al. 2010). As already mentioned, these changes become manifested on molecular level by the gain of epithelial markers like EpCAM (Huang et al. 2011) or CDH-1 (Chen et al. 2010; Li, R. et al. 2010) as well as the loss of somatic cell signature. With the goal to predict subpopulations of cells undergoing reprogramming and separate them from intermitted cells, several surface molecules but also genomic markers were analyzed. Studies demonstrated that early reprogramming initiation is accompanied by Thy1 down-regulation, SSEA-1 and AP

increase followed by Oct4 activation. Two waves of molecular changes were discovered in cells regarding their Thy1, SSEA-1 and Oct4-GFP status. The first wave takes place between day zero and day three, is driven by Klf4 and c-Myc and leads to an up-regulation of genes involved in cell metabolism, proliferation and cytoskeletal organization. Although Thy1-positive cells initiate the first wave, they fail to go through the second wave. The second wave is initiated by Oct4, Sox2, and Klf4 and is associated with the onset of pluripotency-related genes (Polo et al. 2012). It begins at day nine exclusively in SSEA-1-positive cells, even though the majority of SSEA-1 expressing cells never generated iPSCs. This indicates the need for more precise markers like ICAM1, which is homogeneously expressed in pluripotent stem cells. Nevertheless, the discovery that two distinct waves of transcriptional changes are responsible for the cell fate change towards pluripotency has been confirmed using a combination of ICAM1, CD44 and a Nanog reporter system (O'Malley et al. 2013). On day six after transgene induction $CD44^-/ICAM1^+$ cells replace $CD44^+/ICAM1^-$ cells and activate the Nanog reporter. Here, $CD44^-$ and $ICAM1^+$ cells closely correlate to Nanog expression which has been discovered to be a rate-limiting step. Furthermore, the same study proves the up-regulation of particular endogenous pluripotency markers by the time CD44 disappears, and discovers the transient up- and down-regulation of several epidermal genes in intermediate cells (O'Malley et al. 2013). These results have also been described in previous publications (Mikkelsen et al. 2008; Sridharan et al. 2009), highlighting the complexity of the reprogramming process. Furthermore, even in human cells the transition from initiation to maturation phase has been demonstrated to represent the “bottleneck” regarding the acquisition of pluripotency. Although the majority of reprogrammed human somatic cells expresses the pluripotency marker Tra-1-60, only a fraction of ~1% actually becomes pluripotent (Tanabe et al. 2013). The last step during the reprogramming process describes the transition to stable pluripotent stem cells undergoing self-renewal independent from transgene expression. Therefore, the repression of the Yamanka factors is a critical step towards the stabilization phase. Genes involved in this process are classified into two groups: one being responsible for stem cell maintenance and one selective for transition, indicating that the transition and maintenance of pluripotency are regulated by different mechanisms. In more detail, a siRNA screen of late reprogramming cells proved the importance of pluripotency factors for the maintenance of iPSCs, while the network regulating the acquisition of the competent state contained molecules distinct from the Yamanka factors. Among those have been regulators of cytoskeletal remodeling, chromosomal organization and segregation as well as distinct signaling pathways, indicating the importance of other molecules than the core pluripotency markers for survival upon transgene suppression *(Golipour et al. 2012).* Once cells transit to the last phase, the stabilization phase, they acquire full pluripotent potential shortly after transgene suppression (Okita et al. 2007; Stadtfeld et al. 2008b). Single cell analysis by Fluidigm and single-molecule-

mRNA-fluorescence-*in-situ*-hybridization (sm-mRNA-FISH) revealed endogenous Sox2 to be a discriminating marker of this event, regulating the Bayesion network (Buganim et al. 2012). This last phase of the reprogramming process is time-consuming and slow. It is characterized by numerous epigenetic modifications such as changes in DNA methylation. Especially human cells require extensive passaging until their epigenetic memory is lost and iPSCs become more and more similar to human ESCs (Chin et al. 2009). Furthermore, stabilization of pluripotency is associated with telomere elongation back to embryonic length (Marion et al. 2009) and the reactivation of inactivated X-chromosomes in female cells of mouse and human (Lengner et al. 2010; Maherali et al. 2007; Tomoda et al. 2012).

VI.1.7. Kinetics during the reprogramming process

Different hypothesis arose about the predictability of the reprogramming process based on discoveries that iPSC generation follows distinct kinetic events. The stochastic model assumes that every single cell can potentially reprogram and acquire a pluripotent state. Nevertheless, the modality differs considerably, and random rate-limiting incidents are responsible for the variable latencies. Accelerations of the kinetics can be achieved by modifications affecting the rate of cell-divisions, like the overexpression of Lin28, or the disruption of the p53/p21 complex, or cell-division-independent changes like Nanog overexpression (Hanna et al. 2009c). Time course investigations in single cells derived from a secondary system suggest that stochastic processes and deterministic phases alternate. Interestingly, the studies from *Buganim et al.* suggest an early stochastic phase and late Sox2-dependent deterministic progress (Buganim et al. 2012). In contrast, the molecular roadmap defined by *Polo et al.* assumes two transcriptional waves separated by a stochastic middle part (Polo et al. 2012). In both models, the stochastic events represent rate-limiting roadblocks. The elimination of methyl-binding-protein 3 (Mbd3) accelerates reprogramming speed and raises efficiency up to nearly 100% identifying the nucleosome remodeling and deacetylation (NuRD) complex as major reprogramming roadblock (Rais et al. 2013). Mbd3 has been shown to bind target sites of Oct4, Sox2, Klf4 and c-Myc after transcription factor induction, therefore suppressing the initiation of reprogramming. Accordingly, Mbd3 knockdown cells generated iPSC-like colonies within six days after transgene induction. In contrast, other studies of genetic and small-interfering RNA (siRNA)-mediated knockdown and knockout of Mbd3 (Dos Santos et al. 2014) as well as short-hairpin RNAs (shRNA)-mediated suppression (Onder et al. 2012) have been shown to have no effect on the reprogramming kinetics of mouse fibroblasts. Noteworthy, also a subpopulation of privileged cells has been demonstrated to reprogram according to the deterministic model (Guo et al. 2014). Within granulocyte/monocyte-progenitors a small number of cells characterized by ultra-fast cycling account for the majority of iPSCs after 4 to 5 cell divisions. In addition, expression of reprogramming factors can induce privileged cells

within a fibroblast population, suggesting a rather dynamic state. In accordance with these data, reprogramming barriers can be eliminated increasing the reprogramming efficiency not only by genetic manipulation but also with the help of small molecule inhibitors, challenging the assumption of the stochastic model.

VI.1.8. Epigenetic modifications during the reprogramming process

In contrast to nuclear transfer based reprogramming, the DNA demethylation process initiated *via* transcription factor overexpression, requires a longer period of time. For this reason, low-passage iPSCs retain residual methylation marks at distinct sequence sites leaving iPSCs with an epigenetic memory (Kim, K. et al. 2010). These specific molecular signatures substantially affect their differentiation potential, highlighting the influence of the parental cells on the phenotype of their reprogrammed daughter cells. Consequently, low-passage iPSCs predominantly differentiate into the lineage of their parental cells (Bar-Nur et al. 2011; Kim et al. 2011). Nevertheless, with continuous passaging epigenetic marks are gradually resolved and molecular as well as functional differences abrogate, resulting in indistinguishable iPSCs independent of their origins (Polo et al. 2010). In pluripotent stem cells the epigenetic reorganization is highly dynamic, indicated by an upraised turnover rate compared to somatic cells. Indeed, antagonistic effects balance, and therefore stabilize the canonical epigenetic profile (Shipony et al. 2014). In contrast to stem cells, somatic cells inherit epigenetic information to their daughter cells, making them vulnerable for the manifestation of epigenetic alterations. Accordingly, comparative chromatin modification and transcription program analysis between ESCs and iPSCs show little differences (Bock et al. 2011; Chin et al. 2010; Doi et al. 2009; Guenther et al. 2010). Nevertheless, functional analysis revealed that iPSCs differ from ESCs in their developmental potential, as only few were able to produce all-iPSC mice (Boland et al. 2009; Kang et al. 2009; Zhao et al. 2009). In order to investigate this observation in more detail, murine ESC transduced with the Yamanaka factors have been used to generate viable mice. From these transgenic mice cells derived of various somatic tissues were reprogrammed into iPSCs and analyzed for molecular differences compared to their genetically identical ESC *(Stadtfeld et al. 2010)*. The comparison demonstrated the differential expression of exclusively two molecules; the non-coding RNA *Gtl2* and the small nucleolar RNA *Rian*. Treatment of iPSCs with ascorbic acid or alternatively with the histone deacetylase inhibitor valproic acid reactivated the silent gene cluster, resulting in murine iPSCs with an equal molecular profile as ESCs and the potential to efficiently generate all-iPSC mice (Stadtfeld et al. 2012). Noteworthy, to date similar detailed molecular analysis between human ESC and iPSCs are ethically not feasible. Nevertheless, vitamin C also seems to improve the induction of pluripotency in human cells. Here, it decreases cellular senescence during the process and therefore increases reprogramming efficiency (Esteban et al. 2010). Furthermore,

these studies demonstrated that alterations in environmental conditions during the reprogramming process affect the epigenetic and biological outcome.

VI.1.9. Application of induced pluripotent stem cells for investigating molecular mechanisms in disease pathology and regenerative medicine

The iPSC technology holds great promises for future medical improvements. The cells can be expanded and cultured indefinitely providing a never-ending source for cell replacement attempts. Their differentiation into insulin-producing β-cells (Hua et al. 2013; Jeon et al. 2012), functional hepatocytes (Chen, Y. F. et al. 2012; Takayama et al. 2012), cardiomyocytes (Itzhaki et al. 2011; Kim, C. et al. 2013), and dopaminergic neurons (Devine et al. 2011; Jiang et al. 2012; Swistowski et al. 2010) give a reason to believe that cell replacement strategies for diabetes, liver cirrhosis, cardiovascular disease and neurodegenerative diseases like Parkinson's disease are within reach without the concern of immune rejection. Nevertheless, the tumor-forming potential of iPSCs or ESCs forms the major hurdle of transplantation studies to go into clinical application and request strict differentiation protocols with defined selection methods for the isolation of differentiated cells. Therefore, cell replacement strategies are currently of minor importance while iPSCs are already well-recognized for disease modeling, drug screens and toxicity tests. Their greatest advantage is that studies can be performed directly with the cell-type of interest derived from individual patients without fearing the problem of limited sources. There are numerous examples of drug tests that have failed due to the lack of that possibility demonstrating the need for precise models mimicking diseases. Preclinical experiments in mice sometimes do not fulfill this criteria for the reason that drugs were effective in mice but not in human or *vice versa* (Tobert 2003). Furthermore, even genetically manipulated human cells often do not resemble disease mechanisms adequately and demonstrate the demand of drug validations in the system that is actually affected by the disease. For example, the properties of non-steroidal anti-inflammatory drugs (NSADs) as modulators of γ-secretase (GSMs) resulting in reduced levels of Aβ42 have been investigated in APP-transgenic non-neuronal cells. While micromolar concentrations have successfully been able to reduce Aβ42 ratios, neurons derived from patient-iPSCs have been completely resistant (Mertens et al. 2013). Especially the investigation of neuronal diseases like Parkinson, Alzheimer and Huntington's disease benefits from iPSCs-based disease modeling. Defined neural inducers enable researchers to produce glial cells (Wang et al. 2013), dopaminergic (Ha et al. 2011; Ryan et al. 2013) and striatal neurons (An et al. 2012; Nelson et al. 2014) from iPSCs derived from patients carrying disease-causing mutations. These specific neuronal cell populations are then subjected to studies comparing disease affected and healthy cells.

Even molecular insights in oncogenic processes have been found using iPSCs. The group of Ohnishi et al. (2014) connected nuclear factor-based reprogramming and tumorigenic transformation of cells *in vivo*. Using iPSC-derived chimeric mice with doxycycline inducible transcription factors, they demonstrated that the reprogramming factors drive tumor initiation. Short-term induction of the transgenes generates reversible dysplasia in various organs but prolonged exposure resulted in tumor formation with de-differentiated phenotypes distinct from teratomas. Performing methylation analysis between ESCs/iPSCs, normal kidneys and kidney tumors, they identified that failed repression of ESC-polycomb repressive complex (PRC) targets is responsible for the generation of transgene-independent tumors. Furthermore, they found a PRC signature similar to Wilms' tumors, a pediatric kidney cancer and have been able to show an identical methylation profile within samples of this specific tumor (Ohnishi et al. 2014).

In spite of unresolved questions concerning the tumorigenic potential of iPSCs, the first clinical study started in august 2014 using autologous iPSC-derived retinal pigment cells (RPCs) for transplantation into patients suffering from exudative age-related macular degeneration (Sipp 2013).

VI.1.10. Induced pluripotent cancer cells

Reversible epigenetic modifications regulate the differentiation process generating specialized cells of an organism. Additionally, epigenetic alterations combined with irreversible genetic mutations also play a role in the transformation of tumor cells. Accordingly, several hints exist that tumors arise selectively in the context of a specific developmental state. This suggests that on the one hand chromatin modifications regulate differentiation processes, on the other hand they can affect the tumorigenic potential of cells. A way to gain insight into this interplay of epigenetic modifications and transforming events is based on the utilization of cancer-derived iPSCs, so called induced pluripotent cancer cells (iPCCs). The generation and validation of iPCCs is similar to somatic iPSCs. However, the generation of iPCCs is more difficult to achieve, probably due to their various epigenetic and genetic aberrations. The first report of reprogrammed cancer cells came from the Jaenisch laboratory. They generated murine ESC cell lines derived from the RAS-inducible melanoma cell line R545 by nuclear transfer (Hochedlinger et al. 2004). Without H-RAS overexpression, the R545-ESCs have been able to contribute to chimeras, to restore lymphocytes in *Rag2* deficient mice and to produce ES-cell-derived embryos up to E9.5 upon tetraploid complementation. Additionally, the same cell line was amenable for transcription factor-induced reprogramming with only Oct4, Klf4 and c-Myc (Utikal et al. 2009a). Similar to SCNT-ESCs resulting melanoma iPCCs contributed to chimeric mice after blastocyst injection indicating that the R545 melanoma

genome remains the potential to contribute to a living organism. Since then, a wide variety of murine and human tumor cells were reprogrammed using different techniques.

Mouse lung carcinoma cells reprogram into murine iPCCs after transient transfection of the Yamanaka factors and selection for Nanog-expressing cells (Lin & Chui 2012). Human prostate and colon cancer cells display marker expression and methylation profiles of PSCs through miR-302 transfection (Lin et al. 2008). Data from human chronic myeloid leukemia (CML) cells of blast crisis stage demonstrated successful induction of pluripotency with Oct4, Sox2, Klf4 and c-Myc retroviruses. Here, deprivation of c-Myc resulted in cell death while Oct4, Sox2, and Klf4 were necessary to generate fully reprogrammed cells (Carette et al. 2010). According to the mutational status of the parental cell line the resulting iPCCs differ from normal iPSCs in the expression of the *BCR-ABL* oncogene. In contrast to CML cells the derived iPCCs escape oncogene addiction and become resistant to BCR-ABL inhibitor treatment with imatinib. Data from CML-iPSCs derived from a primary patient sample uncovered that phosphorylation of ERK1/2, AKT and c-Jun N-terminal kinases (JNK) has not been affected by imatinib but has been decreased in differentiated hematopoietic cells (Kumano et al. 2012). In contrast, the phosphorylation state of CRKL and STAT5, which are specific for CML-iPCCs, have been reduced in CML-iPCCs and their differentiated hematopoietic progenies. Consequently, the authors suggested that CRKL and STAT5 are not essential for survival, instead signaling pathways for iPSC maintenance compensated for the imatinib induced BCR-ABL inhibition. Furthermore, it was shown, that oncogenic mutations did not prevent differentiation so that neurons, neuronal epithelium, fibroblast-like cells and hematopoietic cells could be differentiated from CML-iPCCs (Carette et al. 2010). These showed no response to imatinib treatment while CD34, CD43 and CD45 positive cells were reduced by the inhibitor indicating that *BCR-ABL* oncogene dependence correlated with the epigenetic differentiation status of cells. CML cells were also reprogrammed using episomal vectors for Oct4, Sox2, Klf4, Nanog, Lin28 and c-Myc. Generated that way, iPCCs lacked transgene integrations, were positive for the BCR-ABL fusion gene and differentiated in granulocytes, erythrocytes, monocytes and megakaryocytes (Hu et al. 2011). The first human iPCCs of solid tumors cells were derived from four gastrointestinal cancer cell lines selected for low Nanog expression. The cells were transduced with the four Yamanaka factors by retroviral delivery and showed signs of pluripotency *in vitro* (Miyoshi et al. 2010). Derived differentiations were characterized by increased sensitivity to chemotherapeutics and decreased tumor-initiating properties *in vivo,* which might be explained by reactivation of tumor suppressor genes through reprogramming. The same group also discovered a re-sensitization to drugs of miR-302 reprogrammed hepatocellular carcinoma cells and the link to the miRNA target AOF2. miRNA mediated repression of AOF2 decreases c-Myc expression levels, thereby elevating miR-29b expression and sensitizing cells to Mcl-1 induced apoptosis (Koga et al. 2014). The influence

of epigenetic changes on drug response has been confirmed by methylome analysis of reprogrammed human non-small cell lung cancers (NSCLCs). After reprogramming, aberrantly methylated promoters of NSCLCs become hypomethylated in iPCCs, containing genes associated with development but also tumor suppressor genes like *APC*, *TIMP3* and *WRN*. Accordingly, between 59 and 110 genes from a list of 391 unique genes commonly up-regulated in NSCLCs were down-regulated upon reprogramming (Mahalingam et al. 2012). Notable, only a small percentage of tumor suppressor genes were elevated in iPCCs compared to the number of oncogenes which were down-regulated after reprogramming. Combined analysis of gene expression data and methylation analysis verified that dysregulation of tumor suppressor genes and oncogenes in NSCLCs is partially reversed in cancer-derived iPCCs (Mahalingam et al. 2012). A comparable study from Zhang et al. (2013) in human sarcoma cells found global hypomethylations upon reprogramming. Greatest changes were observed equally in tumor suppressor genes and oncogenes. In order to determine the grade of epigenetic resetting in iPCCs, the group used a set of 50 genes previously identified to discriminate mouse ESCs, MEFs and MEF-iPSCs. The expression levels suggested that the sarcoma cells were only reprogrammed slightly beyond a mesenchymal stem cells (MSCs) state which might indicate the existence of reprogramming roadblocks that prevent the acquisition of full pluripotency. Accordingly, *in vitro* experiments demonstrated the successful differentiation into fat and bone producing cells as well as in blood cells. These terminal differentiated cells of connective tissue or blood abolished the tumor initiating potential of the parental sarcoma cells. Nevertheless, the group used lentiviral vectors constitutively expressing the Yamanaka factors indicating a metastable pluripotent state of the sarcoma-iPCCs. More likely, the cells represent partly reprogrammed intermediates which could be in line with the discovery that these cells lacked teratoma formation and rather generated undifferentiated sarcomas *in vivo (Zhang et al. 2013)*. Further results from glioblastoma cells strengthened the hypothesis that the malignancy of a tumor genome correlates to its methylome. Comparison of methylation profiles between glioblastoma neural stem cells (GNS) and neural stem cells identified cancer-specific methylation variable positions (cMVPs). A high percentage of PRC2 target genes including tumor suppressors like cyclin-dependent kinase inhibitor 1C (CDKN1C) and TES were found to be hypermethylated in GNS but demethylated upon reprogramming. Furthermore, demethylation of the tumor suppressor genes persisted during the differentiation into non-neuronal cells but became hypermethylated in neural progenitors which was associated with loss of protein expression and an increased tumorigenic potential (Stricker et al. 2013). Nevertheless, the results also demonstrate that the majority of normalized cMVPs remain hypomethylated even during neuronal differentiation. On the one hand epigenetic resetting can lead to a stable loss of cancer specific methylation marks but on the other hand distinct sites are susceptible to reacquire aberrant chromosomal modifications.

Another study utilized cancer cells as a heterogeneous starting population in order to investigate whether the reprogramming process selects for subpopulations. NSCLCs harboring mutations for TP53 and CDKN2A and CDKN2B were subjected to nuclear reprogramming and subsequently analyzed for occurring mutations. In contrast, to the parental cell lines, none of the known mutations were detectable. Therefore, the group proposed that an undetectable small subpopulation carrying no mutations was amenable to reprogramming and giving rise to iPCCs with a homogenous karyotype. Consequently, the process of reprogramming cancer cells might be illustrated by the so called elite model suggesting the existence of a favored cell population which is more susceptible to become pluripotent (Lai et al. 2013). On the other hand, tumor-derived pluripotent stem cells are applicable to model tumorigenesis. Especially when mouse models rather resemble advanced tumors, reprogramming of tumor cells might represent an elegant technique to investigate early neoplastic processes. Kim, J. et al. (2013) compared pancreatic intraepithelial neoplasias (PanINs), which progressed to invasive pancreatic ductal adenocarcinomas (PDACs) with reprogrammed human ductal adenocarcinomas derived from primary resections (Kim, J. et al. 2013). Although the reprogrammed cancer cells shared characteristics with iPSCs they required low doxycycline concentrations indicating that the cells were not fully reprogrammed. Nevertheless, upon subcutaneous injection the iPCCs gave rise to teratoma-like structures with a high portion of endodermal tissue with PanIN1-, PanIN2- and PanIN3-like structures. These are able to progress to invasive human pancreatic cancer and therefore resemble early-stages of PDAC. On the basis of cultured organoids derived from PanIN-like cells, they found several proteins and markers associated with early-stage pancreatic cancer and additionally identified the HNF4α network to play a key role in the late PanIN stages (Kim, J. et al. 2013).

VI.2. Melanoma

Melanomas arise from pigment-producing melanocytes and represent the most serious form of skin cancer. In 2014, melanomas were estimated to account for only 2% of all skin tumors in the US but to cause 80% of skin cancer-related deaths. The five-year survival rate of patients suffering from metastatic melanoma reaches merely 15% (American Cancer Society, 2013). In order to understand the biological processes that are characteristic for growth and progression of melanomas, it is necessary to gain insights into the early embryonic development of melanocytes.

VI.2.1. The origin of melanoma

During neurulation the neural plate border elevates and folds into the neural tube. Depending on the organism, neural crest cells (NCCs) delaminate and migrate from the dorsal neural tube along distinct routes into the periphery where they differentiate into a wide range of lineages

such as peripheral neurons, endocrine cells, bone, cartilage, connective tissue and melanocytes (reviewed in Gammill & Bronner-Fraser 2003). Thereby, the anatomic location of the neural crest derivatives determines their fate by environmental conditions. The cellular developmental potential and plasticity of trunk and cranial neural crest cells (Baker et al. 1997) indicate that these cells generate bipotent glial-melanocytic lineage progenitors (Dupin et al. 2000), which are committed to differentiate into non-pigmented melanoblasts. These migrate along the dorsolateral trail and invade the epidermis where they expand extensively to distribute equally (Yoshida et al. 1996). In hairless regions of the skin, melanoblasts reside at the basement membrane and differentiate when stimulated by keratinocytes. Melanoblasts located in hairy regions of the skin localize either at hair bulbs as differentiated melanocytes or at hair follicle bulges as immature melanocyte stem cells (McSCs) (Nishimura et al. 2002). Maintained in their specific compartment, melanocytes mainly produce melanin in order to protect our skin from ultraviolet (UV) radiation-induced damage. Furthermore, recent studies propose that McSCs play an important role in the regeneration of wounded skin epithelium. Here, McSCs leave their stem cell niche to migrate into wounded regions in order to support the repopulation and repigmentation of the injured region (Chou et al. 2013).

The exact mechanism behind the transformation of melanocytes or their progenitor cells into tumorigenic melanoma cells is not fully understood. Interestingly, UV irradiation of melanoma cells induces a higher genetic mutation rate than in any other solid tumor. Improved molecular techniques allowed to investigate genomic alterations that drive tumor transformation and to discriminate them from potential UV-induced passenger mutations. The most prominent mutation found in melanomas (63%) affects the *BRAF* gene followed by mutations in neuroblastoma RAS viral oncogene homolog (*NRAS*), tumor protein p53 (*TP53*), phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*), and in the gene locus *CDKN2A* (Hodis et al. 2012). In the correct cellular context these genomic changes can promote a stepwise process leading to the development of malignant melanoma. In histological sections, this process is classified by the so-called Clark model. According to this classification, malignant melanomas develop from a benign melanocytic nevus with aberrantly proliferating melanocytes. The identification of mutations in *BRAF* or *NRAS* and its correlation with an abnormal mitogen-activated protein kinase (MAPK) signaling in benign nevi suggest that this overactivation initially stimulates melanocyte proliferation and thereby represents an early event in melanomagenesis (Pollock et al. 2003; Wu et al. 2007; Yazdi et al. 2003). Interestingly, oncogenic *BRAF* or *NRAS* induce cellular senescence in melanocytes by induction of p16 inhibitor of cell cycle kinase 4A ($p16^{INK4A}$) (Gray-Schopfer et al. 2006; Michaloglou et al. 2005). Animal models with endogenous *Braf* mutations demonstrated that some melanocytes escape the protective cell cycle arrest probably by the acquisition of additional mutations (Dhomen et al. 2009) resulting in dysplastic nevi. Nearly 50% of all melanomas carry a mutation in one of

the tumor suppressor genes *TP53*, *PTEN* or *p16*^{INK4A} (Hodis et al. 2012) affecting DNA damage repair, cell growth or sensitivity to apoptosis. Accordingly, single mutations in *RAF*, *RAS*, *PTEN* or *p16*^{INK4A} fail to initiate melanomas but combinations of these mutations facilitate the progress towards *in situ* melanomas. Resulting lesions initially remain confined to the epidermis but expand laterally. Therefore this phase is defined as the radial growth phase. Afterwards the vertical growth phase follow, which is crucial for the formation of malignant melanoma and associated with poor clinical outcome. Here, cells gain invasive properties, penetrate the basal membrane and infiltrate the surrounding tissue in order to generate metastasis at distant sites. Therefore, melanoma cells down-regulate junctional E-cadherin and gain N-cadherin resulting in a migratory phenotype (Alonso et al. 2007). This process describes an epithelial-to-mesenchymal transition (EMT) and is linked to a hyperactivation of MAPK, nuclear factor kappa B (NF-κB) and phosphoinositide 3-kinase (PI3K)/AKT signaling resulting in transcription of target molecules like *SNAI1*, *TWIST* and *SLUG* (Caramel et al. 2013). These genes bind promoter regions of E-cadherin leading to its repression (Cano et al. 2000; Ohkubo & Ozawa 2004; Poser et al. 2001; Weiss et al. 2012). Moreover, oncogenic ERK activation initiates a switch in the expression pattern of genes acting as tumor suppressors in melanocytes like ZEB2 and SLUG towards the expression of ZEB1 and SNAIL1, further triggering EMT and thereby driving melanomagenesis (Caramel et al. 2013).

VI.2.2. Mutations involved in melanomagenesis and their therapeutic significance

VI.2.3. RAS-RAF-MEK

MAPK signaling is initiated by the binding of a wide variety of growth factors, hormones and differentiation-inducing molecules to their receptors resulting in the activation of rat sarcoma oncogene (RAS). GTP-bound RAS triggers a cascade of phosphorylation steps initiated *via* interactions with the RAS-binding-domain (RBD) of membrane-recruited RAF proteins. Phosphorylation of two amino acids within in the kinase domain are required for full activation of BRAF, in contrast to A- and C-RAF, which additionally require phosphorylation within the negative-charge regulatory domain (N-region) (Fabian et al. 1993; Mason et al. 1999) explaining why BRAF plays a predominant role in activating downstream mitogen activated protein kinase 2 (MAP2K1, MEK) (reviewed in Matallanas et al. 2011). BRAF phosphorylation allows formation of a specific dimerization mode called side-by-side dimerization, facilitated either by homodimers consisting of two BRAF molecules or heterodimers consisting of one BRAF molecule and one molecule of kinase suppressor of RAS (KSR), a RAF-related pseudo-kinase (Rajakulendran et al. 2009) In this conformation, these molecules promote phosphorylation of dual-specificity kinases MAP2K1/MAP2K2 (MEK1/2), which in turn

subsequently activate extracellular signal-regulated kinase 1/2 (ERK1/2). Phosphorylated ERK targets proline-neighboring serine or threonine residues of cytoplasmic and nuclear molecules resulting in the induction of cell proliferation, survival and differentiation. Genetic examinations of MAPK signaling members revealed mutations of BRAF in 60% of all melanoma cases emphasizing its role as key player in melanomagenesis (Davies et al. 2002). Additionally, mutated BRAF plays a role in several other tumors types including colorectal, lung, thyroid, ovarian, pancreatic and prostate cancer (Brose et al. 2002; Cho et al. 2006; Ishimura et al. 2003; Nikiforova et al. 2003; Oliveira et al. 2003; Perren et al. 2004; Wang et al. 2003). Thus, understanding the mechanism of oncogenic BRAF signaling is of essential interest for multiple tumor types. In 80% of all cases a single nucleotide substitution within the catalytic domain results in replacement of valine (V) with glutamic acid (E) at position 600 leading to increased kinase activation (Lovly et al. 2012) by mimicking phosphorylation of the activation loop. Substitutions at the same position with lysine (K), arginine (R), methionine (M) and aspartic acid (D) are less frequent but also result in a BRAF hyperactivation illustrating the regulatory importance of this position (Heinzerling et al. 2013; Lovly et al. 2012; Menzies et al. 2012). Novel studies using RAF specific inhibitors revealed the mechanisms behind $BRAF^{V600E}$ activity pointing towards a RAS-independent function of mutated BRAF. Furthermore, elevated ERK levels induce a negative feedback loop repressing RAS in melanoma cells. Blocking RAF signaling using small molecules inhibitors release this feedback triggering RAS activation. Hence, RAF inhibition is accompanied by a rebound in ERK activation resulting in a new steady state (Lito et al. 2012). There is evidence that only copper-bound MEK is able to interact with ERK (Turski et al. 2012). According to that, prevention of copper-MEK interaction decreases oncogenic BRAF signaling in mice and reduces tumor growth of $BRAF^{V600E}$ positive melanoma cells. Therefore, copper deprivation using chelators applied for the treatment of Wilson's disease might be supportive in MAPK targeting therapies (Brady et al. 2014). In more than 22% of melanoma cases (Ball et al. 1994; Hodis et al. 2012) the RAS family member neuroblastoma rat sarcoma oncogene (NRAS) harbors activating mutations predominantly Q61R or Q61L transitions in exon two. Interestingly, mutations in BRAF and NRAS are exclusive (Daniotti et al. 2004; Hodis et al. 2012). Mutant BRAF itself only triggers growth of benign nevi but its activation together with loss of p53 transforms subpopulation of melanocytes (Patton et al. 2005; Yu et al. 2009) into malignant melanoma cells. Murine $BRAF^{V600E}$ models confirm the transforming potential of the gene and nicely demonstrate the role of additional mutational events in other molecules and their contribution to malignant transformation. In this manner loss of PTEN in combination with oncogenic BRAF signaling has been identified to generate melanomas with 100% incidence whereas $BRAF^{V600E}$ mutations induced skin hyperplasia but not melanomagenesis in this model (Dankort et al. 2009). In contrast, the second mouse model demonstrated that $BRAF^{V600E}$ mutations alone

were sufficient to induce tumor formation in 70% of mice. Beside these differences, both models confirmed appearance of benign nevi and senescent melanocytes upon induction of mutated BRAF. Interestingly, p16INK4a was expendable for melanocyte senescence and tumor progression but influenced tumor latency and increased numbers of developing tumors per mouse (Dhomen et al. 2009). Knockdown of $BRAF^{V600E}$ using small interfering RNA (siRNA) in melanoma cells suppressed typical tumor characteristics like invasiveness, cell proliferation (Sumimoto et al. 2004), and tumor vascularization (Sharma et al. 2005). Furthermore, simultaneous expression of INK4a and inhibition of BRAF potently induced apoptosis in melanoma cells indicating a functional interaction of BRAF and INK4a mutations (Zhao et al. 2008b). Additionally, knockdown experiments point towards a role of mutated BRAF as a negative regulator of protein kinase B (Akt) signaling *via* activation of mammalian target of rapamycin complex 2 (mTORC2), thereby suggesting a negative feedback mechanism between MAPK and AKT signaling (Chen, B. et al. 2012). Taken together, the high frequency of BRAF mutations in melanoma and their putative role as one of the driver mutations highlights mutated BRAF as a promising target in melanoma therapy.

The first clinically tested RAF inhibitor was sorafenib (Wilhelm et al. 2004). This small compound inhibitor failed to provide survival benefits in clinical trials most probably due to its low specificity to $BRAF^{V600E}$ and additional reaction against BRAF, CRAF, vascular-endothelial growth factor (VEGF) and platelet-derived growth factor receptor tyrosine kinases (PDGF RTKs). Vemurafenib (PLX4032) is a type I ATP-competitive $BRAF^{V600E}$ inhibitor and was the first small molecule approved by the FDA in 2011 selectively targeting $BRAF^{V600E}$ (Joseph et al. 2010). In a phase I clinical trial with patients carrying V600E mutated BRAF, vemurafenib treatment led to tumor shrinkage in 81% increasing median overall survival to 15.9 months. Since then, other BRAF inhibitors like dabrafenib or MEK inhibitors such as trametinib and selumetinib were developed representing the most important MAPK signaling inhibitors.

Although these compounds are very promising drugs for treating RAS-RAF-MEK-ERK-driven tumors their application is limited due to resistance mechanisms and paradoxical MAPK pathway activation. In BRAF wildtype melanoma cells carrying a RAS mutation, inhibition of BRAF activity results in hyperactivation of ERK. Application of BRAF inhibitors (Halaban et al. 2010) induce stimulation of CRAF-MEK-ERK leading to increased cell proliferation and migration. These results have been confirmed by an oncogenic RAS mouse model with inducible expression of a kinase-inactive form of BRAF ($BRAF^{LSL\text{-}D594A}$), that mimics consequences of BRAF inhibition leading to the generation of rapidly growing melanomas (Heidorn et al. 2010). Paradoxical activation describes a mechanism of ERK activation in BRAF wildtype cells after administration of BRAF inhibitors. These block one protomer of the RAF homo- or heterodimers, thereby inducing RAS-dependent transactivation of the other protomer (Poulikakos et al. 2010). In accordance to this hypothesis cells harboring $BRAF^{V600E}$ mutations

are characterized by an inactive RAS proto-oncogene required for paradoxical activation, thereby leading to a predominant inhibition of V600E monomers. BRAF and CRAF mutants that are defective in dimerization fail to induce ERK overactivation confirming that dimerization plays a key role for paradoxical activation (Hatzivassiliou et al. 2010). Further insights were unraveled in a recently published study discovering ATP-dependent auto-regulatory mechanisms of RAF wildtype proteins (Holderfield et al. 2013). Autophosphorylation within the P loop stabilizes an enzymatic inactive conformation of RAF proteins, abolishing its activation. Consequently, $BRAF^{V600E}$ melanoma cells bypass auto-regulatory mechanisms similar to other P loop mutation-bearing cells.

The drug-induced hyperactivation of RAS-RAF-MEK-ERK signaling is not restricted to the melanocytic lineage but also affects cells of other lineages. Consequently, it causes severe side-effects like formation of squamous cell carcinomas (SCCs) that often harbor RAS mutations (Oberholzer et al. 2012; Su et al. 2012). Nevertheless, SCCs are slightly invasive and therefore easy to remove.

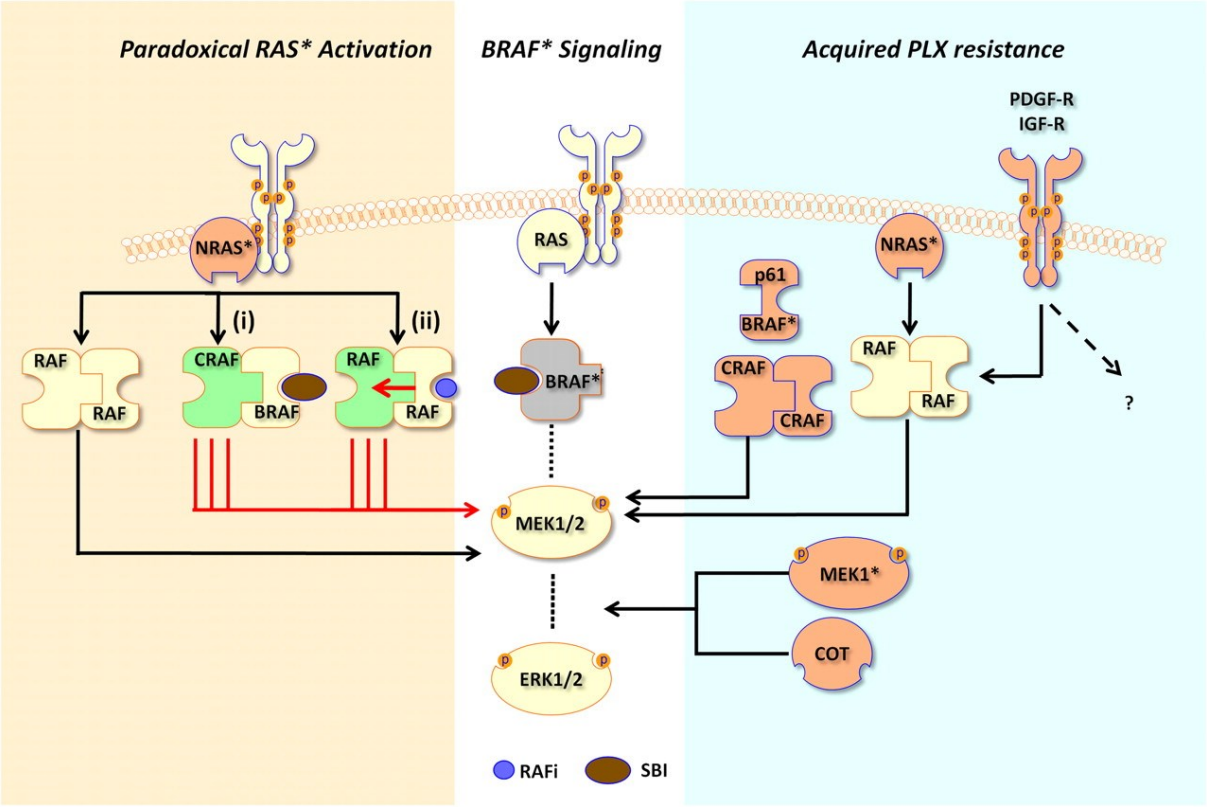


Figure 2 | Inhibition of MAPK signaling members and pharmacological consequences. Adapted with permission from Tsao et al. (2012).

The high percentage of relapse during targeted melanoma treatment within a year is one of the most severe issues indicating that tumor cells quickly adapt to BRAF inhibition (Flaherty et al. 2010). Studies with dabrafenib, trametinib and selumetinib demonstrated that acquired

resistance mechanisms are not vemurafenib-specific but present general strategies of melanoma cells to escape MAPK pathway inhibition. Nevertheless, many experiments were performed with vemurafenib so that mechanisms leading to therapy resistance are most explicitly investigated for this drug. Molecular events conferring resistance to inhibitor treatment either restore signaling activity or circumvent inhibition by switching to other pathways. Stimulating RTKs of tumor cells with corresponding ligands, also commonly found to be up-regulated in tumors or surrounding tumor stromal cells, can rescue drug-induced growth arrest *in vitro.* This also demonstrates an important role of the tumor microenvironment in the development of drug resistance. In $BRAF^{V600E}$ mutated melanoma cells stimulation with hepatocyte growth factor (HGF) mediates resistance by activation of the PI3K and MAPK pathway (Straussman et al. 2012; Wilson et al. 2012). This was also shown for some cell lines using neuregulin 1 (NRG1) as stimulating agent. Inhibition of the HGF receptor MET by crizotinib re-sensitizes tumor cells to RAF inhibition and increases vemurafenib efficacy Additionally, HGF expression was detected in pre-treated patient samples correlating inversely to drug response. Other groups confirmed that increased RAF dimerization by activation of upstream signaling molecules like PDGFR or NRAS can mediate resistance against novel RAF inhibitors (Nazarian et al. 2010). Furthermore, loss of the MAPK negative regulator NF1 is associated with de-repression of RAS signaling providing a stimulus leading to activation of CRAF (Whittaker et al. 2013) or K- and HRAS (Maertens et al. 2013). Signaling through alternative RAF family members restores MAPK signaling activity, therefore providing an effective resistance strategy against RAF isotype-specific monotherapies since respective clinical data demonstrated that successful inhibitor treatment requires nearly complete MAPK signaling inhibition (Bollag et al. 2010). Surprisingly, no acquired secondary mutations in vemurafenib-resistant melanomas were found in the $BRAF^{V600E}$ gene indicating that cells retain their ability to respond to vemurafenib (Nazarian et al. 2010). This observation was confirmed by discontinuous dosing strategies. Tumors which acquired drug resistance through elevated MAPK signaling became dependent on continuous BRAF inhibition. Therapy cessation resulted in an initial tumor regression probably due to excessive ERK activation followed by cell cycle arrest or induction of apoptosis. Furthermore, discontinuous dosing eliminated the survival advantage of resistant clones preventing therapy resistance in mice (Das Thakur et al. 2013). Nevertheless, in some patients with acquired vemurafenib resistance a splice variant of $BRAF^{V600E}$ lacking the RAS-binding domain was detected. The 61 kDa variant excluded exon 4-8 and was characterized by increased RAF dimerization even in a RAS-independent manner. A dimerization-deficient mutant restored sensitivity against vemurafenib indicating that drug resistance can be generated by increased dimerization ability (Poulikakos et al. 2011). Furthermore, occurrence of BRAF oncogene amplification can facilitate resistance by increasing expression levels in melanoma (Shi et al. 2012) and also colon cancer (Corcoran

et al. 2010). Studies in BRAF-mutated colon cancer identified resistance mechanisms also observed in melanoma cells like the expression of epidermal growth factor receptor (EGFR) as an alternative activation mechanism of MAPK signaling. In six out of 16 melanoma patients with acquired resistance to BRAF treatment a gain in EGFR expression was initiated by TGF-β signaling inversely correlating to Sox10 levels (Sun et al. 2014). Furthermore, this study confirmed previous results of drug-resistant cell populations with survival advantages under therapy which are reverted during drug holidays.

Melanoma-driving mutations affecting the MAPK pathway and leading to therapy resistance can also occur downstream of RAF. Several studies recently identified MAP2K1 (MEK1) and its mutation hotspot at codon P124 as a feasible melanoma oncogene (Hodis et al. 2012; Krauthammer et al. 2012; Nikolaev et al. 2012). Furthermore, MEK inhibition by trametinib can cause mutations in MEK1 (Emery et al. 2009) as well as MEK2 (Villanueva et al. 2013) resulting in resistance to trametinib itself and other MEK inhibitors. Previously, we reported a case of a trametinib-resistant melanoma patient well responding to vemurafenib treatment indicating that resistance mechanisms against MEK inhibition do not necessarily facilitate cross-resistance to BRAF-targeting therapy (Bernhardt et al. 2014). These data were supported by a retrospective study of 23 melanoma patients with inverse sequential therapy (MEKi treatment followed by BRAFi in case of progression) showing increased disease control rates (Goldinger et al. 2014). However, patients quickly develop novel resistances against vemurafenib. Besides others, mutations in the genes for MEK1 and MEK2 (Emery et al. 2009; Villanueva et al. 2013) were observed to be responsible for insensitivity against BRAF and MEK inhibitors in parallel. Nevertheless, co-targeting both molecules (Emery et al. 2009) or even in combination with inhibition of the PI3K/mTOR axis prevented the appearance of multi-resistant clones (Shi et al. 2011; Villanueva et al. 2013). Screening pharmacological substances in BRAF inhibitor-resistant cells identified an insulin-like growth factor receptor 1 (IGF-1R)-dependent drug resistance. In samples of recurrent melanomas elevated levels of IGF-1R and phosphorylated AKT were detected. Accordingly, combination therapy targeting MEK and IGF-1R in parallel resulted in increased cytotoxicity (Villanueva et al. 2010). Besides already discussed pathways involved in melanoma formation also modulation of pathways that are not associated with melanoma progression can de-sensitize melanoma cells to drugs. The overexpression of 600 kinases and kinase-related open-reading frames (ORFs) identified MAP3K8 as a potential mediator of resistance (Johannessen et al. 2010). This kinase is also known as COT and is able to induce JNK and MAPK signaling upon stimulation by molecules associated with inflammatory processes like tumor necrosis factor (TNF), interleukin-1 (IL-1) or CD40 (Vougioukalaki et al. 2011). Thereby, COT actives MEK and ERK in a RAF-independent way and was detected in melanoma patients with acquired BRAF resistance (Johannessen et al. 2010). Analysis of deregulated networks in various cancer types identified

the translation-initiation complex to integrate several cancer-related signal pathways like MAPK and PI3K/mTOR signaling. The eukaryotic initiation factor 4F (eIF4F) controls this process by regulating the step of initiation (reviewed inBitterman & Polunovsky 2012) and is commonly deregulated in tumors (De Benedetti & Graff 2004). A recent publication shows that the eIF4F complex contributes to the development of resistance against BRAF and MEK mono- as well as combination therapy in BRAF V600 mutated melanoma, colon and thyroid cancer cell lines (Boussemart et al. 2014). Combined inhibition of eIF4F and BRAF V600 synergistically induces cell death in tested cancer cells, suggesting eIF4F to be an interesting target to prevent innate and *de novo* resistance. Mainly, cell culture systems were used to study the appearance of *de novo* mutations as a result of therapy resistance in $BRAF^{V600E}$ mutated cells. In order to detect molecular lesions appearing *in vivo* 100 samples of 44 melanoma patients with acquired resistance against vemurafenib or dabrafenib were sequenced. In more than 52% mutations exclusively appeared in the MAPK pathway associated to reactivation of ERK signaling (NRAS mutations 18%, KRAS mutations 7%, BRAF-mutant amplification 19%, BRAF splice variants 13%). Furthermore, all tumor samples were still harboring $BRAF^{V600E}$ but did not gain secondary mutations in the BRAF gene locus. This lead to the disproof that BRAF wildtype cells remain silent in melanoma generating resistant tumors during treatment with V600E-specific inhibitors. Instead, the detection of $BRAF^{V600E}$ in all samples demonstrated that the cells fulfill the genetic criteria to respond to therapy. In 4% of all tested melanoma samples only PI3K/AKT signaling was affected while 18% developed resistant cells due to mutations in MAPK and PI3K/AKT signaling. Therefore, reactivation of MAPK signaling and activation of the PI3K/AKT pathway represent the core mechanism of BRAF inhibitor resistance. In addition, analysis of several metastases from patients of the same cohort demonstrated that often multiple driver mutations are acquired in recurrent tumors.

VI.2.4. PTEN

Phosphatase and tensin homolog (PTEN) is lost in about 10% of all melanoma cases (Stahl et al. 2003). With its bi-specific function as protein and lipid phosphatase, PTEN is involved in the regulation of cell growth, proliferation and survival. As dual specificity protein phosphatase PTEN dephosphorylates tyrosine as well as serine/threonine residues. The identification of phosphatidylinositol (3,4,5,)-trisphosphate (PIP_3) as target of PTEN led to the discovery of its negative regulatory function in PI3K/AKT signaling (Maehama & Dixon 1998). Loss of PTEN leads to the accumulation of PIP_3, the product of the PI3K, which recruits AKT to the plasma membrane where AKT it is activated by phosphorylation. As AKT is a known oncogene promoting cell survival, proliferation and migration, PTEN functions as tumor suppressor gene in melanoma but also in other tumors like breast cancer where it mediates cell cycle arrest

(Weng et al. 2001). More important, there is evidence that PTEN is directly involved in regulating MAPK signaling. In breast and glioblastoma tumor cells, it inhibits EGFR- and PDGFR-mediated MAPK activation and decreases MEK and ERK phosphorylation (Gu et al. 1998; Weng et al. 2001). Interestingly, PI3K itself is rarely mutated in melanoma compared to other cancer types (Omholt et al. 2006). In combination with the suggested cooperation of PTEN and BRAF activation (Tsao et al. 2004) it indicates that the protein phosphatase activity of PTEN or its direct interaction - for example with p53 (Tang & Eng 2006) - are the dominant mechanisms behind its tumor suppressive function in melanoma compared to its role in AKT signaling.

VI.2.5. TP53

The role of p53 in melanoma is still controversially discussed. Molecular analysis in a large cohort of primary melanoma species only detected few reoccurring mutations resulting in amino acid substitution (Albino et al. 1994; Houben et al. 2011; Papp et al. 1996) although mutation rates up to 20% are also frequently reported (Hodis et al. 2012; Ragnarsson-Olding et al. 2002; Stretch et al. 1991; Weiss et al. 1995). One possible explanation for this discrepancy was given by $BRAF^{V600E}$ -driven mouse models in which significant differences were observed between sun-exposed and unexposed tumors (Viros et al. 2014). UV-induced cytosine-to-thymine transitions at the 3' end of pyrimidine dimers were responsible for p53 mutations that could be detected in 40% of the tumors indicating that p53 is a major target of UV radiation (Viros et al. 2014). The protective effect of sunscreen against ultraviolet radiation (UVR) delays the onset of melanoma development but is not able to completely prevent UVR-induced tumorigenesis. Nonetheless, no significant accumulation of p53 mutations was detectable in sun-exposed human cutaneous melanomas in comparison to sun-shielded mucosal melanomas (Ragnarsson-Olding et al. 2002). Noteworthy, melanomas with p53 mutations did not accumulate additional mutations in CDKN2a suggesting that the need for p53 loss of function mutations in melanoma is reduced compared to other cancer types due to the high prevalence of CDKN2a mutations (Hodis et al. 2012). Additionally, further mechanisms inhibiting p53 function in melanoma were recently identified. Phosphorylation of inhibitor of apoptosis-stimulating protein of p53 (iASPP) by cyclin B1/CDK1 prevents iASPP dimerization so that monomers translocate to the nucleus where they bind p53 and decrease its activity (Lu et al. 2013). In addition MDM4 was found to suppress p53 activity in the majority (~65%) of human melanoma samples by its direct interaction with the transcriptional activation domain of the tumor suppressor (Gembarska et al. 2012). This leads to the conclusion that recurrent genetic alterations in melanoma affect p53 wildtype functionality so that p53 restoration represents a promising therapeutic strategy. Nutlin3, a p53 agonist slightly restored p53 function and delayed tumor growth (de Lange et al. 2012; Ji et al. 2012) but failed to initiate

p53-dependent apoptosis (Tseng et al. 2010). Disruption of MDM4-p53 interaction increased p53 activity and resulted in enhanced response to chemotherapeutic treatment (Gembarska et al. 2012). Similarly, MDM2 knockdown experiments or administration of JNJ-7706621, a potent pan-CDK inhibitor, prevent p53 inhibition leading to reduced melanoma growth. In combination with Nultin3, inhibition of MDM2 and iASPP suppress human melanoma growth in melanoma xenografts by p53-dependent apoptosis. Additionally, together with vemurafenib, reconstructed and functionally active wildtype p53 achieved synergistic effects and decreased melanoma proliferation and tumor growth up to 80% (Lu et al. 2013).

VI.2.6. CDKN2A

The CDKN2A locus on chromosome 9p21 contains four exons and encodes for two tumor suppressor proteins $p16^{INK4a}$ (exons 1a, 2, 3) and $p14^{ARF}$ (exons 1b, 2, 3) by alternative splicing. Therefore, loss or hypermethylation leading to repressed expression of this particular locus highly correlates with tumor susceptibility and is detected in a wide variety of tumor cell lines (Kamb et al. 1994). About 12-20% of all melanomas harbor mutations in the CDKN2a locus resulting in homozygous or heterozygous loss of CDKN2a (Castellano et al. 1997; Young et al. 2014). Although both proteins represent tumor suppressors, they possess distinct functions. $p16^{INK4a}$ directly interacts with the cyclin D1-cyclin dependent kinase four (CDK4) and six (CDK6) complex inhibiting phosphorylation of retinoblastoma protein (RB) and thereby the transition from G1 to S phase. Consequently, mutations in $p16^{INK4a}$ convey re-entry into cell cycle. In contrast, $p14^{ARF}$ regulates p53 degradation *via* the regulation of MDM2. Key target of MDM2 is p53, which is ubiquitinated and therefore marked for degradation. Mutations in $p14^{ARF}$ or hypermethylation of its promoter lead to destabilization and degradation of p53. (Pomerantz et al. 1998; Zhang et al. 1998). Taken together, loss of CDKN2a affects two main tumor suppressor pathways, the RB and p53 pathway. Several reports of melanoma-prone families and clinical atypical moles identified CDKN2a germline mutations supporting its role as *bona fide* susceptibility gene (FitzGerald et al. 1996; Soufir et al. 1998).

VI.2.7. Novel melanoma driver mutations distinct from UV-induced passenger mutations

Two large studies compared whole-exome sequencing data from tumor and normal tissue pairs of melanoma patients and selected non-silent mutations. 85% of these mutations, enriched in more than 20% of melanomas, represented YC->YT transitions. These transitions derive most likely from UV-light induced damage indicating that frequent melanoma mutations might represent passenger mutations that do not contribute to the malignant phenotype (Hodis et al. 2012). Excluding loci with generally high mutational events, researchers identified exonic mutations positively selected during melanomagenesis. Among the statistically significantly

enriched mutations were BRAF, NRAS, PTEN, TP53, MAP2K1 and the $p16^{INK4a}$ gene, but also five novel candidates were detected (Hodis et al. 2012). Both studies identified PPP6C, RAS-related C3 botulinum toxin substrate 1 (RAC1) and inactivating mutations in AT rich interactive domain 2 (ARID2). RAC1 belongs to the Rho family of small GTPases and harbored a P29S mutation next to its catalytic site facilitating a stronger binding to GTP and stabilization of this active state leading to stimulation of MAPK signaling, increased migration as well as proliferation. Another newly discovered melanoma-associated gene is PPP6C, a serine/threonine protein phosphatase involved in signaling pathways controlling cell cycle progression in part through negative regulation of cyclin D1. Results from both groups included R264C mutations in PPP6C pointing towards a tumor suppressive function of this protein in melanoma. Similarly, ARID2 was affected by loss-of-function mutations, indicating that the subunit of the human SWIth/Sucrose NonFermentable chromatin-remodeling complex (SWI/SNF) as wells as other subunits of this multiprotein complex act as tumor suppressors (Hodis et al. 2012).

VI.2.8. Melanoma mutations outside coding sequences

Exome-based sequencing identified many mutations driving melanoma initiation and progression. Nevertheless, this approach misses the influence of non-coding regions within the genome. Recently, researchers studying deregulated microRNAs upon oncogenic BRAF or NRAS expression detected miR-146a. This particular miRNA interferes with the NOTCH repressor NUMB. Localized on the cell surface NUMB inhibits NOTCH expression on neighboring cells and hence plays an important role during differentiation as a key player of dual cell fate determination (reviewed in Schweisguth 2004). According to previous data, NOTCH promotes melanomagenesis (Pinnix et al. 2009) as well as melanoma progression (Asnaghi et al. 2012; Howard et al. 2013; Liu et al. 2006). Hence, inhibition of NUMB by miR-146a mediates Notch activation and promotes melanoma cell proliferation and tumor initiation (Forloni et al. 2014). More strikingly, an enriched single-nucleotide polymorphism (SNP) increasing the expression of mature miR-146a correlate with melanoma progression from patient-matched nevi, primary tumors and metastases. This might indicate the existence of melanoma driver mutations offside the commonly investigated coding sequences. In addition, whole genome sequencing data identified mutations within the core promoter region of the human telomerase reverse transcriptase (TERT) in 71% of melanomas examined (Huang et al. 2013). These cytidine-to-thymidine transitions (C228T and C250T) induced by UV-radiation lead to an additional E-twenty-six (ETS) transcription factor binding site resulting in 1.5- to 4-fold up-regulation of transcriptional activity (Horn et al. 2013; Huang et al. 2013). Analysis of 140 melanoma cases and 165 healthy controls revealed that these mutations do not represent common germline variants but rather are specific for tumor cells (Horn et al. 2013). The

prevalence of the mutation is similar to the combined two most frequent (BRAF and NRAS) exon mutations suggesting that melanoma cells are exposed to selective pressure for TERT promoter mutations.

VI.2.9. The role of MITF and differentiation-associated regulators in melanoma

MITF plays a key role in melanogenesis and melanocyte development but its role in melanoma development and progression is controversial. Dysfunctional MITF results in pigmentation defects (Hodgkinson et al. 1993) and disruption of the retinal epithelium development (Capowski et al. 2014) indicating that MITF widely affects survival of the complete melanocytic lineage. In the presence of constitutive BRAF signaling, MITF acts as an oncogene (Garraway et al. 2005) and accordingly, in 20% of melanomas, MITF is amplified correlating to elevated mRNA levels. Nevertheless, in transformed melanocytes BRAF-induced cell proliferation is antagonized by MITF (Selzer et al. 2002; Wellbrock & Marais 2005) indicating that differentiation mechanisms regulated by MITF are partially reverted during the transformation process (Landsberg et al. 2012; Vachtenheim et al. 2001). Consequently, molecules that regulate MITF are likewise important in melanoma. Four different promoters - A, H, B and M - control the expression of corresponding MITF isoforms, therefore allowing their tissue-specific expression. M-MITF is exclusively present in melanocytes and controlled by Sox10, Pax3, WNT, and cAMP/CREB (Shibahara et al. 2001). Sox10 is expressed at first in NCCs and regulates multipotency, proliferation and survival leading to the initiation of MITF expression in melanocytic-differentiated cells (Potterf et al. 2001). Various studies demonstrated Sox10 expression in melanoma cells (Agnarsdottir et al. 2010; Bakos et al. 2010) showing that the transcription factor initiates NCC-like properties. Ablation of Sox10 reduce invasion and tumorigenesis of melanoma cells and result in increased cellular senescence (Cronin et al. 2013; Graf et al. 2014; Shakhova et al. 2012).

Pax3 acts similar to Sox10. During development its expression is located at regions of the neural tube and mutations in the human gene result in pigmentation and hearing defects (Waardenburg syndrome) (Tassabehji et al. 1994). Furthermore, Pax3 is expressed in nearly all analyzed melanoma samples and contributes to melanoma cell survival. (Plummer et al. 2008; Scholl et al. 2001).

Wnt signaling is frequently dysregulated in melanoma but activation of the canonical or non-canonical signaling pathway leaves this pathway difficult to investigate. This might be one reason why many different studies with conflicting data exist. For example, activation of β-catenin in human and murine melanoma models demonstrated an anti-tumorigenic effect of Wnt signaling characterized by decreased tumor proliferation, better survival and increased sensitivity to drug induced apoptosis (Chien et al. 2009; Zimmerman et al. 2013). In contrast,

in BRAF mutated PTEN-deficient tumors loss of β-catenin/Wnt is associated with increased patient survival and decrease melanoma metastasis (Damsky et al. 2011).

VI.2.10. Subpopulations of melanoma cells with altered tumorigenic activity

In order to understand the interplay of therapy resistance and acquisition of distinct mutations melanomas were intensively characterized. Novel molecular and cellular techniques, like single-cell expression analysis, identified the existence of frequently occurring melanoma subpopulations (Quintana et al. 2008; Vidwans et al. 2011; Yancovitz et al. 2012) by demonstrating a high heterogeneity within melanoma tumors (Ennen et al. 2014). Due to this heterogeneity consequences for therapy prognosis and treatment arise. Single agent monotherapy targeting specific subpopulations might leave others unharmed resulting in therapy failure. Furthermore, subpopulations differ in their ability to initiate new tumors and metastasis suggesting that specific cells are tumor drivers and need to be targeted for successful cancer eradication. In order to identify potential tumor-initiating cells several markers were described in order to define distinct melanoma subpopulations.

VI.2.11. CD271

Since progenitors of melanocytes develop from neural crest origin, expression of neural crest stem cell markers was examined in melanoma cells. Therefore, melanoma bulk populations were sorted according to low-affinity nerve growth factor receptor (LNGFR or CD271) expression in order to enrich melanoma-initiating cells (Beretti et al. 2014; Boiko et al. 2010; Civenni et al. 2011; Redmer et al. 2014). Subcutaneous injection of the $CD271^+$ and $CD271^-$ subpopulations into *Rag2*$^{-/-}$*γc*$^{-/-}$ mice and NOD/SCID mice generated tumors, derived in 90-100% from the $CD271^+$ subpopulation (Boiko et al. 2010; Civenni et al. 2011), while $CD271^-$ melanoma cells were unable to initiate tumor growth. Recent studies contributed to these results as CD271 knockdown reduced the tumorigenic potential of melanoma cells (Redmer et al. 2014). Furthermore, $CD271^+$ melanoma cells showed decreased MITF expression levels correlating to increased levels of stemness-associated genes like Oct4, Nanog and NES (Cheli et al. 2014), thereby supporting the idea that highly tumorigenic melanoma cells display a dedifferentiated phenotype with reduced expression of melanocytic markers. Nevertheless, CD271 was also detected in a fast cycling but less tumorigenic population and its expression could be reacquired by initially negative populations indicating a phenotypic plasticity in melanoma cells. In line with that, others observed a high tumor-initiating potential at the single cell level. Every forth cell generated tumors in immunocompromised NOD/SCID interleukin-2 receptor *γc*$^{-/-}$ mice independently from any marker expression highlighting the influence of the mouse system on the tumor-initiating potential (Quintana et al. 2008). Furthermore, potential heterogeneously expressed stem cell markers like CD271, ABCB5, CD166, A2B5, CD151,

CD54, CD44, CD9, CD29, N-cadherin, CD49e, CD49f, L1-CAM, E-cadherin, and c-kit were investigated in patient-derived parental, marker$^{+/high}$- and marker$^{-/low}$-sorted secondary tumors. All secondary tumors recapitulated the heterogenic subpopulations of their parental tumors without any significant differences in tumorigenicity indicating a reversible expression signature (Quintana, Shackleton et al. 2010(Quintana et al. 2010).

VI.2.12. ABCB5

Some melanoma subpopulations are highly resistant to chemotherapeutic treatments through the expression of efflux transporter. ATP-binding cassette transporter B5 (ABCB5) was demonstrated to be expressed in clinical melanoma samples and involved in doxorubicin resistance in multiple melanoma cell lines. ABCB5$^+$ cells co-express activated leukocyte adhesion molecule (ALCAM/CD166), a biomarker of progressive melanoma, and CD133 (Frank et al. 2005). Both markers are transmembrane glycoproteins associated to a stem cell signature. In xenograft experiments ABCB5$^+$ cells have been shown to form tumors more frequently than bulk tumor cells. In contrast to these results another group claimed that ABCB5$^-$ tumor cells were unable to reconstitute the ABCB5$^+$ population and to generate secondary tumors, which were exclusively derived from ABCB5$^+$ melanoma cells (Schatton et al. 2008). Temozolomide-treated melanoma-bearing mice and also dacarbazine-treated tumor patients were enriched for ABCB5$^+$ cells providing evidence that multi drug resistance is associated with its function as an efflux transporter (Chartrain et al. 2012). Additionally, there is evidence that ABCB5 controls IL-1β secretion, thereby inducing a slow-cycling drug resistant phenotype (Wilson et al. 2014). Of note, ABCB5 as well as other discussed markers can be used in order to enrich tumor-initiating cells. Nevertheless, only half of ABCB5 expressing cells initiate primary and secondary tumor formation.

VI.2.13. CD133

Lessons from leukemia research encouraged to quest the role of CD133 in melanoma. CD133 was first recognized in hematopoietic stem cells (Yin et al. 1997) and identified in numerous reports as a potential marker for cancer-initiating cells of a wide variety of tumor types (reviewed in Grosse-Gehling et al. 2013). Patient-derived melanomas as well as melanoma cell lines harbor a small fraction of CD133 expressing cells, which are associated with increased tumorigenicity (Lai et al. 2012; Monzani et al. 2007), enhanced drug resistance (El-Khattouti et al. 2014) and elevated metastatic potential (Rappa et al. 2008). CD133 is expressed predominantly in actively cycling cells of several tumor types including melanoma and ESCs (Jaksch et al. 2008). According to its function as a stem cell marker CD133 is co-expressed with a subset of molecules also serving as pluripotency and progenitor markers

(Zimmerer et al. 2013), and cells expressing these markers are able to differentiate into mesenchymal cells or astrocytes (Monzani et al. 2007).

VI.2.14. ALDH

Another marker associated with drug resistance and detected in human melanoma cells is aldehyde dehydrogenase (ALDH). A subset of tumor cells derived from patient samples and from *in vivo* xenografts showed ALDH activity and could further be characterized by increased tumorigenic and self-renewal potential as demonstrated by serial transplantation assays (Boonyaratanakornkit et al. 2010; Luo et al. 2012). Only ALDH-expressing cells derived from initially $ALDH^+$ tumors, were able to generate secondary and tertiary melanomas. Inhibition of ALDH reduced cell proliferation and tumorigenicity, induced apoptosis and increased response towards paclitaxel treatment. On the contrary, another study failed to find any correlation between tumor-initiating potential and ALDH expression questioning whether selective markers for tumor-initiating cells exist in melanoma (Prasmickaite et al. 2010). Alternatively, subpopulations with enhanced tumorigenicity can be enriched under distinct culture conditions like non-adherent spheroid culture (Fang et al. 2005) proposing that functional assays might be more helpful in order to select melanoma-initiating subpopulations than the selection by surface marker.

VI.2.15. Sox2

Besides skepticism about markers for tumor-initiating cells many groups mutually agree on a correlation of putative stem cell marker expression and enhanced tumorigenic potential. Therefore, this remains to be investigated in more detail. Lately, the pluripotency factor Sox2 has been shown to facilitate self-renewal in melanoma spheres (Santini et al. 2014a). Silencing by knockdown experiments in patient-derived melanoma cells resulted in cell cycle arrest and induction of apoptosis. Interestingly, melanoma cells with high ALDH activity expressed two to three fold higher Sox2 levels, which is possibly regulated by HEDGEHOG-GLI (HH-GLI) signaling (Santini et al. 2012; Santini et al. 2014a). Recent data from lung squamous cell carcinoma (LSCC) highlighted the contribution of Sox2 in the set-up of a cell-autonomous HH signaling axis. On the one hand this supports the role of Sox2 in establishing features of HH-mediated stemness, on the other hand it suggests that Sox2 rather controls HH signaling than *vice versa* (Justilien et al. 2014). In addition, Sox2 was observed to play a role in melanoma cell invasion (Girouard et al. 2012). It localized predominantly at the invasive front of melanomas and controlled expression of matrix metallo-protease-3 (MMP-3). Consequently, overexpression in melanoma cells resulted in increased invasiveness.

VI.2.16. JARID1B

The idea that subpopulations are responsible for the initiation of tumors was derived from hierarchical-organized tumors like leukemia (Bonnet & Dick 1997) or gastro-intestinal cancers (Hirsch et al. 2014; Kobayashi et al. 2012). In these tissues, distinct stem cell populations exist and replenish the organ. When transformed, these cells give rise to aberrantly proliferating progenitor cells resulting in hyperplasia and cancer. In melanomas the situation is different as they do not follow a strictly hierarchical organization. This is supported by previous studies observing revertible proliferative and invasive states of melanoma cells (Hoek et al. 2008). Fast-growing cells expressed high levels of MITF and formed tumors after about 14 days in contrast to low-proliferative but highly invasive melanoma cells that needed 59 days for tumor generation. Regardless of which cells were injected, appearing tumors were indistinguishable in their molecular and phenotypical signature indicating the potential of cells to switch between the different states. The group assumed that microenvironmental factors were responsible for specifications of the phenotype. Recently, detection of jumonji AT-rich interactive domain 1B (JARID1B) expression in melanoma cells allows the identification of similar subpopulations of slow-cycling cells that can dynamically switch their phenotype. JARID1B catalyzes demethylation of histone 3 K4, thereby altering the expression of developmental genes (Albert et al. 2013; Dey et al. 2008) involved in proper neural differentiation (Schmitz et al. 2011) but plays also a role in different cancer types (Barrett et al. 2007; Kano et al. 2013; Ohta et al. 2013; Xiang et al. 2007; Yamamoto et al. 2014). *In vivo* JARID1B is highly present in melanomas but rarely in melanocytes of benign nevi (Kuzbicki et al. 2013; Radberger et al. 2012). Separation according its expression levels revealed no difference in the tumor-initiating potential of JARID1B subpopulations, although only JARID1B positive cells were able to continuously repopulate tumors (Roesch et al. 2010). These results suggest that the cellular heterogeneity of a single melanoma either arises from the accumulation of distinct genetic events or individual tumor cells adopt different epigenetic states. Furthermore, there is evidence that dynamic regulation of JARID1B involves bidirectional regulatory functions of Notch signaling. Enrichment of JARID1B$^+$ cells under cisplatin or vemurafenib application *in vitro* and *in vivo* and enhanced therapy response after JARID1B knockdown demonstrated the ability of this histone demethylase to mediate multi drug resistance (Roesch et al. 2013). The mechanism behind this multi-drug resistance is completed understood. Nevertheless, the investigators observed a switch of the bioenergetics metabolism towards an elevated mitochondrial respiratory chain activity. Inhibition of key enzymes of the respiratory chain reduced the JARID1B subpopulation and pushed the cells into an active cell cycle overcoming drug resistance.

VI.2.17. Inflammatory events in melanoma and their role in tumor- and metastasis development

Immune responses represent an effective defense system against infections and damaged cells prone to develop tumors. Mediators of inflammatory events are cytokines and chemokines that attract and activate other immune cells in order to restrain cancer growth. Already in 1863, Virchow suggested that there is a link between inflammatory processes and tumor development, in other words: tumors arise at sides of inflammation. Nevertheless, a direct link remained hidden until recent studies found a remarkable effect of chemokines on melanoma cells. Immune cells secrete TNF-α, stimulated melanoma cells *via* NGFR and led to a dedifferentiated amelanocytic phenotype with reduced expression of the melanoma antigens glycoprotein 100 (gp100) and tyrosine-related protein 2 (TRP2) (Landsberg et al. 2012). Down-regulation of melanoma epitopes prevent successful recognition of melanoma cells by antigen-specific cytotoxic T-cells from an adoptive T-cell transfer. Similar to data obtained from the JARID1B study, this dedifferentiated phenotype is revertible and after removal of the inflammatory environment, cells acquire an equilibrium state similar to pretreatment. Participation of immune cells as mediators of an inflammatory environment play a critical role as demonstrated by UV–radiation-induced neutrophilic responses. Neutrophil-conditioned medium as well as TNF from activated neutrophils promoted melanoma cell adhesion to endothelial cells resulting in angiotropism and perivascular invasion (Bald et al. 2014). Thus, UV-radiation of HGF-CDK4 mice did not affect the incidence or growth kinetics of developing melanomas compared to non-radiated mice but strikingly increased the numbers of their lung metastasis. Suppression of inflammatory events using non-steroidal anti-inflammatory drugs like aspirin seems to decrease the incidence of melanoma (Gamba et al. 2013).

Hence, inflammatory events are of special importance as melanoma cells respond to chemokines and cytokines by switching their epigenetic state. Taken together, studies corroborate the developing concept of highly plastic melanoma cells that quickly adopt to environmental factors resulting in altered phenotypes as one potential way to escape therapy and to promote tumor development.

VII. Materials and Methods

VII.1. Materials

Reagents and kits	Company	Branch
Alkaline Phosphatase Staining Kit II	Stemgent	San Diego, USA
DNeasy Blood & Tissue Kit	QIAGEN	Hilden, Germany
DAPI	Roche Diagnostics	Mannheim, Germany
Dako Fluorescent Mounting Medium	Dako	Hamburg, Germany
TritonX-100	Carl Roth	Karlsruhe, Germany
Albumin Fraction V	Carl Roth	Karlsruhe, Germany
Skim Milk Powder	Sigma-Aldrich	Steinheim, Germany
Tween20	Applichem	Darmstadt, Germany
cOmplete Mini Protease Inhibitor Cocktail	Roche Diagnostics	Mannheim, Germany
PhosphoStop Phosphatase Inhibitor Cocktail	Roche Diagnostics	Mannheim, Germany
Pierce BCA Protein Assay Kit	ThermoScientific	Karlsruhe, Germany
Immobilion PVDF membrane pore size 0.45 µm	Merck Millipore	Darmstadt, Germany
Amersham ECL Prime Western Blotting Detection Reagent	GE Healthcare	Freiburg, Germany
Rotiphorese Gel 30	Carl Roth	Karlsruhe, Germany
Tetramethylethylenediamine	Carl Roth	Karlsruhe, Germany
Ammonium Persulfate Solution (APS)	Carl Roth	Karlsruhe, Germany
Arcturus PicoPure RNA isolation Kit	Applied Biosystems	Foster City, CA, USA
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific	Karlsruhe, Germany
RNase-Free DNase Set	Qiagen	Hilden, Germany
SYBR Green PCR Master Mix	Applied Biosystems	Warrington, UK
X-tremeGene 9 DNA transfection Reagent	Roche Diagnostics	Mannheim, Germany
Plasmid Maxi Kit	Qiagen	Hilden, Germany
alamarBlue	Invitrogen	
Ibidi Culture-Insert 500 µm	ibidi	München, Germany
CytoSelect 96-Well Cell Invasion Assay	CELL BIOLABS	Heidelberg, Germany

Cell culture reagents	Company	Branch
Neurobasal medium	Gibco® Life Technologies	Darmstadt, Germany
DMEM AQmedia	Sigma-Aldrich	Steinheim, Germany
mTesR1	Stem Cell Technologies	Köln, Germany
Medium 254	Gibco® Life Technologies	Darmstadt, Germany
DMEM/F12	Gibco® Life Technologies	Darmstadt, Germany
Human melanocyte growth supplement (HMGS) 100x	Gibco® Life Technologies	Darmstadt, Germany
Fetal Calf Serum (FCS)	Biochrom	Berlin, Germany
Adenine	Sigma-Aldrich	Steinheim, Germany
Penicillin/Streptomycin	Sigma-Aldrich	Steinheim, Germany
Trypsin	Sigma-Aldrich	Steinheim, Germany
Non-essential amino acids	Sigma-Aldrich	Steinheim, Germany
2-Mercaptoethanol	Gibco® Life Technologies	Darmstadt, Germany
Basic fibroblast growth factor (bFGF)	Promokine	Heidelberg, Germany
Bone morphogenic protein 4 (BMP4)	Promokine	Heidelberg, Germany
Leukemia Inhibitory Growth Factor	Sigma-Aldrich	Steinheim, Germany
Epidermal Growth Factor (EGF)	Gibco® Life Technologies	Darmstadt, Germany
N2	Gibco® Life Technologies	Darmstadt, Germany
B27	Gibco® Life Technologies	Darmstadt, Germany
Noggin	R&D systems	Wiesbaden-Nordenstadt, Germany
Insulin	Sigma-Aldrich	Steinheim, Germany
Hydrocortisone	Sigma-Aldrich	Steinheim, Germany
Cholera Toxin	Sigma-Aldrich	Steinheim, Germany
Forskolin	R&D systems	Wiesbaden-Nordenstadt, Germany
Doxycyline	Sigma-Aldrich	Steinheim, Germany
Mitomycin C	Carl Roth	Karlsruhe, Germany
Dimethylsulfoxide	Carl Roth	Karlsruhe, Germany

Small molecule inhibitors	Company	Branch
PLX4032	Selleck Chemicals	München, Germany
GSK1120212	Selleck Chemicals	München, Germany
CHIR99021	Selleck Chemicals	München, Germany
LDN193189	Selleck Chemicals	München, Germany

SB431542	Selleck Chemicals	München, Germany
Y-27632	Stemgent	San Diego, USA

Antibodies	**Company**	**Branch**
Mouse anti-Cytokeratin (AE1/AE3) antibody	Dako	Hamburg, Germany
Rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody	Cell Signaling Technology	Leiden, Netherlands
Rabbit anti-p44/42 MAPK (ERK1/2) antibody	Cell Signaling Technology	Leiden, Netherlands
Mouse anti-BRAF V600E antibody, clone VE1	Biomol	Hamburg, Germany
Mouse anti-Cytokeratin 20 antibody, clone K	Dako	Hamburg, Germany
Mouse anti-MelanA antibody	VECTOR LABORATORIES	Lörrach, Germany
Rabbit anti-Sox2 antibody	Abcam	Cambridge, UK
Rabbit anti-Ki67 antibody	Abcam	Cambridge, UK
Rabbit anti-Nanog	Abcam	Cambridge, UK
Mouse-Tra-1-60	Cell Signaling Technology	Leiden, Netherlands
Mouse-Tra-1-81	Cell Signaling Technology	Leiden, Netherlands
Rabbit anti-Neuron-specific class III β-tubulin (Tuj 1)	Kindly provided by Dr. Sandra Horschitz, Central Institute for Mental Health	Mannheim, Germany
Atto 488 goat anti-rabbit IgG	Sigma-Aldrich	Steinheim, Germany
Atto 647 goat anti-mouse IgG	Sigma-Aldrich	Steinheim, Germany
Atto 647 goat anti-rabbit IgG	Sigma-Aldrich	Steinheim, Germany

VII.2. Buffer solutions:

Phosphate buffered saline (PBS) pH 7.4

3.2 mM Na_2HPO_4
0.5 mM KH_2PO_4
1.3 mM KCl

Tris buffered saline (TBS) pH 7.6

137 mM NaCl
20 mM Tris

Running buffer pH 8.3
25 mM Tris
190 mM glycine
0.1% SDS

Transfer buffer pH 8.3
25 mM Tris
190 mM glycine
20% methanol

Laemmli 2x buffer pH 6.8
4% SDS
10% 2-mercaptoethanol
0.004% bromophenol blue
0.125 M Tris HCl

Washing buffer pH 7.6
137 mM NaCl
20 mM Tris
0.1% Tween20

Cell lysis buffer for protein isolation
1x PhosphoStop
1x cOmplete Mini Protease Inhibitor Cocktail
1% Triton-X
in TBS

Analysis Software	**Source**
ImageJ	National Institute of Health (NIH)
GraphPad Prism 5	GraphPad Prism
NIS-Elements Viewer	Nikon
7500 Software v2.0.5	Applied Bioscience
Chipster	Chipster Open source
DAVID Bioinformatics Resources	NIH
Ingenuity	Qiagen
Leica Application Suite v4.0	Leica

Devices	**Company**	**Branch**
AB 7500 Real Time PCR machine	Applied Biosciences	Darmstadt, Germany
SpectraMax M5	Molecular Devices	Biberach an der Riss, Germany
NanoDrop ND-1000 Spectrophotometer	Peqlab Biotechnologie GmbH	Erlangen, Germany
Nikon Eclipse Ti Fluorescence microscope	Nikon	Düsseldorf, Germany
Nikon Eclipse TS100 microscope	Nikon	Düsseldorf, Germany
Leica DM LS microscope	Leica	Wetzlar, Germany
ImageQuant LAS biomolecular imager	GE Healthcare	Freiburg, Germany

VII.3. Methods

VII.3.1. Cell culture

All cells were grown in a humidified atmosphere at 37°C and 5% CO_2. Individual culture conditions are listed below.

VII.3.1.1. Cell culture of tumor cells and murine embryonic fibroblasts

Tumor cells and murine embryonic fibroblasts were cultivated in Dulbecco's Modified Eagle Medium (DMEM) with 4500 mg/l glucose and 4 mM L-alanyl-L-glutamine supplemented with 10% (v/v) heat inactivated fetal bovine serum (FCS), 1% (v/v) 100x non-essential amino acids (NEAA), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.1 mM β-mercapthoethanol, from here on referred as complete medium. Every 3-5 days when 80% confluence was reached the cells were subcultured using a 21 mM trypsin solution.

VII.3.1.2. Melanocyte cell culture

Human melanocytes were obtained from foreskins, kindly provided by Dr. Uysal, Mannheim. Donor age ranged from new born to three years old patients. Excised foreskins were incubated for 15 min at room temperature in 10% Braunol solution, were washed with PBS and separated from subcutaneous fat followed by cutting into 10 x 4 mm pieces. Specimens were digested in dispase (1 mg/ml) at 4°C overnight followed by 15 min digestion of the epidermis at 37°C in trypsin/EDTA. Primary melanocytes were washed and transferred to 10 cm dishes in medium 254 supplemented with 1% (v/v) 100x human melanocyte growth supplement (HMGS) resulting in a final concentration of 0.2% (v/v) bovine pituitary extract, 0.5% v/v fetal bovine serum, 1 µg/ml recombinant human insulin-like growth factor-I, 5 µg/ml bovine transferrin, 3 ng/ml basic fibroblast growth factor (bFGF), 0.18 µg/ml hydrocortisone, 3 µg/ml heparin and 10 ng/ml phorbol 12-myristate 13-acetate. Cells were subcultured before reaching 90% confluence.

VII.3.1.3. Generation of iPS and iPC cells

For reprogramming of human tumor cells, fibroblasts and melanocytes 10^5 cells per cm^2 were seeded on gelatin-coated plates and transduced with a reverse tetracycline-controlled transactivator (FUdeltaGW-rtTA-zeocin) containing a zeocin resistance gene. Cells were selected with 100 µg/ml zeocin in complete medium generating resistant clones that were manually picked and expanded. The clones were co-infected with a doxycycline-inducible vector expressing a stem cell cassette (STEMCCA) encoding for the transcription factors Oct4, Sox2, Klf4 and a puromycin resistance or alternatively for Oct4, Sox2, Klf4 and c-Myc. The next day, superinfection was performed to reach higher efficiencies. All transductions were

conducted by incubation of cells with virus for 24 h at 37°C in DMEM medium supplemented with 10 µg/ml polybrene. 24 h after the last infection 10^5 cells in complete medium were plated onto six well tissue culture plates coated with gelatin. After cell attachment doxycycline was added to the medium to induce transgene expression. From here on, medium was changed every second day. After 30-40 days first colony-forming cells originated. In order to create reprogrammed clones derived from single cells individual colonies were manually transferred onto fresh feeder cells in DMEM/F12 with 20% (v/v) knockout serum replacement (KOSR), 2 mM L-glutamine, 1% (v/v) NEAA, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.1 mM ß-mercaptoethanol, 1 µg/ml doxycycline and supplemented with 10 ng/ml human LIF, from here on referred to as naïve hES medium, until homogenous colonies were established.

VII.3.1.4. Human induced pluripotent stem cell culture

Stable clones of human iPSCs were cultivated under xeno-free cell culture conditions using a synthetic surface matrix. Therefore, one day prior use six well tissue culture plates were coated with Matrigel for one hour at room temperature and stored at 4°C. Human iPSCs were washed and undifferentiated parts were manually dissociated into cell clusters of 50-100 cells. These small cell aggregates were transferred to Matrigel-coated plates in mTeSR1 medium containing 20% (v/v) mTeSR1 supplements of bovine serum albumin, recombinant human bFGF, recombinant human TGF-β, lithium chloride, pipecolic acid and γ-aminobutyric acid. Every other day medium was changed and differentiated parts manually removed. Alternatively, human iPSCs were cultivated on feeder cells in DMEM/F12 with 20% (v/v) knockout serum replacement, 2 mM L-glutamine, 1% (v/v) NEAA, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.1 mM β-mercaptoethanol and 10 ng/ml bFGF, from here referred as human ES medium.

VII.3.1.5. Culture of human iPS and iPC cells in the alternative pluripotent state

Mitotic inactivated feeder cells were plated on gelatin-coated six well tissue culture plates in complete medium and incubated for two days to ensure proper attachment and spread. Then iPS cells were transferred onto the feeder cells and medium was changed to naïve hES medium. For passaging cells were harvested every 4-7 days using trypsin and replated at 1:30 to 1:100 ratios in naïve hES medium containing 10 µM ROCK inhibitor (Y27632). In order to separate iPS or iPC cells from feeder cells by preplating, cells were harvested using trypsin, dissociated into single cells, washed and resuspended in naïve hES medium containing ROCK inhibitor. Then, the cell suspension was transferred onto gelatin-coated tissue culture plates and incubated for 2 hours at 37°C. Afterwards undifferentiated cells floating in the supernatant were collected and prepared for further experiments.

VII.3.1.6. Preparation of murine embryonic fibroblast

Day 12.5 to 13.5 embryos postcoitum of C57BL/6 mice were dissected from the uterus and incubated for five minutes in 10% Braunol. Embryos from one mouse were rinsed in PBS followed by the mechanical removal of internal organs and the head. The carcass was manually minced in trypsin/EDTA solution using scalpels and incubated at 37°C for ten minutes. Afterwards the cell suspension was neutralized in complete medium, washed and resuspended in complete medium. Subsequently, the cells of one embryo were transferred to one 150 mm tissue culture dish and cultivated until confluence.

VII.3.1.7. Mitotic inactivation of feeder cells

Murine embryonic fibroblasts were expanded until passage three in either T175 cell culture flasks or 150 mm cell culture dishes. Dense fibroblasts were incubated with 10 µg/ml mitomycin C for 4 hours and washed with Ca^{2+} and Mg^{2+} containing PBS for three times followed by rinsing the cells with Ca^{2+} and Mg^{2+} free PBS. The postmitotic cells were trypsinized for 5-7 min at 37°C until the cell detached followed by neutralization of the enzymatic digestion with complete medium. After centrifugation the cells were resuspended in 80% FCS with 20% (v/v) dimethyl sulfoxide (DMSO) and aliquoted at a concentration of $1x10^6$ cells per vial. Until thawing the vials were stored in liquid nitrogen.

VII.3.1.8. Fibroblast differentiation

For the differentiation into fibroblast-like cells HT-144-iPCCs were seeded onto 80% confluent mitotic-inactivated feeder cells in naïve hES medium with 10 µM ROCK inhibitor and 1 µg/ml doxycycline and cultivated for two to five days until small colonies were formed. In order to establish the clones A-C, different protocols were followed. For clone A medium was switched to complete medium until stably expandable colonies emerged. Clone B was generated by changing the medium to DMEM/F12 1:1 with Neurobasal medium containing 1% B27 and 0.5% (v/v) N2 supplement (Gibco) for 3 days. Then the medium was also switched to complete medium with 20% (v/v) FCS. For clone C iPCC colonies were cultivated in DMEM/F12 3:1 supplemented with 10% (v/v) FCS, 0.18 mM adenine, 0.5 µg/ml hydrocortisone, 100 pM cholera toxin, 10 ng/ml EGF, 5 µg/ml insulin for 10 days and supplemented on days four to ten with 0.5 nM BMP-4. Afterwards fibroblast-like cells were split and maintained in T75 cell culture flasks with complete medium.

VII.3.1.9. Neuronal differentiation

For neuronal induction, $2x10^4$ cells per cm^2 were seeded on Matrigel-coated dishes in human naïve ES medium supplemented with 10 µM ROCK inhibitor. When small colonies of 5-10 cells appeared medium was changed to DMEM/F12 and Neurobasal mixed at a 1:1 ratio with 1% (v/v) B27 and 0.5% (v/v) N2, 100 ng/ml noggin, 0.5 µM LDN-193189, 10 µM SB-491542, 2 µM

CHIR-99021, 10 µM forskolin and 10 ng/ml bFGF for 3-10 days. Subsequently, the cells were cultivated for additional 5-10 days without small compound inhibitors but in the presence of 10 ng/ml bFGF.

VII.3.1.10. Small molecule inhibitors

All small molecule inhibitors were obtained from Selleck Chemicals as powder. Vemurafenib (PLX4032), trametinib (GSK1120212), LDN193189 and SB431542 were dissolved in DMSO to a stock concentration of 10 mM, CHIR99021 was dissolved in DMSO to a 30 mM stock solution. Aliquots of the inhibitors solutions were stored at -20°C and applied at the indicated concentrations.

Inhibitor	Target	Targeted pathway	Resolvent
PLX4032	$BRAF^{V600E}$	MAPK	DMSO
GSK1120212	MEK1/MEK2	MAPK	DMSO
CHIR99021	GSK-3α/β	GSK-3β	DMSO
LDN193189	ALK2, ALK3	BMP4	DMSO
SB431542	ALK5, ALK4, ALK7	TGF-β	DMSO

Table 2 | Small molecule inhibitors used in the study.

VII.3.2. Transformation and plasmid isolation

For plasmid amplification Dh5α competent E.coli cells were heat shocked at 42°C for 90 s in the presence of the plasmid of interest followed by a resting phase on ice for 2 minutes. 500 µl LB media was added and bacteria were shaken for 60 minutes at 37°C. Transformed bacteria were plated on LB agar plates with 100 µg/ml ampicillin for selection and incubated overnight at 37°C. Single colonies were manually picked and transferred to sterile culture tubes containing 5 ml LB media supplemented with 100 µg/ml ampicillin and incubated for 12 h at 37°C. From the bacteria suspension plasmid DNA was isolated and the vector confirmed by restriction digestion. Sterile flasks containing 200 ml LB medium supplemented with 100 µg/ml ampicillin were inoculated with bacteria containing verified plasmids and shaken in an orbital shaker at 37°C overnight. The next day bacteria were pelleted, lysed and plasmid DNA purified using the QIAGEN Plasmid Purification Maxi Kit according the manufacturer's protocol. After isopropanol ethanol precipitation air-dried plasmid DNA was redissolved in buffer TE. Quality and quantity was analyzed using a NanoDrop ND-1000 Spectrophotometer.

VII.3.3. Viral vector production

Viral vector production was performed using a three plasmid transfection system in HEK 293T producer cells. Therefore, 60-70% confluent 293T cells in c medium were transfected with the

expression vectors encoding for the packing proteins *gag*, *pol* and *rev* and the envelop plasmid VSV-G in addition to the plasmid of interest using FuGENE transfection reagent according to the manufacturer's manual. After 12 h medium was changed to complete medium without antibiotics. For four consecutive times viral supernatant was collected every 12 h starting 24 h after infection. The pooled supernatants were filtered and directly used for infection or stored at 4°C for up to two weeks. For the infection of melanocytes the viral supernatant was concentrated by ultracentrifugation at 40.000xg for 2 h at 4°C, resuspended in PBS and stored at -80°C.

VII.3.4. Teratoma formation assay and immunohistochemical staining

For teratoma formation assay 10^6 naïve iPCCs were resuspended in 50% Matrigel and subcutaneously injected into each flank of NOD/SCID mice. After 8-10 weeks teratomas were isolated, washed and fixed in 4% paraformaldehyde overnight at room temperature. Paraffin-embedded samples were cut and sections were deparaffinized in xylene followed by rehydration. After antigen retrieval the following targets were stained: S100, Ki67, PanCK, $BRAF^{V600E}$, CK20, MelanA, Sox2, p44/42, phospho-p44/42. Afterwards samples were counterstained with haematoxylin. For histological analysis of tumors and evaluation of teratomas, sections were stained with haematoxylin and eosin (H&E). Cutting, deparaffinization and stainings were kindly performed by Sayran Arif-Said, Clinical Cooperation Unit Dermato-Oncology. All samples were analyzed under a Leica DM LS light microscope.

VII.3.5. RNA isolation and cDNA transcription

RNA extraction was performed using the RNeasy kit (Qiagen) or PicoPure RNA isolation kit (Life Technologies) according to the manufacturer's instructions. Briefly, pelleted cells were lysed and RNA extracted using a column based purification. Every sample was DNase I treated for 15 minutes at room temperature on the purification column followed by two washing steps and the elution in RNase-free H2O.

RNA concentration and quality was measured using a NanoDrop ND-1000 Spectrophotometer. Exclusively samples fulfilling the quality recommendations were further analyzed. From each sample 500 ng RNA were incubated with oligo $(dT)_{18}$ primers in a volume of 12 µl for five minutes at 65°C. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's advice. Before use cDNA was diluted 1:5 in nuclease-free H2O.

VII.3.6. Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed using SYBR Green PCR Master Mix and an Applied Biosystems 7500 Real-Time PCR System. Results were either normalized to two endogenous controls (GAPDH

and 18s RNA or GAPDH and ß-actin) or for low differently regulated expression levels between the samples confirmed by analyzing three technical replicates. Each primer pair was evaluated and amplification efficiency confirmed to lay in a range from 85-110%. Gene quantification was calculated using the Pfaffl method (Bustin et al. 2009) calculating the delta-delta Ct. Statistical analysis was carried out in Excel and visualization of graphs in GraphPad Prism 5. Primers used in this study are listed in table 000.

	Forward primer	Reverse primer
Sox2_endo	*GCTAGTCTCCAAGCGACGAA*	*GCAAGAAGCCTCTCCTTGAA*
Oct4_endo	*GACAGGGGGAGGGGAGGAGCTAGG*	*CTTCCCTCCAACCAGTTGCCCCAAAC*
Nanog_endo	*CAGTCTGGACACTGGCTGAA*	*CTCGCTGATTAGGCTCCAAC*
Sall4	*ATTCCCTGGGTGGTTCACT*	*AGCACATCAACTCGGAGGAG*
Tet1	*CGCTACGAAGCACCTCTCTTA*	*CTTGCATTGGAACCGAATCATTT*
DPPA4	*GACCTCCACAGAGAAGTCGAG*	*TGCCTTTTTCTTAGGGCAGAG*
Nodal	*CAGTACAACGCCTATCGCTGT*	*TGCATGGTTGGTCGGATGAAA*
Lefty1	*AGGAGCTGGTCATCCCCAC*	*GCCACCTCTCGGAAGCTCT*
Lefty2	*TGGACCTCAGGGACTATGGAG*	*CCGAGGCGATACACTGTCG*
DNMT3L	*TGAACAAGGAAGACCTGGACG*	*CAGTGCCTGCTCCTTATGGCT*
Pax6	*AACGATAACATACCAAGCGTGT*	*GGTCTGCCCGTTCAACATC*
MAP2	*CGAAGCGCCAATGGATTCC*	*TGAACTATCCTTGCAGACACCT*
RBFOX3	*TCGTAGAGGGACGGAAAATTGA*	*GCCGTTGGTGTAGGGGTTC*
MITF-M	*AGAGGGAGGGATAGTCTACCG*	*ACTTGGTGGGGTTTTCGAGG*
AP2	*GGAGACGTAAAGCTGCCAAC*	*GGTCGGTGAACTCTTTGCAT*
Sox10	*AGCCCAGGTGAAGACAGAGA*	*ATAGGGTCCTGAGGGCTGAT*
TRP1	*AGCAGTAGTTGGCGCTTTGT*	*TCAGTGAGGAGAGGCTGGTT*
E-Cadherin	*AGCCAACCTTAACTGAGGAGT*	*GGCAAGTTGATTGGAGGGATG*
FSP1	*CTGCCCAGCTTCTTGGGG*	*TGGGCTGCTTATCTGGGAAG*
Vimentin	*ACACCCTGCAATCTTTCAGACA*	*GATTCCACTTTGCGTTCAAGGT*
GAPDH	*GAAGGTGAAGGTCGGAGTC*	*GAAGATGGTGATGGGATTTC*
β-Actin	*GGATGCCACAGGATTCCATACCCA*	*TCACCCACACTGTGCCGATCTACGA*

Table 3 | qPCR primer pairs used in the study.

VII.3.7. Genomic DNA isolation and cell authentication

Genomic DNA was isolated from HT-144 and their reprogrammed counterparts by a column-based purification method using the QIAGEN DNeasy Blood & Tissue Kit according to the manufacturer's instructions. Briefly, cells were lysed and digested with proteinase K for ten minutes at 56°C. Genomic DNA was isolated from the mixture through column purification and eluted in buffer AE. DNA concentration and quality was measured using a NanoDrop ND-1000 Spectrophotometer. A 24-plex single nucleotide polymorphism profiling assay was performed by MULTIPLEXION as described in Castro et al. (2013) to confirm their common identity. Additionally, cell authentication was performed by DMSZ applying short tandem repeat DNA typing and comparison to the DNA reference database of human cell lines, thereby verifying the HT-144 cell line.

VII.3.8. Immunofluorescence and alkaline phosphatase staining

Tumor and feeder cells were seeded on gelatin coated coverslips while neuronal-differentiated cells were seeded on Matrigel-coated coverslips. For nuclear staining cells were fixed in methanol for 7 min at -20°C and subsequently rinsed with -20°C cold acetone. Samples for surface marker staining were fixed in 4% paraformaldehyde (PFA) for 5 min at room temperature and permeabilized with 0.1% Tween 20 in PBS for additional 5 min. Blocking was performed with PBS containing 0.5% (w/v) BSA, 1% (v/v) FCS and 0.1% (v/v) Triton X-100 for 30 min at room temperature. Then samples were incubated with primary antibodies overnight at 4°C in blocking solution with the indicated dilutions: rabbit anti-Nanog 1:150, mouse anti-TRA-1-60 1:250, mouse anti-TRA-1-81 1:250 and rabbit anti-β3-tubulin (Tuj-1) 1:250. Samples were washed twice with blocking solution and incubated with either Atto 488 goat anti-rabbit IgG 1:500, Atto 647 goat anti-mouse IgG 1:500 or Atto 647 goat anti-rabbit IgG in blocking dilution for 4 h at 4°C. Afterwards samples were washed twice, counterstained with 100 ng/ml DAPI in PBS and mounted with Dako Fluorescent Mounting Medium (Dako, S3023). The next day samples were analyzed with a Nikon ECLIPSE Ti fluorescent microscope.

Staining for alkaline phosphatase activity was performed using the Stemgent Alkaline Phosphatase Staining Kit II according to the manufacturer's protocol. Following fixation, cells were washed with PBS and incubated with AP staining solution for 15 minutes at room temperature. The reaction was stopped by washing with PBS and pictures were taken using a Nikon ECLIPSE TS 100 light microscope.

VII.3.9. Western blot analysis

Cells were washed twice with ice-cold PBS and proteins were isolated in 1% Triton X-100 supplemented with Roche cOmplete Mini Protease Inhibitor Cocktail and Roche PhosphoStop Phosphatase Inhibitor Cocktail in PBS. Cell debris was pelleted and protein concentration

estimated using Pierce BCA Protein Assay Kit. Under denaturating conditions 30 µg protein was fractionated by SDS-PAGE. 12% gels were produced according the scheme below. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes with 30V for 10 h at 4°C. Membranes were blocked in 5% non-fat dried milk in TBST for 1h at room temperature. Primary antibodies were diluted in 5% (w/v) BSA, 0.1% (v/v) Tween 20 in TBS in the following dilutions: rabbit anti-phospho-p44/42 1:2000, rabbit anti-p44/42 1:1000, anti-rabbit GAPDH and incubated overnight at 4°C. Membranes were washed three times in TBST and subsequently incubated with a goat anti-rabbit horseradish peroxidase linked antibody diluted 1:10.000 in 5% non-fat dried milk in TBST at room temperature for 2 h. Proteins were visualized by enhanced chemoluminescence (ECL) reagent as peroxidase substrate.

Resolving gel

3.3 ml	H2O
3 ml	30% acrylamide/bisacrylamide solution
2.5 ml	1.5 M Tris pH 8.8
100 µl	10% SDS solution
100 µl	10% ammonium persulfate solution (APS)
20 µl	tetramethylenediamine (TEMED)

Stacking gel

3.4 ml	H2O
0.83 ml	30% acrylamide/bisacrylamide solution
0.63 ml	1 M Tris pH 6.8
50 µl	10% SDS solution
50 µl	10% ammonium persulfate solution (APS)
5 µl	tetramethylenediamine (TEMED)

VII.3.10. 24-colour FISH

24-multi colour FISH to detect chromosomal rearrangements was kindly performed by Prof. Dr. Jauch, Department of Human Genetics, Heidelberg University Hospital. Each probe binds specifically one of the human chromosomes. After washing and probe detection reactions the chromosome spreads were counterstained with DAPI and analyzed with a fluorescence microscope.

VII.3.11. Invasion assay

Cell invasiveness was determined by the use of the CELL BIOLABS 96-well cell invasion assay precoated with an extracellular basement membrane according to the manufacturer's

instructions. 150 µl medium supplemented with 10% FCS was added to the lower feeder tray of the invasion plate. Cells were collected by trypsinization, washed with PBS and resuspended in glucose-free, serum-free DMEM medium and 100 µl of the cell suspension containing $5x10^4$ cells distributed to each insert. Afterwards invasion plates were incubated for 24 h at 37°C. The membrane chamber was then transferred to the harvesting tray containing 150 µl Cell Detachment Solution and incubated for 30 minutes at 37°C. Migrated cells on the lower surface of the membrane were detached by gently tilting the membrane chamber followed by adding 50 µl Lysis Buffer/CyQuant GR dye solution. After 20 minutes incubation at room temperature, 150 µl solution were transferred into a black 96-well plate and analyzed using a SpectraMax M5 multimode plate reader.

VII.3.12. Migration assay

To avoid a variable physical manipulation of cells, a cell chamber-based migration assay was performed. Therefore, sterile cell culture inserts were placed into six well cell culture plates and incubated until air-dried inserts remained fixed to the plate. Cells were harvested by trypsinization, washed and resuspended at a concentration of $5*10^5$ cells/ml followed by the transfer of 70 µl cell suspension into each chamber of the insert. Afterwards cells were allowed to attach overnight before the culture inserts were removed. Directly light microscopy images were acquired with a Nikon ECLIPSE Ti microscope at the indicated time points using an automated positioning system to ensure recording of precisely the same locations. Images were analyzed using Nikon NIS-Elements AR 4.00.00 image analysis software.

VII.3.13. Cell viability assay

Cell viability was analyzed using alamarBlue Cell viability assay. The method monitors metabolic activity based on the reducing conditions generated by growing cells. 1.000, 3.000 and 5.000 cells were plated in triplicates in black 96-well plates in 100 µl complete medium. After 24 h at 37°C 100 µl of complete medium was added supplemented with DMSO or inhibitors at two fold concentrations ranging from 1 nm to 1 µM. At indicated timepoints 20 µl alamarBlue were added and cells were allowed to reduce the reagent for two to four h at 37°C followed by fluorescence measurement using a SpectraMax M5 microplate reader at an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Cell viability was calculated from the resulting change in fluorescent intensity normalized to cells exposed to the vehicle only.

VII.3.14. Whole genome expression analysis

Whole genome expression profiling was performed at DKFZ Core Facility for Genomics and Proteomics. Samples were analyzed on a HumanHT-12 v4 Expression BeadChip (Illumina) that is able to quantify expression levels of 48107 human genes. Therefore, 1 µg total RNA

was isolated as described in VII.1.14. (RNA isolation and cDNA transcription) in triplicates from each sample. Members of the core facility performed quality control, reverse transcription with labeling, chip hybridization and calculation of mean averages for each probe according to the manufacturer's instructions. Resulting raw data were exported to Chipster software. First, array data were log2 transformed and quantile normalized. For assessing differentially regulated genes two group test or several group test using empirical Bayes method were performed with a p value adjustment according to Benjamini-Hochberg (BH). P value threshold was set to 0.05 to filter for significantly deregulated genes. Differentially expressed genes were clustered using Spearman correlation as a distance measure visualizing up-regulated genes in red and down-regulated genes in green. Dendrograms as result of the hierarchical clustering were constructed by the average linkage method. Gene sets were analyzed for enrichment of distinct pathways using DAVID software from the Bioinformatics Resources from the National Institute of Allergy and Infectious Diseases (NIAID), NIH (http://david.abcc.ncifcrf.gov/) and pathway analysis was performed by MetaCore, THOMSON REUTERS (https://portal.genego.com/).

VIII. Aims of the thesis

The technique to convert somatic cells of various origins into a pluripotent state by overexpression of transcription factors offers a model to analyze molecular changes, which are associated with the switch from one cell fate to another. This process is induced by epigenetic modifications, providing the potential to study implications of cell type-specific epigenetic marks on cellular fates.

In cancer cells, genomic modifications like hypermethylations of DNA and chromatin are aberrantly regulated. Their epigenetic signature comprises marks derived from the cell of origin and cancer-specific modifications. These cancer-specific epigenetic modifications are associated with tumor-promoting functions.

Although novel melanoma therapies targeting MAPK signaling have the capacity to provide significant clinical benefit, their therapeutic efficacy is limited due to the development of acquired resistance. Besides secondary mutations, epigenetic modifications seem to play a major role in the appearance of resistant melanoma cells.

Therefore, this thesis addresses the following questions:

a) Can human melanoma cells be converted into pluripotent stem cells by nuclear-based reprogramming?
b) Do epigenetic changes, associated with nuclear reprogramming, influence the development of resistances against novel targeted melanoma therapies?

Using iPSCs technology is an elegant way to study molecular processes in human cancer cells. The question, how epigenetic mechanisms regulate tumorigenicity and sensitivity to therapies, is instrumental for the development of novel cancer therapeutics. Furthermore, anti-cancer drugs affecting the epigenetic cell state are already used in the clinic.

IX. Results

IX.1. Generation of induced pluripotent cancer cells

Reprogramming of cancers cells was investigated using four human melanoma cell lines of different mutational status, a primary human melanoma cell line derived from a trametinib-resistant melanoma metastasis (Ma-1) (table 4), the human cervical cancer cell line HeLa, and primary melanocytes as control. Cells were co-infected with the lentiviral expression vector TetO-human-STEMCCA and a Zeocin-selectable rt-TA transactivator (FUdeltaGW-rtTA-Zeocin). The stem cell cassette (STEMCCA) encodes for the reprogramming factors Oct4, Sox2, Klf4 and c-Myc separated by a combination of internal ribosome entry sites (IRES) and self-cleaving 2A oligopeptides. Data from whole gene expression analysis demonstrated that the melanoma cell lines HT-144 and Ma-1 expressed c-Myc at levels similar to iPSCs (figure 3B). Therefore, the STEMCCA vector was modified by replacing c-Myc with a puromycine resistance gene to reprogram tumor cells without the oncogene (figure 3C). In cells expressing the transactivator, transgene expression was induced by the addition of doxycycline (figure 3A&C). 30-40 days after the induction of exogenous transcription factors, cells were cultivated on postmitotic murine fibroblasts (feeder cells) and two days later medium was replaced with naïve ESC-medium. Depending on the cell type, 20-40 days later first colony-forming cells appeared, which were manually picked and transferred onto fresh feeder cells. Surprisingly, the emerging cells shared morphological features of murine ESCs. In contrast to bFGF-dependent human iPSCs or ESCs, these colonies were maintained in the presence of doxycycline and human LIF without bFGF. Cells were resistant to single-cell dissociation without Rho kinase inhibitor (Y-27632), although its addition significantly increased cell survival. After re-plating trypsin-digested cells, single cells formed tightly packed, doom-like shape colonies on feeder cells and were positive for the expression of alkaline phosphatase (AP), an early marker of reprogramming (figure 3D). The origin of the HT-144-iPCCs cells was confirmed analyzing 24 single nucleotide polymorphisms, followed by comparison to their parental cell line (figure 3F). The consensus sequence proved the purity and authentication of the reprogrammed and parental cells. Furthermore, it was of interest whether normal human iPSCs derived from somatic cells (NHiPSCs) could be cultivated under the same conditions suitable for tumor-derived iPSC-like colonies. Therefore, feeder-free NHiPSCs were picked and transferred to feeder cells in the presence of doxycycline and human LIF. After two to three passages with trypsinization and single cell replating colonies appeared, which were morphologically indistinguishable from murine ESCs. Consequently, iPSCs could be switched between different pluripotency-states. In the alternative state, iPSCs gained features of murine

ESCs, capable to form tightly packed three-dimensional colonies derived from single cells on dense feeder cells (figure 3D&E). NHiPSCs that were switched into the alternative state of pluripotency are hereafter referred to as NHiPSCs AS. In summary, tumor cells were reprogrammed towards a state similar to murine ESCs, generating AP positive colonies by consistently overexpressing the reprogramming factors.

Human melanoma cell lines	***BRAF***	***NRAS***
HT-144	V600E	wt
WM266.4	V600D	unknown
Mewo	wt	wt
SKMEL147	wt	mut
Ma-1	V600E	wt

Table 4 | Mutational status of human melanoma cell lines.

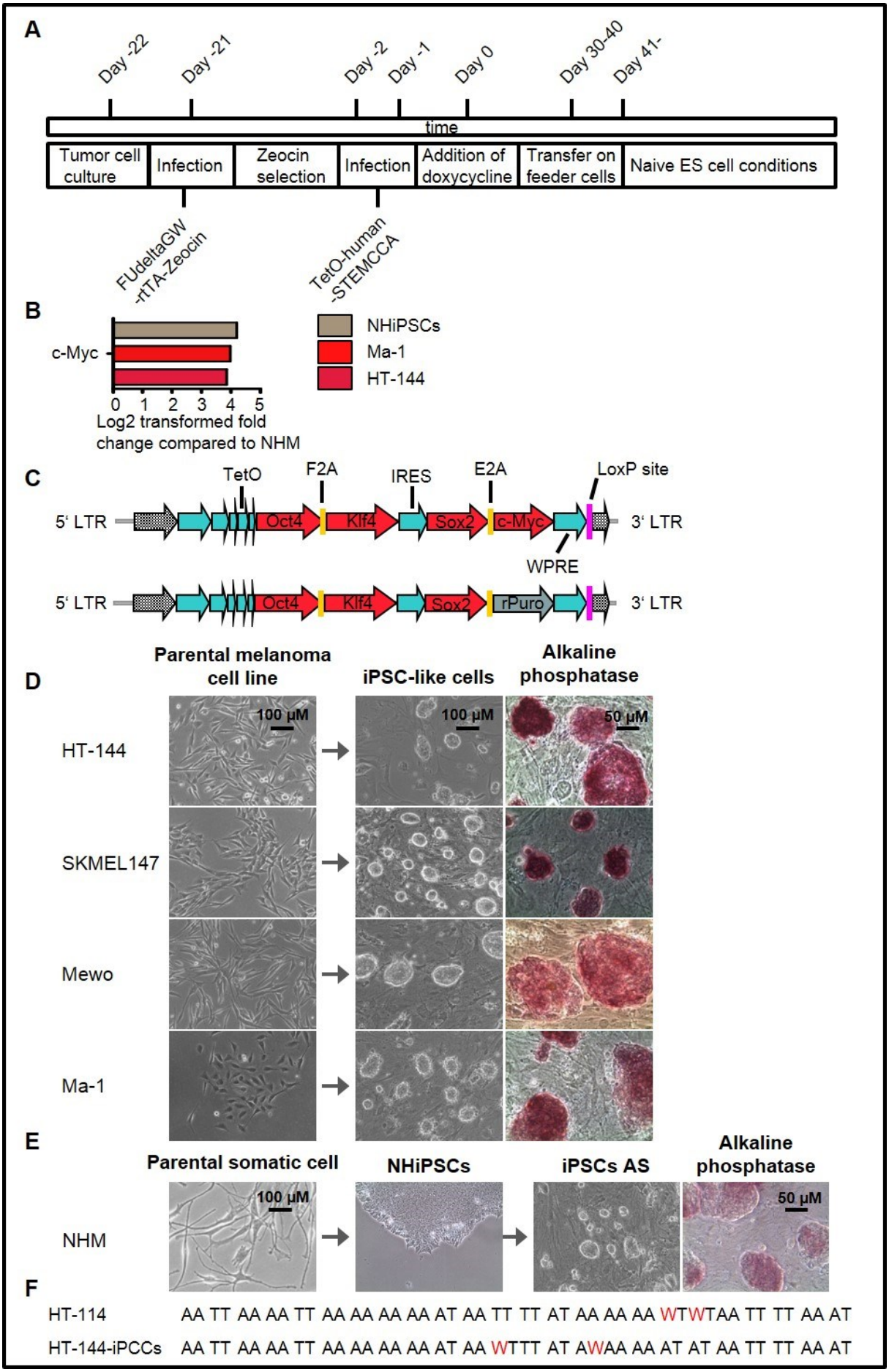

A
Day -22
Day -21
Day -2
Day -1
Day 0
Day 30-40
Day 41-
time
Tumor cell culture
Infection
Zeocin selection
Infection
Addition of doxycycline
Transfer on feeder cells
Naive ES cell conditions
FUdeltaGW -rtTA-Zeocin
TetO-human -STEMCCA
B
c-Myc
0 1 2 3 4 5
Log2 transformed fold change compared to NHM
NHiPSCs
Ma-1
HT-144
C
TetO
F2A
IRES
E2A
LoxP site
5' LTR
Oct4
Klf4
Sox2
c-Myc
3' LTR
WPRE
5' LTR
Oct4
Klf4
Sox2
rPuro
3' LTR
D
Parental melanoma cell line
iPSC-like cells
Alkaline phosphatase
100 µM
100 µM
50 µM
HT-144
SKMEL147
Mewo
Ma-1
E
Parental somatic cell
NHiPSCs
iPSCs AS
Alkaline phosphatase
100 µM
50 µM
NHM
F
HT-114 AA TT AA AA TT AA AA AA AA AT AA TT TT AT AA AA AA WTWTAA TT TT AA AT
HT-144-iPCCs AA TT AA AA TT AA AA AA AA AT AA WTTT AT AWAA AA AT AT AA TT TT AA AT

Figure 3 | Reprogramming of human tumor cells into induced pluripotent cancer cells (iPCCs). A) Schematic time line for the reprogramming of tumor cells. B) Expression analysis of c-Myc in melanoma cell lines HT-144 and Ma-1 compared to NHiPSCs by gene expression microarray. Expression levels were normalized to normal human melanocytes (NHM). Fold change was log2 transformed. C) Illustration of the STEMCCA vector used for reprogramming, encoding for the transcription factors Oct4, Sox2, Klf4 and a puromycin resistance for selection. D) Microscopical analysis of reprogrammed tumor cells (iPSC-like cells) and of alkaline phosphatase (AP) expression. E) NHiPSCs can be switched in an alternative state similar to iPCCs. F) Single nucleotide polymorphism analysis confirmed the authentication of HT-144-iPCCs. W indicates unclear data position.

IX.2. Reactivation of endogenous pluripotency markers in human melanoma-derived iPCCs

One hallmark of fully reprogrammed iPSCs is the reactivation of endogenous pluripotency markers. In particular, Nanog, Oct4, and Sox2 are key mediators of pluripotency (Boyer et al. 2005), regulating the expression of target genes to facilitate self-renewal and to promote an undifferentiated cell state. As previously shown, pluripotency factors form a hierarchical organized network centered on Sox2, which orchestrates downstream targets (Buganim, Faddah et al. 2012) during the reprogramming process. Therefore, endogenous expression of key pluripotency markers on mRNA and protein level was investigated in the presence of doxycycline. Nanog and Sox2 were up-regulated in all six reprogrammed tumor cell lines to levels similar to fibroblast-derived human iPSCs (figure 4A). The emerging colony-forming cells are from here on called induced pluripotent cancer cells (iPCCs). In comparison to all pluripotency markers investigated, Oct4 showed least changes. Although it was increased significantly in nearly all iPCCs lines from a technical point of view, it remained questionable whether the levels reached were biologically relevant. While Oct4 expression in iPCCs derived from Ma-1, SKMEL147 and HT-144 reached around 1% compared to NHiPSCs (previously demonstrated to be pluripotent, http://www.ub.uni-heidelberg.de/archiv/17870), other iPCCs were even below 1%. Exclusively, HT-144-iPCCs expressed Oct4 at nearly 10% of the level observed in NHiPSCs. According to the Bayesian transcription factor model (figure 1), Sall4 in part regulates Oct4. Here, increased Sall4 mRNA expression in all tested iPCC lines was observed compared to the parental tumor cell lines. In HT-144-, Ma-1-, SKMEL147- and HeLa-derived iPCCs the expression of Sall4 reached levels similar to those of NHiPSCs. WM266-4- and Mewo-iPCCs also increased expression of Sall4 but only to about 1 to 15% compared to the expression level in NHiPSCs. Due to low Oct4 expression levels observed in iPCCs, expression levels of factors potentially substituting Oct4 were analyzed. Many previous studies shed light on the reprogramming process, identifying substitutes for the original reprogramming factors Oct4, Sox2, Klf4 and c-Myc. To date, each factor can be replaced by one or more

factors. Nevertheless, the only transcription factors that can replace Oct4 are Tet1 and Nr5a2 (Gao et al. 2013; Heng et al. 2010). Therefore, Tet1 mRNA transcription levels were investigated and found to be increased in iPCCs (figure 4B). HT-144 cells initially expressed high levels of Tet1, so that the increase upon reprogramming was not significant. In contrast, Ma-1-iPCCs showed a high up-regulation of Tet1 expression levels compared to its parental cell line and to NHiPSCs. In order to confirm expression of pluripotency markers on protein levels, immunofluorescence staining was performed. As Oct4, Sox2, and Klf4 were expressed by the STEMCCA cassette, protein expression of Nanog and the surface marker Tra-1-60 were investigated. While original melanoma cell lines and also the cervical cancer line HeLa were negative, all tumor-derived iPCCs showed nuclear expression of the transcription factor Nanog, homogenously distributed in the colonies (figure 5A), and were positive for the glycoprotein Tra-1-60 covering the colony surfaces (figure 5B). Taken together, these data indicate that continued expression of reprogramming factors successfully induced pluripotency in cancer cells with reactivation of endogenous regulators.

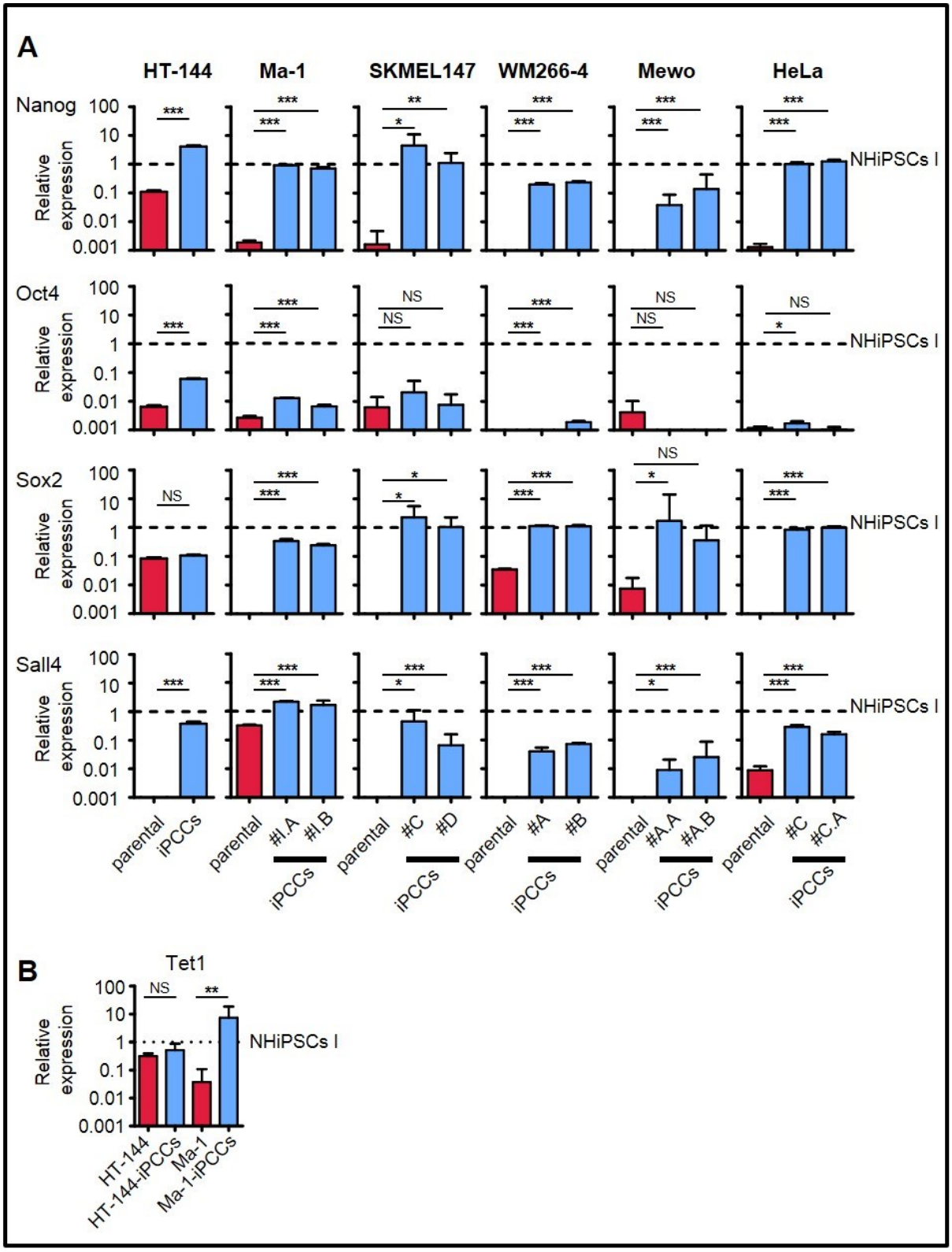

A
HT-144
Ma-1
SKMEL147
WM266-4
Mewo
HeLa
Nanog
Oct4
Sox2
Sall4
Relative expression
100
10
1
0.1
0.01
0.001
NHiPSCs I
***
**
*
NS
parental
iPCCs
#I.A
#I.B
#C
#D
#A
#B
#A.A
#A.B
#C.A
B
Tet1
HT-144
HT-144-iPCCs
Ma-1
Ma-1-iPCCs

Figure 4 | Reactivation of the endogenous loci of pluripotency genes in iPCCs derived from melanoma and cervical cancer cell lines. Total RNA from parental cancer cells, reprogrammed iPCCs and human fibroblast-derived iPSC (NHiPSCs) was isolated, reverse transcribed and analyzed by qPCR for relative transcript levels of the endogenous pluripotency markers Nanog, Oct4, and Sox2. In addition, expression levels of total Sall4 were measured. B) Gene expression levels of Tet1 in HT-144 and Ma-1 tumor cells and their reprogrammed counterparts. Expression levels were normalized to GAPDH. Error bars indicate 95% confidence intervals. P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (**: p value ≤ 0.01; ***: p value ≤ 0.005), NS indicates not significant.

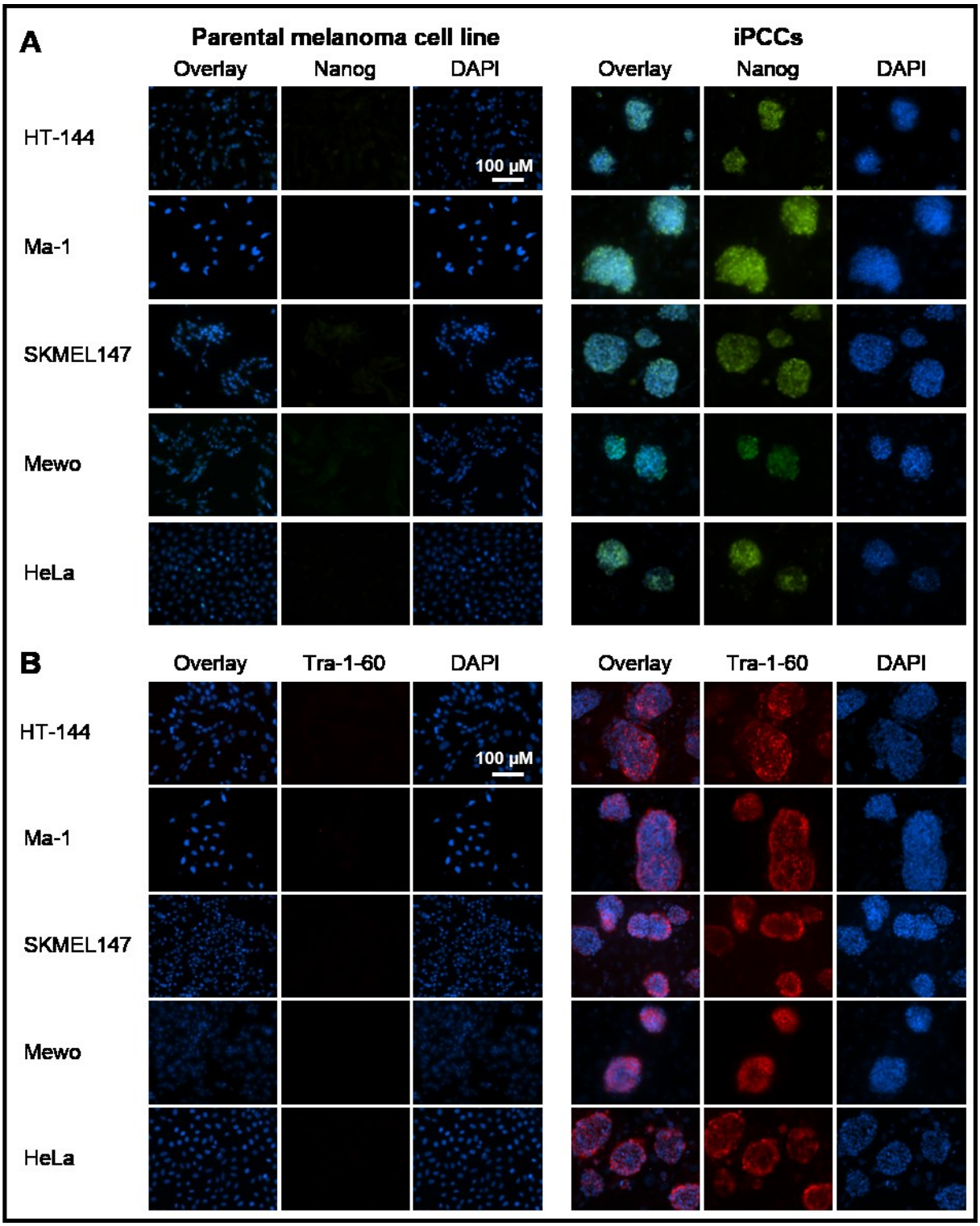


Figure 5 | Immunofluorescence staining of Nanog and Tra-1-60 in iPCCs and original tumor cells. The reprogrammed cancer cells form tightly packed colonies on feeder cells (right panel) and strongly expressed Nanog (A) and Tra-1-60 (B) in contrast to the negative parental cell lines (left panel). Mitotic inactivated murine embryonic fibroblasts served as feeder cells. DAPI was used for nuclear counterstaining.

IX.3. iPCCs demonstrate signs of early embryogenesis and epigenetic remodelling

To determine the transcriptional similarities of iPCCs with iPSCs compared to the parental melanoma cell lines, mRNAs from each sample were analyzed using an Illumina gene expression microarray. Using a p value ≤ 0.05, calculated by empirical Bayes moderated t-test, statistically differentially expressed genes were identified between the parental cell lines (HT-144 and Ma-1), their iPCCs, and iPSCs derived from somatic cells either cultivated on Matrigel (NHiPSCs) or cultivated under the same conditions used for iPCCs (NHiPSCs AS). Hierarchical clustering of the probes demonstrated a higher degree of similarities between iPCCs and iPSCs than between iPCCs and their parental cells (figure 6A). However, NHiPSCs cultivated under the same conditions as the reprogrammed tumor cells still share more molecular similarities with NHiPSCs cultivated under feeder-free conditions than with the iPCCs. These data demonstrate the resetting of the melanoma transcription program.

Next, differentially expressed genes between the parental melanoma cells and their reprogrammed counterparts were analyzed. 2561 commonly expressed genes were identified (figure 6B) and used for pathway analysis, applying Metacore (from GeneGo, https://portal.genego.com/). The differentially expressed genes were found to be enriched in pathways associated with development and cytoskeleton remodeling (figure 6C).

The process of reprogramming results in a stepwise resetting of epigenetic modifications, i.e. methylation marks. In pluripotent stem cells, especially the DNA (cytosine-5)-methyltransferase 3-like (DNTM3L) was shown to play an important role (Ooi et al. 2010). Recent findings demonstrated that DNMT3L is highly expressed in ESCs to prevent hypomethylation at promoter regions of bivalent developmental genes, protecting cells from differentiation (Neri et al. 2013). Here, DNMT3L was found to be expressed in iPCCs at levels comparable to NHiPSCs. Next, the expression of the cell surface marker E-Cadherin (CDH1) was analyzed. Successful induction of a pluripotent state requires a completed mesenchymal-to-epithelial (MET) transition. Therefore, CDH1 represents one of few targets on the cell surface, which allows to monitor single cells during early events in their transition to pluripotent stem cells (Li, R. et al. 2010). HT-144, Mewo and Ma-1 were derived from melanoma metastasis and together with SKMEL147, the cell lines display a metastatic phenotype *in vitro* and *in vivo* (Alla et al. 2010; Gouon et al. 1996; Janji et al. 1999; Pencheva et al. 2014; Wolter et al. 2007). In accordance with their metastatic potential, the majority of the parental cell lines did not express CDH1 at significant levels. Previous studies observed that *in vitro* CDH1 is expressed in melanocytic cells, down-regulated in non-invasive melanoma cells and lost in malignant melanoma cell lines (Danen et al. 1996). Exclusively Mewo cells expressed detectable CDH1 mRNA levels (figure 6A). Stepwise dedifferentiation initiated by the

expression of reprogramming factors induced regulators of early developmental processes. Nodal and its inhibitors Lefty1 and Lefty2 belong to the TGF-β superfamily regulating the axial left-right determination during early embryogenesis. Furthermore, TGF-β signaling directly regulates Nanog in human ESCs, thereby maintaining pluripotency (Vallier et al. 2009; Xu et al. 2008). Here, it was show that iPCCs-derived from HT-144 and also from the primary human melanoma line Ma-1 expressed Lefty1, Lefty2, and Nodal at mRNA levels similar to NHiPSCs (figure 6B). Also DPPA4, a marker exclusively found in pluripotent stem cells and restricted to cells of the pre-implantation embryo (Maldonado-Saldivia et al. 2007), was significantly enhanced upon reprogramming. In reprogrammed HT-144 and Ma-1, DPAA4 expression reached levels around 90% and 110% compared to NHiPSCs (figure 6C). In the reprogramming progress of somatic cells DPPA4 was shown to be involved in the stabilization of the pluripotent stem cell state as one of the molecules outside the Bayesian network, mediating the transition from the maturation to the stabilization phase (Golipour et al. 2012). Thus, this result led to the question whether iPCCs acquired a stable pluripotent stem cell state independent from transgene expression.

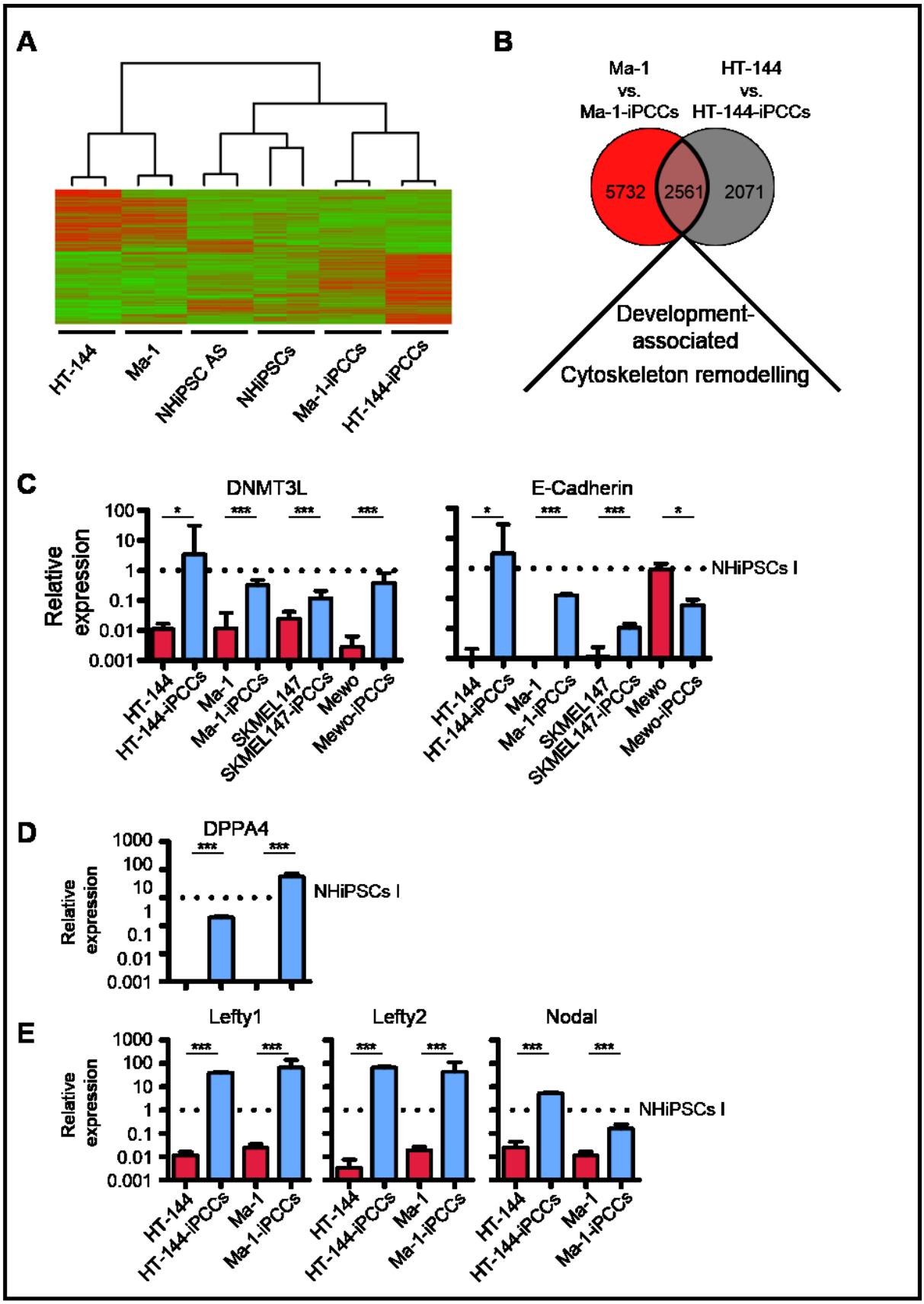

A
HT-144
Ma-1
NHiPSC AS
NHiPSCs
Ma-1-iPCCs
HT-144-iPCCs
B
Ma-1
vs.
Ma-1-iPCCs
HT-144
vs.
HT-144-iPCCs
5732
2561
2071
Development-
associated
Cytoskeleton remodelling
C
DNMT3L
E-Cadherin
Relative
expression
100
10
1
0.1
0.01
0.001
*
***
NHiPSCs I
HT-144
HT-144-iPCCs
Ma-1
Ma-1-iPCCs
SKMEL147
SKMEL147-iPCCs
Mewo
Mewo-iPCCs
D
DPPA4
1000
100
10
1
0.1
0.01
0.001
NHiPSCs I
E
Lefty1
Lefty2
Nodal
NHiPSCs I

Figure 6 | iPCCs share characteristics of normal pluripotent stem cells. A) Heat map and dendrogram generated by unsupervised hierarchical clustering of differentially regulated genes (empirical Bayes moderated t-test $p \leq 0.05$) in tumor cell lines, their reprogrammed daughter cells and iPSCs derived from somatic cells, cultivated either on Matrigel (NHiPSCs) or converted in the alternative state (NHiPSCs AS). Samples were analyzed and displayed as duplicates. B) Venn diagram showing differentially expressed gene probes (empirical Bayes moderated t-test $p \leq 0.05$) between iPCCs and their parental cell lines. 2561 overlapping genes were analyzed for enrichment of signaling pathways using MetaCore pathway analysis. C-E) qPCR analysis of expression levels of the epigenetic modifier DNMT3L and the MET marker CDH1 (C), the stabilization marker DPPA4 (D) and members of the TGF-β superfamily (Nodal, Lefty1 and Lefty2) (E). GAPDH expression was used for normalization to endogenous genes. Error bars indicate 95% confidence intervals. P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (**: p value ≤ 0.01; ***: p value ≤ 0.005). Error bars indicate 95% confidence intervals NS indicates not significant.

IX.4. Melanoma cell reprogramming leads to dedifferentiation with loss of terminal differentiation markers

Nuclear reprogramming is a stepwise process of dedifferentiation, whereby unipotent cells acquire the potential to differentiate in multiple cell types. One sign of this epigenetic remodeling process is the loss of terminal differentiation markers, indicating specialized functions. Here, the melanoma markers MITF, TYR, TRP1, DCT, MC1R and Sox10 were compared between parental melanoma cells and the reprogrammed iPCCs by microarray data (figure 7A). Expression levels were normalized to NHM and displayed as log2 transformed fold change. It was demonstrated that HT-144 cells and Ma-1 differ in their expression levels of melanoma markers. In contrast to Ma-1 cells, HT-144 showed high expression levels of all six markers in the parental cell line, whereas these were significantly down-regulated in their respective iPCCs. Furthermore, a pathway analysis using differentially expressed genes between the parental HT-144, Ma-1 cells and their reprogrammed iPCCs was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) in order to identify signaling pathways that were down-regulated upon reprogramming. Most prominently, key mediators of melanogenesis like MITF and MAPK signaling members were found to be significantly decreased (figure 7B). Hence, expression levels of typical melanoma markers were investigated by qPCR in a panel of parental melanoma cell lines and compared to their respective iPCCs in order to confirm the previous data (figure 7C). MITF mRNA expression was detectable in the four human melanoma cell lines HT-144, Ma-1, SKMEL147 and Mewo whereas expression levels were below the detection limit in all iPCCs tested. In addition, TRP1 was decreased to 1% or less in melanoma-derived iPCCs referred to the initial mRNA levels. Only Ma-1 cells, which were derived from a primary melanoma metastasis, showed similar TRP1 expression in iPCCs and in the parental cell line. However, these data only reflected expression levels compared to the parental cell line. In comparison to NHM and HT-144 cells, Ma-1 showed low expression levels of TRP1 and TYR (figure 7A).

Melanoma cells represent transformed melanocytes and share their neural crest origin. In order to exclude, that iPCCs reprogrammed only to a neural crest-like state, mRNA expression levels of neural crest markers including Sox10 and AP2 were compared to the parental cell lines by qPCR. In accordance with the microarray expression results, Sox10 was hardly detectable after reprogramming and AP2 showed more than 100-fold down-regulation in all iPCCs except Ma-1-iPCCs (figure 7D). Here, AP2 was about 50-fold decreased. Taken together, neural crest markers and markers of melanogenesis were strikingly down-regulated upon reprogramming. Collectively, these data show that nuclear reprogramming of human melanoma cells resulted in global gene expression changes, similar to healthy iPSCs accompanied by loss of melanoma markers.

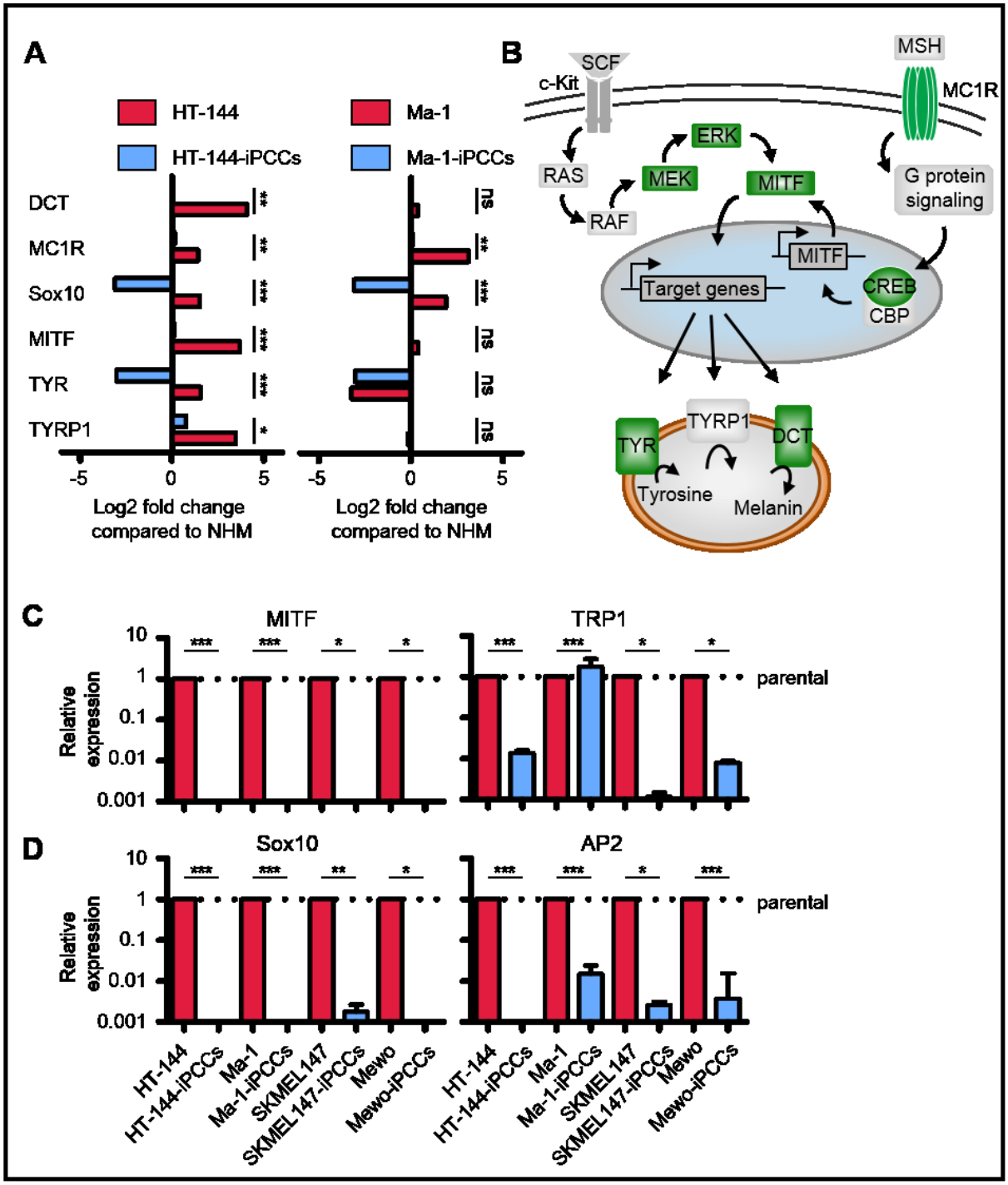

A
HT-144
HT-144-iPCCs
Ma-1
Ma-1-iPCCs
DCT
MC1R
Sox10
MITF
TYR
TYRP1
**
**
***
***
***
*
ns
**
***
ns
ns
ns
-5
0
5
Log2 fold change compared to NHM
Log2 fold change compared to NHM
B
SCF
c-Kit
MSH
MC1R
RAS
RAF
MEK
ERK
MITF
G protein signaling
MITF
Target genes
CREB
CBP
TYRP1
TYR
DCT
Tyrosine
Melanin
C
MITF
TRP1
Relative expression
10
1
0.1
0.01
0.001
parental
D
Sox10
AP2
HT-144
HT-144-iPCCs
Ma-1
Ma-1-iPCCs
SKMEL147
SKMEL147-iPCCs
Mewo
Mewo-iPCCs

Figure 7 | Loss of melanocytic markers in melanoma-derived iPCCs and down-regulation of enzymes involved in melanogenesis. A) Gene expression levels of the melanocytic markers dopachrome tautomerase (DCT), melanocortin 1 receptor (MC1R), Sox10, microphthalmia-associated transcription factor (MITF), tyrosinase (TYR) and tyrosinase-related protein 1 (TRP1) in the parental melanoma cells HT-144 and Ma-1, and their reprogrammed counterparts were determined by gene expression microarray. Fold changes are log2 transformed and calculated compared to NHM. Samples were analyzed in duplicates. B) Global gene expression profiling was used to identify deregulated genes between parental melanoma cells and their iPCCs by two group tests (empirical Bayes moderated t-test $p \leq 0.05$). Down-regulated genes were analyzed using the software tool DAVID, showing enrichment of key molecules involved in melanogenesis (figures in green symbolizing down-regulated genes). Samples were analyzed in triplicates. C) Reprogramming of melanoma cells resulted in loss of melanocytic and D) neural crest markers in qPCR experiments. Parental cell lines served as reference for melanocytic markers. GAPDH expression was used as internal control. Error bars indicate 95% confidence intervals. P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (**: p value ≤ 0.01; ***: p value ≤ 0.005). NS indicates not significant.

IX.5. iPCCs acquire a metastable pluripotent state

In order to elucidate whether reprogramming of tumor cells results in a stable pluripotent state, expression levels of pluripotency markers after doxycycline withdrawal were measured. Importantly, mRNA levels of Nanog were down-regulated after 36 h without transgene expression (figure 8A). After 80 h Nanog expression was 90% decreased compared to initial levels. Usually, human ESCs and iPSCs require bFGF to maintain their pluripotency. Therefore, it was analyzed whether iPCCs can be stabilized by bFGF. Even in the presence of bFGF iPCCs tended to differentiate and could not be cultivated without doxycycline. After seven days iPCCs showed morphological signs of differentiation and no AP positive colonies were detectable (figure 8B). These result indicated that reprogrammed melanoma cells depended on continuous transgene expression to prevent differentiation.

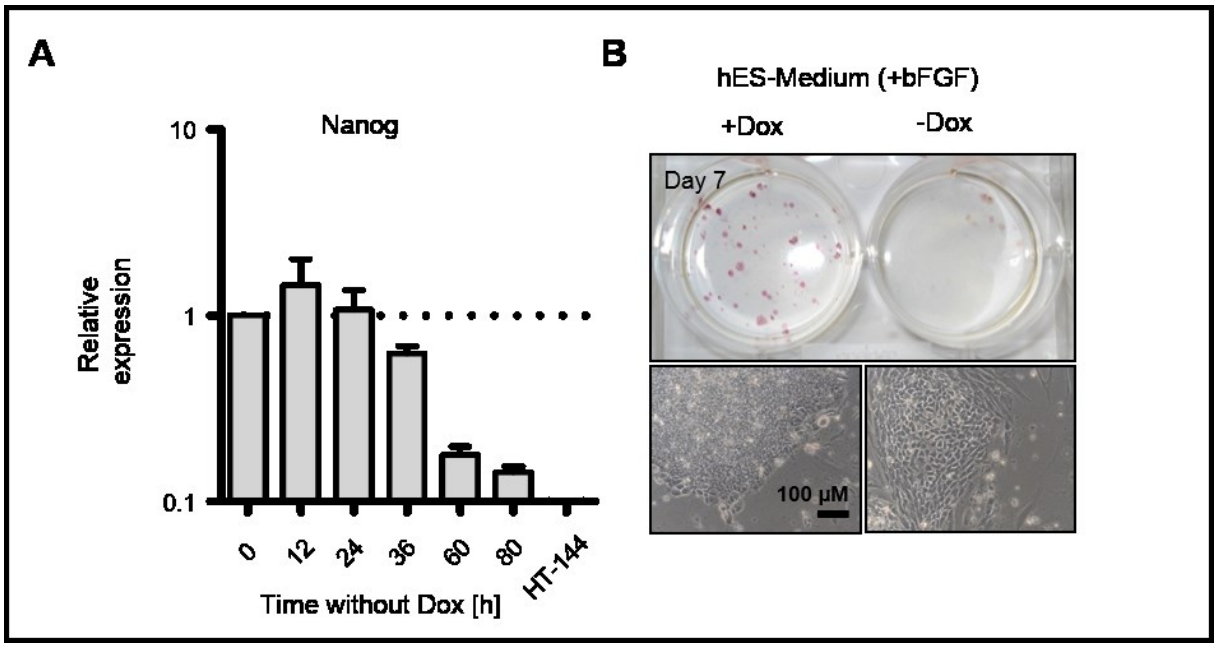


Figure 8 | Reprogrammed tumor cells acquire a metastable pluripotent state. A) Nanog expression levels in HT-144-iPCCs measured by qPCR after doxycycline withdrawal normalized to initial Nanog levels and compared to the parental cell line. Nanog expression was normalized to internal GAPDH. Error bars indicate 95% confidence intervals. B) AP staining of iPCCs cultured for 7 days in medium supplemented with bFGF alone (-Dox) or together with doxycycline (+Dox).

IX.6. HT-144-iPCCs form teratomas *in vivo*

The most stringent assay to test whether a cell is fully pluripotent is the complementation of tetraploid embryos. Fused murine ESCs of the two-cell stadium give rise to tetraploid cells of extra-embryonic tissue and develops to a blastocyst stage. Although tetraploid cells are able to implant in the uterus, they fail to develop into a normal embryo. Under these conditions diploid pluripotent stem cells can normally develop into viable animals. Therefore, this assay allows to determine the developmental potential of the injected cells. Using this technique, publications demonstrated the possibility to generate mice-derived entirely from injected iPSCs (Boland et al. 2009; Kang et al. 2009; Zhao et al. 2009). Ethical reasons imply the need for alternatives when working with human cells. Therefore, the most demanding test for human iPSCs is their capability to differentiate into tissues of the three germ layers, endoderm, ectoderm and mesoderm *in vivo*. Therefore, HT-144-iPCCs were injected subcutaneously in NOD/SCID mice. In all cases tumors developed after 10-12 weeks and contained tissues of all three germ layers (figure 9). Tumor slices stained with haematoxylin and eosin (H&E) demonstrated a high portion of endodermal structures. Taken together, consistent overexpression of Oct4, Sox2, and Klf4 in tumor cells is sufficient to induce the process of reprogramming, resulting in a metastable state with pluripotent characteristics.

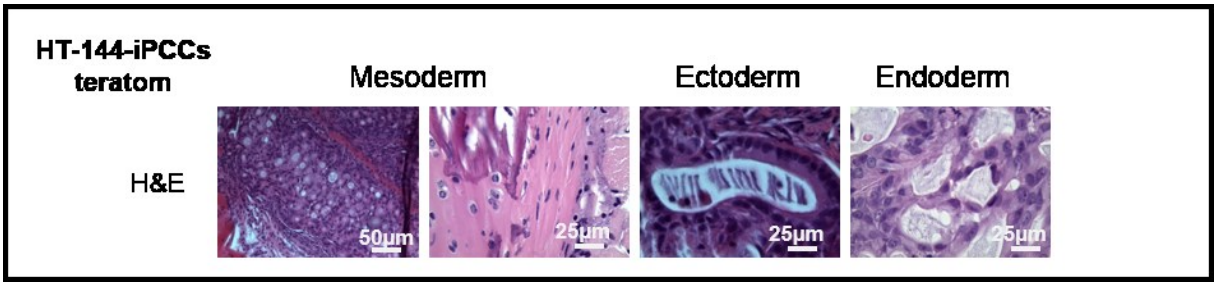
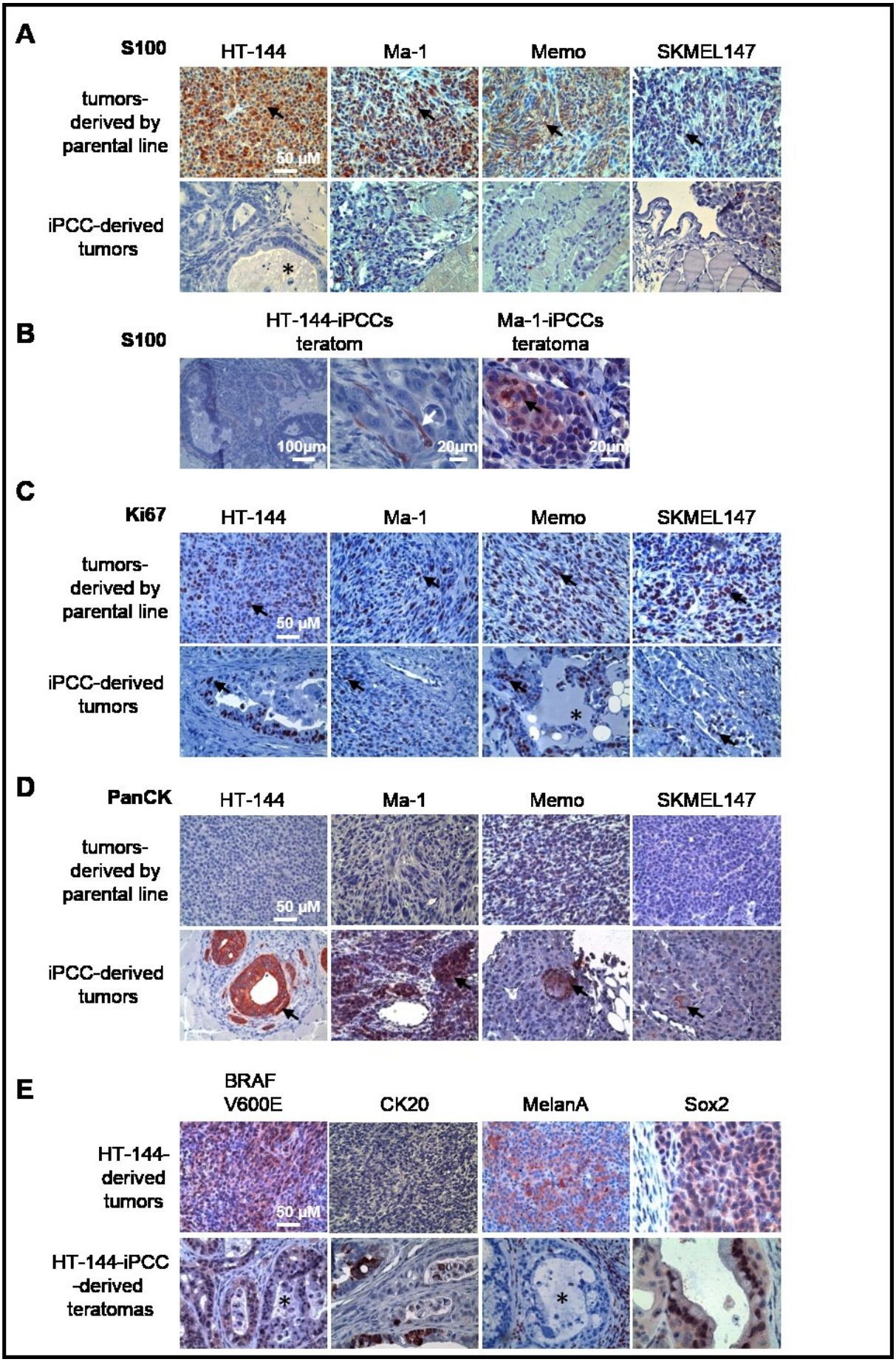


Figure 9 | iPCCs derived from the human melanoma cells HT-144 generated teratomas in vivo. Subcutaneous injection of HT-144-iPCCs generated tumors containing a wide variety of differentiated structures. Paraffine-embedded tumor slices were stained with H&E.

IX.7. iPCCs preferentially differentiate into non-melanocytic lineages

Previous publications showed that tumors derived from iPCCs contained structures from the original tumor (Kim, J. et al. 2013). Consequently, tumors derived from iPCCs and their parental melanoma cells were compared after subcutaneous injection into immunocompromised mice. In total, 26 tumors of four melanoma and iPCC lines were analyzed. Original tumor lines generated melanomas with strong S100 staining, a typical melanoma marker used for diagnostic validations in the clinic (Springall et al. 1983). Furthermore, melanomas were homogenous tumors and exhibited a high nuclear-to-cytoplasmic ratio. In contrast, iPCC-derived teratomas showed multiple areas of differentiated foci that were architecturally organized and contained irregularly shaped cells with enlarged cytoplasm. Formation of gland-like structures were observed in most of the tumors developed from iPCCs but especially HT-144-iPCCs-derived teratomas contained several structures with abundant mucin (* in figure 10A,C,E). Independent of the mutational status, iPCC-derived tumors generated rarely melanoma-like structures (figure 10A). S100 expressing melanoma-like cells were hardly detectable in teratomas derived from Ma-1-, SKMEL147- and Mewo-iPCCs and only few S100-positive cells were derived from HT-144-iPCCs (black arrows in figure 10B). Furthermore, also few S100 positive, neuronal-like cells were found (white arrows in figure 10B) but rarely accumulated HT-144-iPCC-derived S100 expressing cells in nests. Tumors derived from melanoma cell lines expressed the proliferation marker Ki67 homogenously (figure 10C). In contrast, iPCC-derived tumors showed heterogeneous expression of Ki67. Only some cells of distinct structures proliferated actively. A similar heterogeneous pattern was also observed for other markers in tumors derived from reprogrammed cancer cells. In order to identify cells of epithelial origin, a mixture of antibodies recognizing a broad spectrum of cytokeratin proteins was applied. As expected, melanoma cells were pan-cytokeratin negative while iPCC-derived tumors contained epithelial tissues (figure 10D). As HT-144-iPCCs formed the most dedifferentiated tumors characterized by

many varying structures, these tumors were subjected for further analysis. The majority of cells in tumors derived from the HT-144 cell line expressed the BRAF mutation V600E, the melanoma marker MelanA and the transcription factor Sox2 but all were negative for cytokeratin 20. In contrast, HT-144-iPCC-derived tumors showed endodermal-like structures positively stained for $BRAF^{V600E}$ and Sox2, while cells forming connective tissue in between remained negative for these markers (figure 10E). Accordingly, cytokeratin 20 was, similarly to pan-cytokeratins, restricted to distinct tumor sites, while MelanA-positive cells were rather distributed sparsely throughout the tumor (figure 10E). In order to investigate the impact of reprogramming on mitogen-activated protein kinase (MAPK) signaling, samples were additionally stained for ERK and its phosphorylated active form (figure 10F). All melanomas showed high expression levels of ERK but differed significantly regarding the amount of phosphorylated ERK (pERK). HT-144 and SKMEL147-derived tumors contained few pERK-positive cells while Ma-1-derived tumors showed intermediated levels. Exclusively in Mewo-derived tumors the majority of melanoma cells was positive for pERK. In iPCC-derived tumors ERK expression was limited to specific tumor regions. Accordingly, pERK was only detectable in some of the ERK positive areas. Notably, although many cells of Mewo-iPCC-derived tumors expressed ERK, only few cells were positive for its phosphorylated form. In summary, these data suggest that melanoma-derived iPCCs generated teratoma-like tumors containing various differentiated structures that were rarely of melanocytic or melanoma origin. While melanoma markers were homogenously expressed in melanoma cell line-derived tumors, iPCC-derived teratomas demonstrated high heterogeneity regarding their morphological appearance and marker expression.


A
S100
HT-144
Ma-1
Memo
SKMEL147
tumors-derived by parental line
iPCC-derived tumors
50 µM
B
S100
HT-144-iPCCs teratom
Ma-1-iPCCs teratoma
100µm
20µm
20µm
C
Ki67
HT-144
Ma-1
Memo
SKMEL147
tumors-derived by parental line
iPCC-derived tumors
50 µM
D
PanCK
HT-144
Ma-1
Memo
SKMEL147
tumors-derived by parental line
iPCC-derived tumors
50 µM
E
BRAF V600E
CK20
MelanA
Sox2
HT-144-derived tumors
HT-144-iPCC-derived teratomas
50 µM

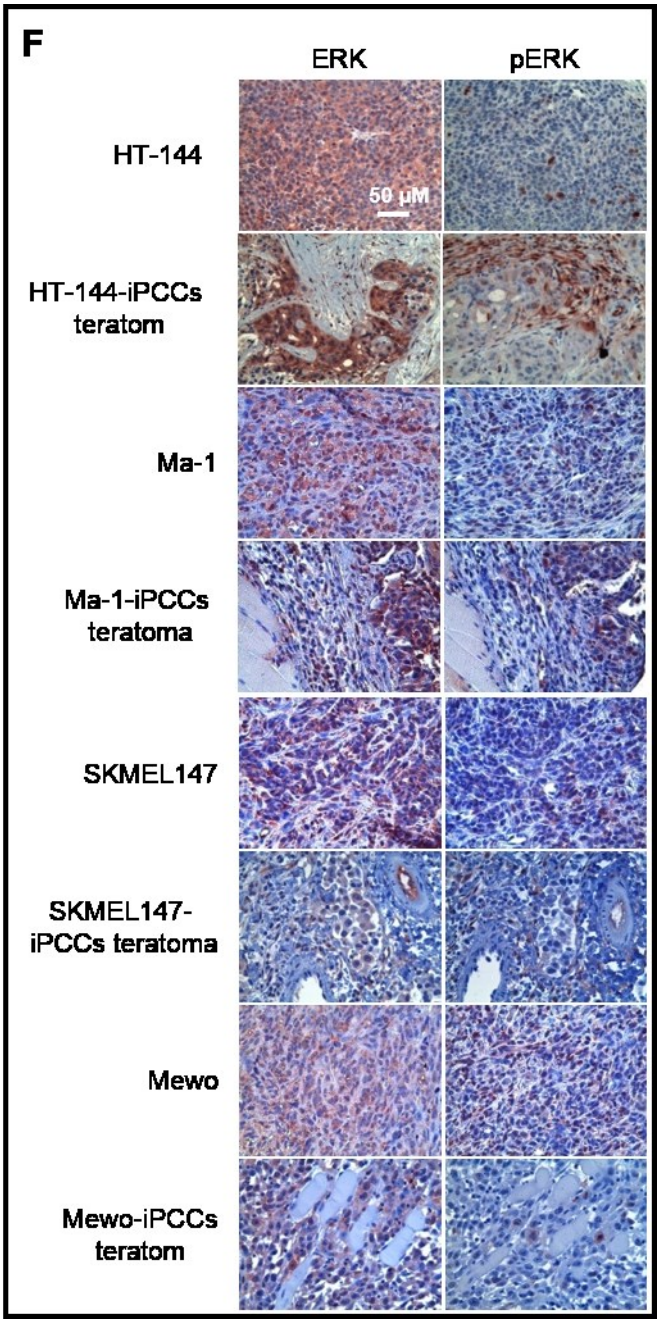


Figure 10 | Teratomas from reprogrammed tumor cells generally do not share characteristics of melanomas. Parental tumor cells and their corresponding iPCCs were subcutaneously injected. Developing tumors were pfa fixed and stained for the melanoma marker S100 (A and B), the proliferation marker Ki67 (B), epithelial cytokeratins (D) and the MAPK signaling molecule ERK and its active form (F). Tumor derived from HT-144 and HT-144-iPCCs were additionally investigated for the expression of the melanoma proteins BRAF V600E and MelanA as well as CK20 and Sox2 via immunostaining (E). Arrows show respective stainings. * demonstrate areas of extensive extracellular mucin.

IX.8. iPCCs achieve terminal differentiation into neuronal cells

Transcription factor-based reprogramming into pluripotent stem cells with subsequent differentiation allows the generation of patient-specific cells. Recent studies reported great success in the generation of terminally differentiated neuronal cell types. Our *in vivo* differentiations of iPCCs showed no characteristics of neuronal differentiation, although spontaneous appearance of neurons in cell culture was occasionally observed. Therefore, neuronal differentiation of iPCCs and iPSCs in the alternative state was investigated *in vitro*. Therefore, a protocol was applied using small molecules inhibiting SMAD and GSK3ß signaling (figure 11A) in combination with low dose of Noggin and the BMP inhibitor LDN-193189 allowing the differentiation of normal human melanocyte-derived hiPSCs (NHiPSCs AS II&III)

(figure 11B) but also tumor cell-derived iPCCs into the neuronal lineage (figure 11C). Within three days after induction of neuronal differentiation first axonal-like structures appeared and elongated with continuing differentiation. In accordance with the differentiation, pluripotency markers Nanog and Oct4 were down-regulated (figure 11D). Nevertheless, expression levels of both transcription factors were clearly higher than in the parental cell line suggesting that the differentiation was not yet completed, although early neuronal markers like microtubule-associated protein 2 (MAP2) and Pax6 increased. In accordance with the observation that differentiated cells stopped proliferation, bundled together (figure 11C) and detached after 20-50 days, the late post-mitotic neuronal marker RBFox3 was found to be significantly up-regulated in HT-144-derived neuronal-like cells (HT-144-dNLCs). In comparison to the iPCCs, HT-144-dNlCs up-regulated neuronal markers more than 1000-fold (figure 11E). As expected, original melanoma cells were negative for all neuronal markers. Immunofluorescence staining of Tuj1 in neuronal differentiated cells derived from HT-144-iPCCs constitutively expressing GFP (figure 11F), confirmed the successful neuronal differentiation. Taken together, these results demonstrate that iPCCs could be differentiated similarly to iPSCs into the neurogenic lineage.

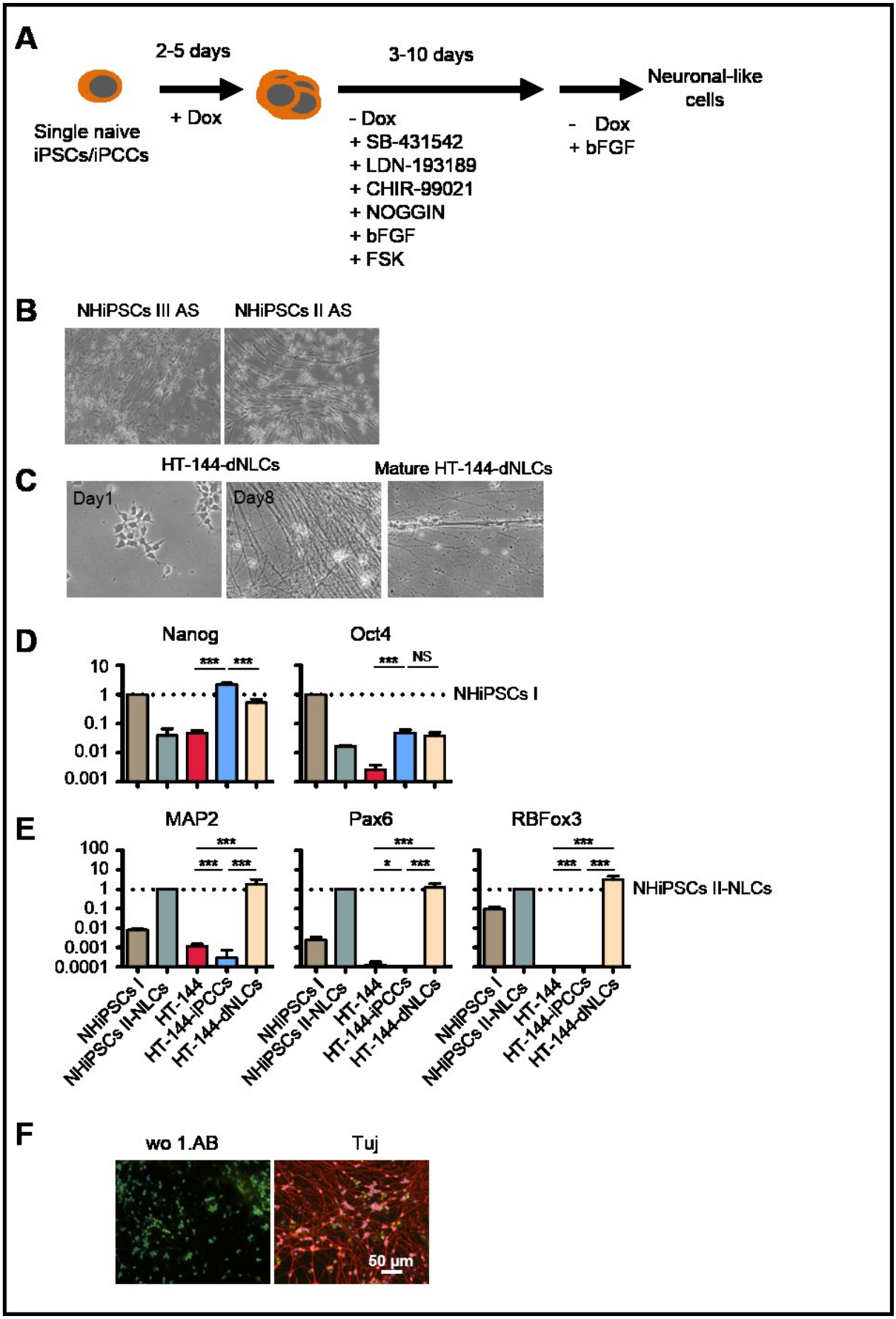

A
2-5 days
+ Dox
Single naive iPSCs/iPCCs
3-10 days
- Dox
+ SB-431542
+ LDN-193189
+ CHIR-99021
+ NOGGIN
+ bFGF
+ FSK
- Dox
+ bFGF
Neuronal-like cells
B
NHiPSCs III AS
NHiPSCs II AS
C
HT-144-dNLCs
Mature HT-144-dNLCs
Day1
Day8
D
Nanog
Oct4
NHiPSCs I
E
MAP2
Pax6
RBFox3
NHiPSCs II-NLCs
NHiPSCs I
NHiPSCs II-NLCs
HT-144
HT-144-iPCCs
HT-144-dNLCs
F
wo 1.AB
Tuj
50 µm

Figure 11 | iPSCs and iPCCs efficiently differentiate into neural cells in the presence of SMAD and GSK3ß inhibitors. A) Scheme for the neural differentiation of iPSCs and iPCCs. Morphological changes including formations of axonal-like structures (white arrows) after the induction of differentiation into the neuronal lineage of NHiPSCs (B) and melanoma-derived iPCCs (C). D-E) Expression levels of pluripotency markers and early and late neuronal markers analyzed by qPCR. Nanog and Oct4 were normalized to NHiPSCs. The neuronal markers MAP2, Pax6 and RBFox3 were normalized to NHiPSCs derived from melanocytes (NHiPSCs II) and differentiated into the neuronal lineage (NHiPSCs II-NLCs). GAPDH served as internal control. Error bars indicate 95% confidence intervals. P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (**: p value ≤ 0.01; ***: p value ≤ 0.005). (F) GFP expressing HT-144-iPCCs were differentiated according the scheme and stained for the neuronal protein Tuj1.

IX.9. HT-144-iPCC-derived fibroblast-like cells *in vitro*

In order to investigate the impact of epigenetic modifications on the melanoma phenotype more detailed, HT-144-iPCCs were randomly differentiated by withdrawal of doxycycline in the presence of FCS, and selected for continuously proliferating, immortal cells. Emerging cells showed a fibroblast-like morphology and could be cultivated in non-stem cell promoting conditions (DMEM medium with FCS) usually used for fibroblast culture. Therefore, these cells were named HT-144-derived-fibroblast-like cells (HT-144-dFLCs) differentiation A. As the differentiation was inefficient, a more defined fibroblast-differentiation protocol was applied. Therefore, doxycycline was withdrawn and iPCC colonies were cultivated in the presence of EGF, insulin, and at later stages with additional BMP-4 (figure 12A). BMP-4 is essential to direct differentiation into mesodermal lineages. After two weeks, appearance of spindle like cells was observed similar to normal human fibroblasts (NHF) (figure 12B). These cells were picked, transferred to new wells and expanded (HT-144-dFLCs B and C). Although these cells proliferated slower than the parental melanoma cell line and the iPCCs, they could be cultured for more than 40 passages. Gene expression profiling was performed and differentially regulated genes between HT-144, HT-144-dFlC A and B and NHF analyzed. Therefore, a several group test using the empirical Bayes moderated t-test method was calculated and the dataset was filtered for genes with a p value ≤ 0.05. Unsupervised hierarchical clustering of these genes was used to construct a dendrogram and a heat map (figure 12C). HT-144-dFlC A and HT-144-dFlC B formed one cluster, indicating that the differentiation was reproducible. Furthermore, the cells shared more transcriptionally similarities with NHF than with the parental cell line.

In order to prove that fibroblast-like cells possess the same genomes like their parental melanoma cells, a 24-multi-colour-fish analysis was performed. Differentiated cells showed the same mutations present in HT-144 cells, indicating their common origin (figure 12D). Furthermore, qPCR analysis revealed loss of Nanog and absence of MITF expression (figure

12E), indicating that the cells differentiated into a non-melanocytic lineage. In addition, fibroblast-like cells up-regulated cell structure proteins like FSP1 and Vimentin compared to iPCCs (figure 12F) and reached expression levels similar to HT-144 cells. Considering this regain of cell structure proteins, the question rose whether fibroblast-like cells also reacquire a migratory and invasive phenotype similar to that observed in melanoma cells. Surprisingly, differences between the distinct differentiations were noted. While HT-144-dFIC from differentiation B showed an increased in the migratory potential compared to parental melanoma cells, cells from differentiation A migrated similarly to HT-144 (figure 12G).
Nevertheless, all fibroblast-like cells showed migratory and invasive properties comparable to the parental melanoma cell line (figure 12G and H). Of note, all cells used for the invasion assay did not show high invasive capacities. Consequently, differences in invasiveness were not significant. In summary, the data point out that epigenetic modifications in melanoma cells, either induced by nuclear reprogramming or differentiation, can alter tumor cell characteristics like morphology, migration and invasion.

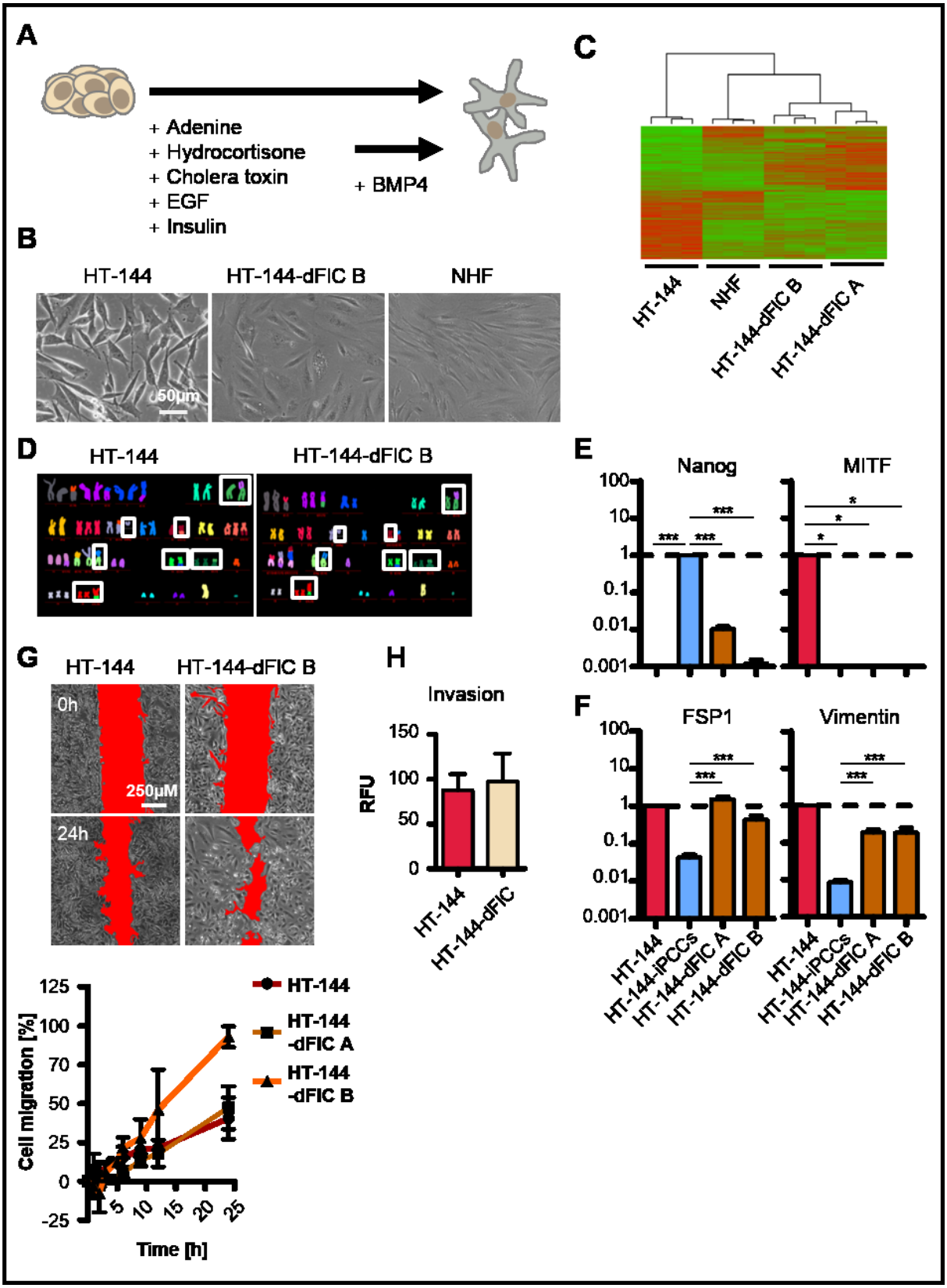

A
+ Adenine
+ Hydrocortisone
+ Cholera toxin
+ EGF
+ Insulin
+ BMP4
B
HT-144
HT-144-dFIC B
NHF
50µm
C
HT-144
NHF
HT-144-dFIC B
HT-144-dFIC A
D
HT-144
HT-144-dFIC B
E
Nanog
MITF
100
10
1
0.1
0.01
0.001
***
*
F
FSP1
Vimentin
HT-144
HT-144-iPCCs
HT-144-dFIC A
HT-144-dFIC B
G
HT-144
HT-144-dFIC B
0h
250µM
24h
Cell migration [%]
Time [h]
HT-144
HT-144 -dFIC A
HT-144 -dFIC B
H
Invasion
RFU
HT-144
HT-144-dFIC

Figure 12 | Epigenetic modifications induced by nuclear reprogramming and differentiation alter melanoma cell identity. A) Schematic protocol for the generation of fibroblast-like cells. Addition of BMP4 directs cells towards a mesodermal fate. B) Morphological comparisons of HT-144-derived fibroblast-like cells (HT-144-dFIC) and normal human fibroblasts. C) Heat map and dendrogram of differentially expressed genes (empirical Bayes moderated t-test p value ≤ 0.05) generated by unsupervised hierarchical clustering of HT-144 melanoma cells, their differentiated daughter cells and NHF. Samples were analyzed in triplicates. NHF were derived from three individual donors. D) 24-multi-colour FISH analysis detects same mutations in the parental melanoma cell line and the derived differentiated cells. E-F) qPCR analysis of the pluripotency marker Nanog, the melanoma marker MITF (E) and the cell matrix proteins FSPα and Vimentin (F) in parental HT-144 cells, their derived iPCCs and fibroblast-like differentiated cells. Nanog was normalized to iPCCs, while the melanoma marker and the cell structure genes were normalized to HT-144 cells. GAPDH was used as internal control. Error bars indicate 95% confidence intervals. P values were calculated by two-tailed, unpaired sample t-test. Asterisk indicates t-test p value of ≤ 0.05 in comparison to the respective reference (**: p value ≤ 0.01; ***: p value ≤ 0.005). G) Parental HT-144 and fibroblast-like cells were seeded in silicone culture-inserts. 24 h later, inserts were removed generating a defined gap. Pictures were taken at indicated time points. Migration potential was calculate as the percentage of gap closure in triplicates. H) G) Triplicates of three independent fibroblast-like differentiations and HT-144 cells were measured for their potential to invade a basement membrane-coated membrane. Relative fluorescent units (RFU) are displayed.

IX.10. Nuclear reprogramming-induced epigenetic modifications mediate therapy resistance in melanoma-derived iPCCs

Recently, it has been demonstrated that leukemia-derived iPCCs lose their BCR-ABL oncogene dependence, restricting targeted therapy to a specific epigenetic cell state (Carette et al. 2010). Considering the activating V600E mutation in HT-144, Ma-1 and SKMEL147 cells, the question was addressed whether epigenetic modifications in iPCCs and iPCC-derived fibroblast-like cells might affect their response to novel targeted melanoma therapy.

Parental melanoma cell lines, their reprogrammed iPCCs and additionally HT-144-dFICs were treated with 1 µM of the MEK inhibitor GSK1120212 (trametinib) and 1 µM of the oncogene-specific BRAF inhibitor PLX4032 (vemurafenib) and compared to vehicle (DMSO) treated-cells. After seven days, vehicle-treated melanoma cells and HT-144-dFICs were grown to confluency, while melanoma-derived iPCCs formed AP positive colonies. Treatment with GSK1120212 reduced cell proliferation in all melanoma cell lines and resulted in the appearance of floating, dead cells (figure 13A). In accordance to their mutational status (as shown in table 4), HT-144 and Ma-1 were sensitive to PLX4032 treatment but not the BRAF wildtype cell lines Mewo and SKMEL147. Compared to the parental cell lines, iPCCs and HT-144-dFICs showed an increased drug resistance. MEK inhibition and BRAF inhibition in iPCCs did not block growth of colonies or their maintenance of pluripotency, indicated by AP activity (figure 13A). In order to extend the analysis, the effect of MAPK pathway inhibition on the cellular metabolic activity was quantified. Therefore, cell metabolic activity was measured by

the colorimetric change of the redox sensitive growth indicator alamarBlue. Cells were treated with increasing concentrations of GSK1120212 and PLX4032, and compared to DMSO-treated control cells (figure 13B). In parental melanoma cells a time- and dose-dependent response was observed. While 1 nM of neither vemurafenib nor trametinib influenced the cell metabolism, 10 nm of both drugs were sufficient to reduce cell activity to approximately one third of the vehicle-treated control after 120 h treatment. Interestingly, administered drugs showed a very narrow therapeutic range in HT-144 cells. Concentrations of 100 nM and 1000 nM did not significantly improve therapy response, indicating that 10 nM triggered nearly the full therapeutic effect. In contrast, all fibroblast-like differentiations (A, B and C) were more resistant to MAPK signaling inhibition (figure 13A&C), although variations among the differentiations were observed. At 1000 nM GSK1120212 fibroblast-like cells from differentiation B responded to drug treatment albeit cells were more resistant than the parental cell line. Similarly, 100 nM PLX4032 resulted in reduced metabolic activity of about 25% after 120 h in differentiation C but hardly effected differentiation B compared to the vehicle treated control while the cellular activity of parental cells decreased to 30%. Fibroblast-like cells derived from differentiation C were the most resistance ones, and did not significantly reduce their metabolic activity independent of the concentration and drug type. Taken together, these data indicate that MAPK signaling is not required for survival in iPCCs and fibroblast-like cells. In contrast, the parental melanoma cell line are addicted to active BRAF signaling, not tolerating any reduction. Activation of MEK and BRAF results predominantly in the phosphorylation of ERK, which then translocates to the nucleus to phosphorylate its nuclear targets. Therefore, phosphorylation levels of ERK correspond to MAPK signaling activity. Performing a western blot against phosphorylated ERK (pERK) demonstrated a strong phosphorylation of the protein in the original melanoma cell line HT-144, but also a clear decrease in HT-144-derived iPCCs and in fibroblast-like cells (figure 13D). Even total ERK protein was lower expressed in fibroblast-like cells compared to the parental cell line. These data suggest that epigenetic modifications, induced by the reprogramming process and subsequent differentiation, led to the loss of BRAF oncogene addiction, resulting in therapy resistance against targeted melanoma therapy.

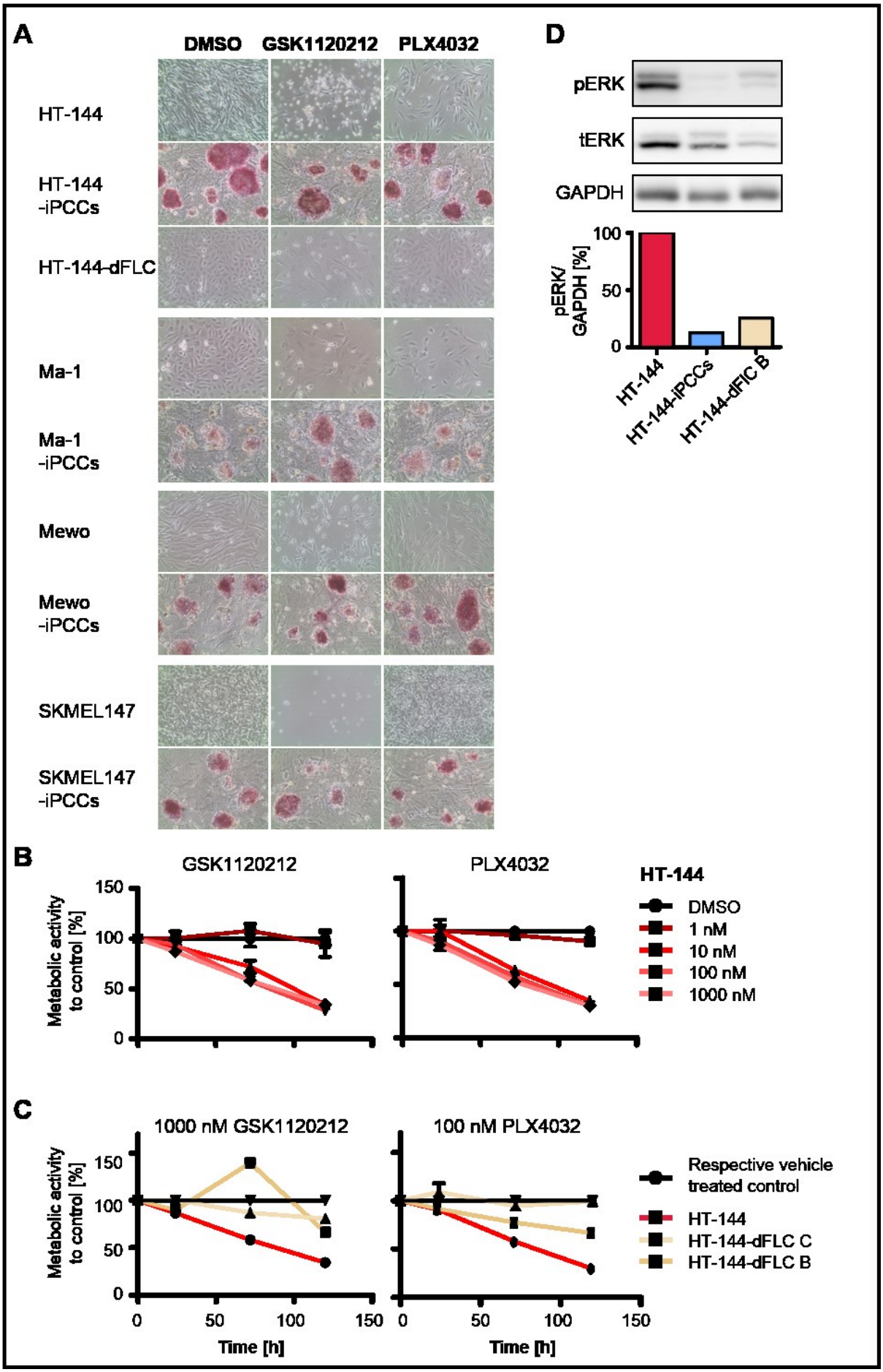

A
DMSO
GSK1120212
PLX4032
HT-144
HT-144 -iPCCs
HT-144-dFLC
Ma-1
Ma-1 -iPCCs
Mewo
Mewo -iPCCs
SKMEL147
SKMEL147 -iPCCs
D
pERK
tERK
GAPDH
pERK/ GAPDH [%]
100
50
0
HT-144
HT-144-iPCCs
HT-144-dFIC B
B
GSK1120212
PLX4032
Metabolic activity to control [%]
150
100
50
0
HT-144
DMSO
1 nM
10 nM
100 nM
1000 nM
C
1000 nM GSK1120212
100 nM PLX4032
Metabolic activity to control [%]
Time [h]
0
50
100
150
Respective vehicle treated control
HT-144
HT-144-dFLC C
HT-144-dFLC B

Figure 13 | Altered melanoma cell identity results in reduced drug response and independence of MAPK signaling. A) The parental melanoma cell lines HT-144, Ma-1, SKMEL147 and Mewo, their reprogrammed counterparts, and HT-144-dFLC B were teated with 1 µM GSK1120212 and 1 µM PLX4032. Seven days after drug application iPCCs colonies were stained for AP activity and pictures were taken. B) HT-144 cells were treated with increasing concentrations of the MEK inhibitor GSK1120212 and the BRAF inhibitor PLX4032 for the indicated time points. Cell metabolic activity was measured by alamarBlue and compared to vehicle treated controls. B) Fibroblast-like differentiated cells B and C were treated with 1000 nM GSK1120212 and 100 nM PLX4032 and compared to the parental cell line. C) The melanoma cells HT-144, their corresponding iPCCs and fibroblast-like cells B were investigated for expression and phosphorylation of ERK by western blot. GAPDH was used as loading control. DMSO served as vehicle control.

IX.11. Tumor suppressor- and oncogene deregulation in distinct differentiation states

In order to evaluate the molecular resemblance of parental melanoma cells, melanoma-derived iPCCs and HT-144-iPCC-derived differentiations, the gene expression signature of deregulated genes was compared using whole genome expression profiling. Unsupervised hierarchical clustering of differentially expressed genes was performed to construct a heat map (figure 14A). Based on the similarities of the expression profile a dendrogram was built. Here, the parental melanoma cells HT-144 and Ma-1 were sharply distinguished from their iPCCs. Ma-1-iPCCs and HT-144-iPCCs cluster together demonstrating that HT-144-dFIC and HT-144-dNIC are transcriptionally distinct from the iPCCs. Nevertheless, HT-144-iPCC-derived differentiations share more molecular similarities with iPCCs than with the parental melanoma cells. In addition, HT-144-dFICs clustered closer to iPCCs than to HT-144-iPCCs differentiated into the neuronal linage.

Previous data demonstrated that nuclear reprogramming leads to epigenetic silencing of oncogens like c-Myc (Zhang et al. 2013). Therefore, expression of tumor suppressor genes and oncogenes in parental melanoma cells, their iPCCs and differentiated cell types was investigated. Lists of oncogenes and tumor suppressor genes were compiled from the UniProt Knowledgebase (http://www.uniprot.org/uniprot/) searching for human genes associated with the keyword oncogene or tumor suppressor gene. 398 reviewed oncogenes and 168 reviewed tumor suppressor genes were identified, and their expression levels in HT-144-iPCCs, HT-144-dFICs A, HT-144-dFICs B and HT-144-dNICs investigated and compared to HT-144 cells. It was assumed that induction of distinct cell fates would lead to a deregulation of distinct sets of tumor suppressor genes or oncogenes. Therefore, tumor suppressor genes exhibiting a fold change ≥ 2 and a p value ≤ 0.01 in at least one sample were filtered. A set of 50 tumor suppressor genes remained (figure 14B). Surprisingly, an equal number of genes were up- and down-regulated in the samples. In accordance to our assumption, it was observed that

between HT-144-dNIC, HT-144-iPCCs, and HT-144-dFICs, tumor suppressor genes were differentially regulated. In order to identify reversibly regulated genes that might alter the tumorigenic phenotype, only up-regulated tumor suppressor genes were further investigated. Both fibroblast-like differentiations shared 16 of the top 20 up-regulated tumor suppressor genes (figure 14C). Therefore, the gene sets of HT-144-dFIC A and B were combined and ranked in accordance to their arithmetic mean. Next, the ten most up-regulated tumor suppressor genes of HT-144-iPCCs, HT-144-dFICs and HT-144-NICs were analyzed for commonly up-regulated genes, identifying bridging integrator 1 (BIN1). BIN1 was the only tumor suppressor gene found to be commonly up-regulated in the samples, compared to the parental melanoma cell line HT-144 (figure 14D). HT-144-iPCCs and HT-144-dFICs shared five reversibly regulated tumor suppressor genes while the neuronal-differentiated cells up-regulated nine exclusive tumor suppressors. Taken together, these data demonstrate that reprogramming alone and re-differentiation leads to an epigenetic change, accompanied with the up-regulation of a small fraction of tumor suppressor genes.

The list containing oncogenes was expanded by MITF and filtered for a fold change ≥ 2.8 and a p value ≤ 0.001 in at least one sample, resulting in a list of 62 genes (figure 15A). Similar to the tumor suppressor genes, an equal number of oncogenes was up- and down-regulated in reprogrammed HT-144-iPCCs and their differentiated daughter cells compared to the parental cell line. Furthermore, oncogene expression differed between the cellular differentiation states. As 17 of the twenty most down-regulated oncogenes were commonly regulated in HT-144-dFIC A and B (figure 15B), the gene sets were combined and ranked according their arithmetic mean. The ten most down-regulated oncogenes of HT-144-iPCCs and HT-144-derived differentiations compared to the parental cell line were analyzed for commonly down-regulated genes. MITF, growth arrest-specific gene 7 (GAS7) and neuroblastoma 1 (NBL1) were identified (figure 15C). HT-144-iPCCs shared four commonly down-regulated oncogenes with HT-144-dNICs and two with fibroblast-like differentiated cells. These also shared two down-regulated oncogenes with HT-144-dNICs. Taken together, the data indicate that similar to tumor suppressor genes also oncogenes are deregulated upon reprogramming of tumor cells into iPCCs and their subsequent differentiation.

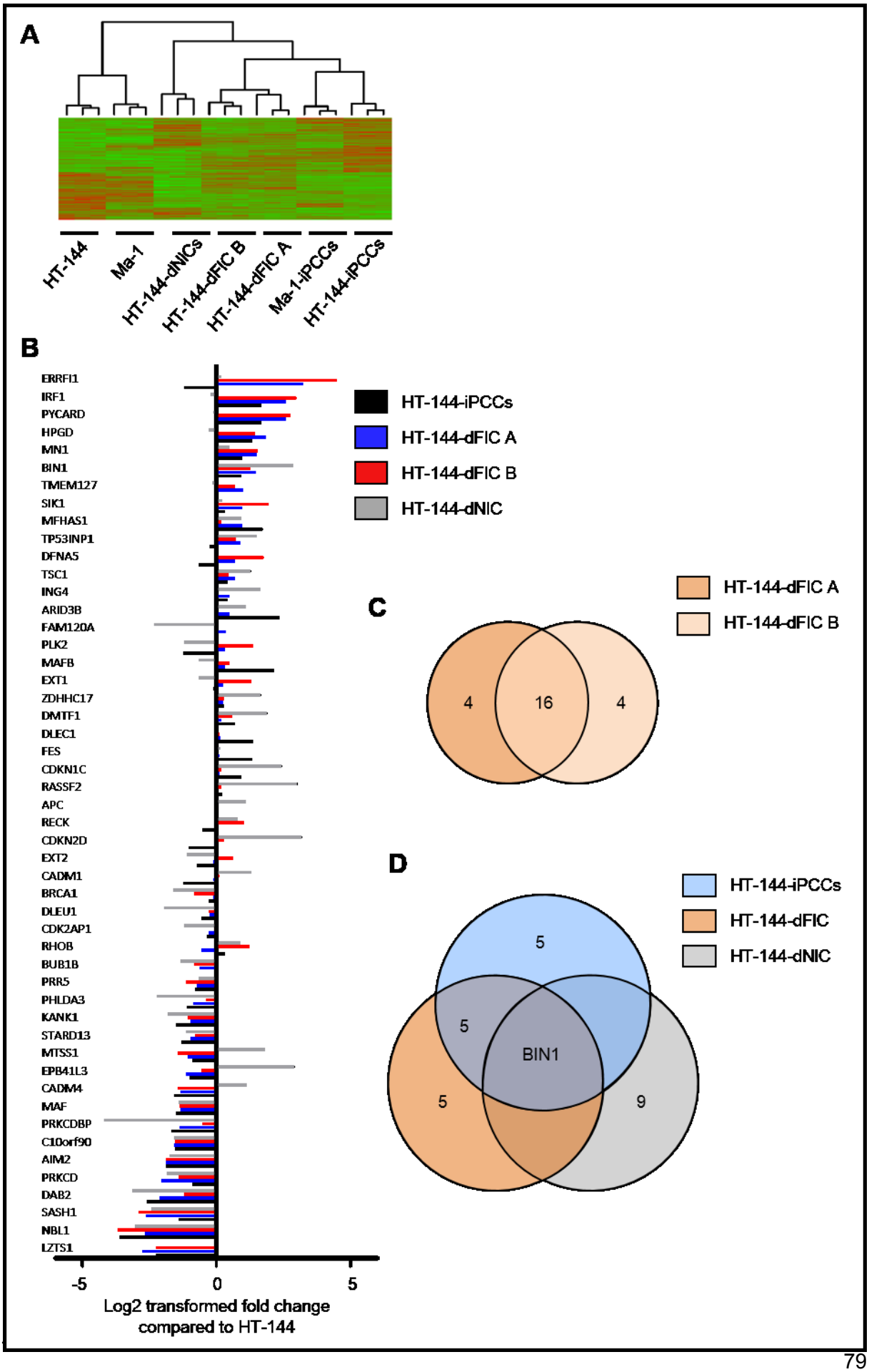

A
HT-144
Ma-1
HT-144-dNICs
HT-144-dFIC B
HT-144-dFIC A
Ma-1-iPCCs
HT-144-iPCCs
B
ERRFI1
IRF1
PYCARD
HPGD
MN1
BIN1
TMEM127
SIK1
MFHAS1
TP53INP1
DFNA5
TSC1
ING4
ARID3B
FAM120A
PLK2
MAFB
EXT1
ZDHHC17
DMTF1
DLEC1
FES
CDKN1C
RASSF2
APC
RECK
CDKN2D
EXT2
CADM1
BRCA1
DLEU1
CDK2AP1
RHOB
BUB1B
PRR5
PHLDA3
KANK1
STARD13
MTSS1
EPB41L3
CADM4
MAF
PRKCDBP
C10orf90
AIM2
PRKCD
DAB2
SASH1
NBL1
LZTS1
-5
0
5
Log2 transformed fold change compared to HT-144
HT-144-iPCCs
HT-144-dFIC A
HT-144-dFIC B
HT-144-dNIC
C
HT-144-dFIC A
HT-144-dFIC B
4
16
4
D
HT-144-iPCCs
HT-144-dFIC
HT-144-dNIC
5
5
BIN1
5
9

Figure 14 | Differentially regulated tumor suppressor genes in iPCCs and their differentiated daughter cells. A) Heat map generated by unsupervised hierarchical clustering of differentially regulated genes (empirical Bayes moderated t-test $p \leq 0.05$) in tumor cell lines, iPCCs, HT-144-dFICs and HT-144-dNICs. The dendrogram added to the heat map cluster samples according their relationship. B) Tumor suppressor genes showing a log2 fold change ≥ 1.5 compared to HT-144 melanoma cells in at least one sample of HT-144-derived iPCCs, fibroblast-like and neuronal-like cells were identified. Fold change is displayed as log2 transformed. The list of tumor suppressor genes was compiled from UniProt Protein knowledgebase (http://www.uniprot.org/). C) Venn diagram of the top 20 differentially regulated tumor suppressor genes between HT-144-dFIC A and B compared to HT-144. Overlapping regions contain commonly up-regulated tumor suppressor genes. D) Venn diagram of the top ten up-regulated tumor suppressor genes in HT-144-iPCCs and their differentiated daughter cells compared to parental cells. Overlapping regions represent commonly up-regulated tumor suppressor genes in indicated samples. HT-144-dFIC contain the top ten up-regulated tumor suppressor genes combined of HT-144-dFIC A and HT-144-dFIC B. Therefore, the arithmetic mean was calculated for each gene and used to rank the genes of the two samples.

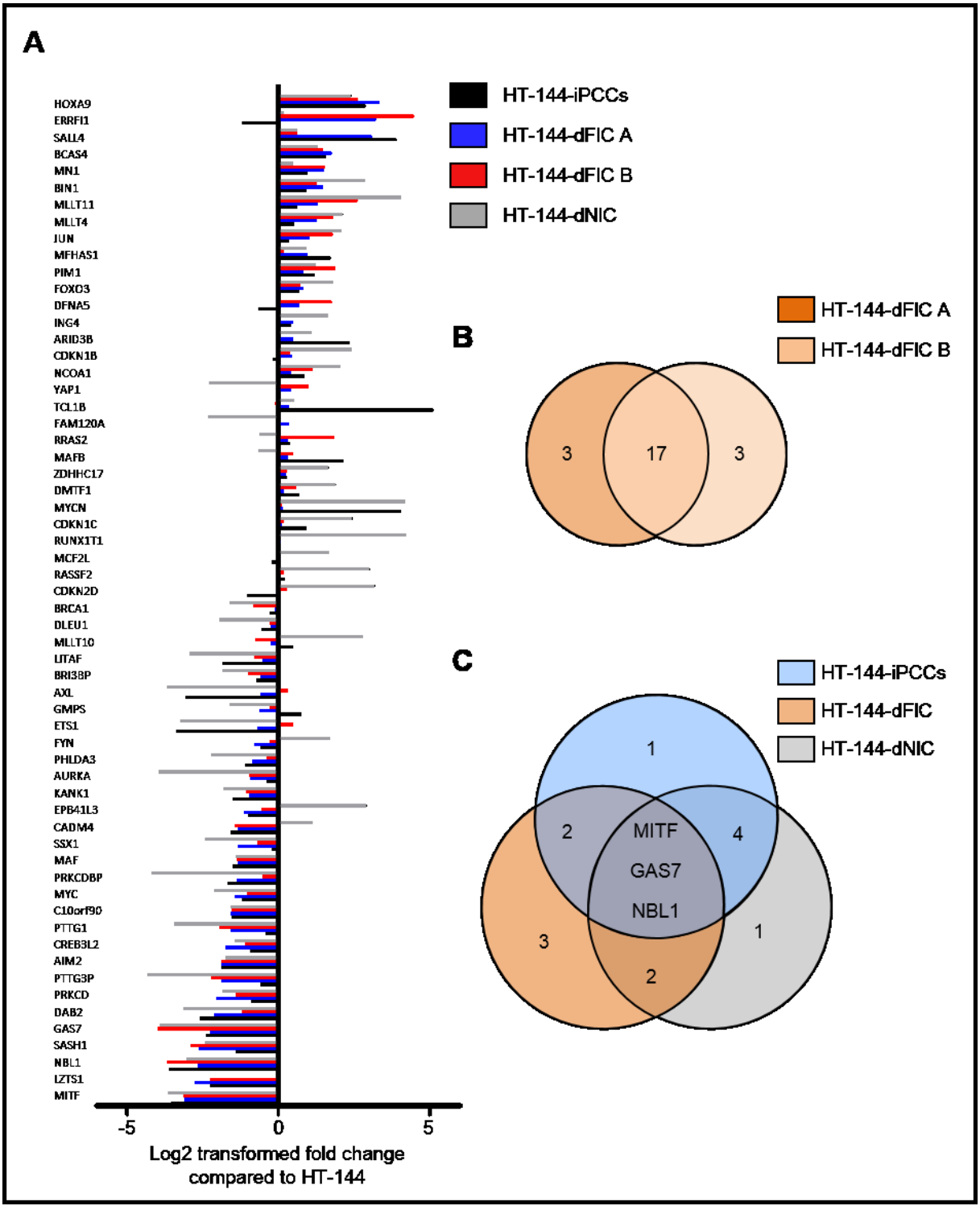

A
HOXA9
ERRFI1
SALL4
BCAS4
MN1
BIN1
MLLT11
MLLT4
JUN
MFHAS1
PIM1
FOXO3
DFNA5
ING4
ARID3B
CDKN1B
NCOA1
YAP1
TCL1B
FAM120A
RRAS2
MAFB
ZDHHC17
DMTF1
MYCN
CDKN1C
RUNX1T1
MCF2L
RASSF2
CDKN2D
BRCA1
DLEU1
MLLT10
LITAF
BRI3BP
AXL
GMPS
ETS1
FYN
PHLDA3
AURKA
KANK1
EPB41L3
CADM4
SSX1
MAF
PRKCDBP
MYC
C10orf90
PTTG1
CREB3L2
AIM2
PTTG3P
PRKCD
DAB2
GAS7
SASH1
NBL1
LZTS1
MITF
-5
0
5
Log2 transformed fold change
compared to HT-144
HT-144-iPCCs
HT-144-dFIC A
HT-144-dFIC B
HT-144-dNIC
B
HT-144-dFIC A
HT-144-dFIC B
3
17
3
C
HT-144-iPCCs
HT-144-dFIC
HT-144-dNIC
1
2
MITF
GAS7
NBL1
4
3
1
2

Figure 15 | Reprogramming deregulates oncogene expression in iPCCs and their differentiated daughter cells compared to parental melanoma cells. A) Oncogenes differentially expressed between iPCCs, HT-144-dFICs and HT-144-dNICs compared to parental melanoma cells. The list of oncogenes was compiled from UniProt Protein knowledgebase (http://www.uniprot.org/) and filtered for genes with a log2 fold change ≥ one and p value ≤ 0.005 (empirical Bayes moderated t-test) in at least one sample of HT-144-derived iPCCs, fibroblast-like and neuronal-like cells. B) Venn diagram of the top 20 down-regulated oncogenes in HT-144-dFIC A and B. Overlapping areas contain commonly deregulated genes of the two samples. C) Venn diagram of the top ten down-regulated oncogenes compared to HT-144 melanoma cells in HT-144-iPCCs, HT-144-dFIC and HT-144-dNIC. Overlapping regions represent genes commonly down-regulated in the indicated samples. HT-144-dFIC contain the top ten down-regulated oncogenes combined of HT-144-dFIC A and HT-144-dFIC B. Therefore, the arithmetic mean was calculated for each gene and used to rank the genes of the two samples.

X. Discussion

In this study the nuclear factor-based reprogramming method was used to analyze the effect of epigenetic remodeling processes, associated with cellular dedifferentiation, on drug sensitivity of human solid tumor cells. First, it was demonstrated that tumor cells are amenable to transcription factor-based reprogramming. Therefore, various melanoma cell lines as well as primary cells-derived from a metastasis and the cervical cancer cell line HeLa were converted into a pluripotent-like state sharing *in vitro* and *in vivo* characteristics of murine ESCs. Finally, data are provided indicating that epigenetic modifications in melanoma-iPCC-derived cells mediate resistance to targeted therapy and decrease their tumorigenic potential.

X.1. Establishment of induced pluripotent cancer cells

X.1.1. Barriers in tumor cells complicate their reprogramming

The induction of pluripotency by ectopic expression of transcription factors is limited by the existence of reprogramming barriers. In tumor cells the first morphological differences after Yamanaka factor induction were observed significantly later than in healthy somatic cells. About 40 days after transgene induction the earliest tumor cells became smaller and generated cell clusters, but did not progress further into large colonies of single cell layers observed in iPSCs derived from human somatic cells. Usually, the reprogramming process of human somatic cells requires 8 to 40 days until colonies emerge using lentiviral expression vectors (Takahashi et al. 2007). Nevertheless, transferring the cells on feeder cells promoted the formation of tightly packed AP positive colonies. The efficiency of the process is limited by the existence of reprogramming barriers. The investigation of those barriers revealed processes like ubiquitination, vesicular transport, endocytosis and cell adhesion to play an important role (Buckley et al. 2012; Qin et al. 2014). Studying the reprogramming process in clonal cell populations demonstrated that every cell can give rise to iPSCs, although significant delays between daughter cells occur (Hanna et al. 2009b). The preference of individual cells to acquire pluripotency indicates that reprogramming roadblocks originate from epigenetic barriers. Resetting the epigenetic profile of differentiated cells into that of ESCs is the challenge of the reprogramming factors. Therefore, epigenetic remodellers and chromatin modifiers must be activated to erase the current epigenetic status. The pluripotency factors Oct4, Sox2, and Klf4, enhance the expression of the DNA methyltransferase DNMT3L compared to the parental cell line (figure 6C). During reprogramming equal numbers of methylated sites are gained and lost, indicating that the balanced methylation and demethylation of loci is comparable important

for the successful acquisition of pluripotency (Lister et al. 2011, Nishino et al. 2011). Genome-wide methylation changes take place in the late phase of reprogramming. Accordingly, the up-regulation of de- or methylating enzymes occurs late in reprogramming (Polo et al. 2012) and correlates with the stabilization phase (Golipour et al. 2012). Although DNMT3L is highly expressed in ESCs, its defined function is not yet clear. There is evidence that the DNA methyltransferase promotes the methylation of housekeeping genes but negatively regulates the methylation at promoters of bivalent genes (Neri et al. 2013). DNMT3L deficiency results in two important observations. First, DNMT3L$^{-/-}$ adult mice are infertile but do not suffer from an additional phenotype (Bourc'his et al. 2001). Secondly, ESCs lacking the enzyme lose DNA methylation over time (Ooi et al. 2010). Therefore, these data demonstrate that the differentiation potential of PSCs is independent of the chromatin modifications induced by DNMT3L, while the DNA methyltransferase plays an essential role in the acquisition or maintenance of a pluripotent state. However, the knockout of the family members DNMT3A and DNMT3B in fibroblasts allows the induction of pluripotency but limits their developmental potential (Pawlak and Jaenisch 2011). Taken together, applying nuclear reprogramming using the Yamanaka factors Oct4, Sox2, and Klf4 to solid human cancer cells suggests that human melanoma and cervical carcinoma cells undergo global epigenetic modifications mediated in part by DNMT3L. Furthermore, these data indicate that the more features a cell already shares with PSCs, the less hurdles exist preventing the induction of pluripotency. Thus, inverse correlations exist between the stage of cellular differentiation of somatic cells, and the frequency and efficiency of iPSCs formation (Eminli et al. 2009; Takahashi & Yamanaka 2006). Also DNA damage sensors like p53, p16^{INK4a}, p14^{ARF}, and p^{CIP1} are induced upon transgene expression representing reprogramming barriers by driving cells into senescence. Therefore, the process can be accelerated by reducing the expression of tumor suppressor genes (Hong et al. 2009; Li et al. 2009; Zhao et al. 2008a) or by the immortalization of cells (Utikal et al. 2009b).

Loss of tumor suppressors and infinite self-renewal is a hallmark of cancer cells, resulting in the hypothesis that those should reprogram faster and more efficiently than somatic cells. Previous studies investigating iPCCs reported controversial observations. In accordance with my observations other reports showed that reprogramming cancer cells is less effective and more time consuming (Lai et al. 2013; Lin & Chui 2012; Utikal et al. 2009a). In contrast, sarcoma and pancreatic cancer cell reprogramming resulted in the slightly earlier formation of ESC-like colonies than usually reported from somatic cells, revealing differences between cancer types (Kim, J. et al. 2013; Zhang et al. 2013). Taken together, these data suggest that tumor-specific epigenetic alterations as well as genomic mutations can represent reprogramming barriers (Mahalingam et al. 2012). These differ between distinct tumor samples explaining the observed differences concerning the reprogramming efficiency. Alternative

reprogramming methods, yielding higher reprogramming efficiencies in general, as well as adding chemical compounds regulating epigenetic modifications might overcome some of the difficulties easier. Furthermore, these might provide further insights into tumor-specific epigenetic barriers.

Reprogramming somatic cells is a very inefficient process separated into distinct molecular phases. Clonal analysis demonstrated that iPSCs appear with varying latencies, while some daughter cells never give rise to iPSCs, indicating that cells pass a stochastic phase during reprogramming (Buganim et al. 2012; Chung et al. 2014). Expression of Oct4, Sox2, Klf4, and c-Myc stochastically induce signaling pathways, resulting in varying possible cell fates like apoptosis, cell senescence, transformation and reprogramming. The stochastic phase ends with the activation of Sox2, initiating a shorter deterministic phase. Here, a hierarchical order of events finally leads to the activation of the pluripotency network starting with the endogenous activation of Sox2.

Somatic cells seem to contain a favored subpopulation of cells that overcome the bottleneck during reprogramming, the stochastic phase, quickly and form iPSCs in a non-stochastic manner according to the elite model (Guo et al. 2014). Similarly, Lai et al. (2013) demonstrated that also cancer cells follow the elite model (Lai et al. 2013), as the analysis of the mutational profile of the reprogrammed cells revealed that nuclear reprogramming selected for a minor subpopulation of cells from the heterogeneous parental cell line. Enriched cells were negative for common mutations of the parental cell line, leading to the suggestion that the iPCCs arise from a progenitor pool of aneuploid cells previous to the acquisition of critical mutations. In order to clarify whether this is also true for melanoma iPCCs, tumors derived from HT-144-iPSCs were analyzed and found to be positive for the $BRAF^{V600E}$ mutation (figure 10E). Furthermore, investigating the genetic profile of fibroblast-like cells differentiated from the same HT-144-iPCCs, demonstrated the presence of common chromosomal aberrations as well as additionally gained mutations. Taken together, melanoma cells might reprogram according to two possible mechanisms. Either the process is similar to somatic cell reprogramming or HT-144-iPCCs are derived from a favored subpopulation, which itself is not depending on less chromosomal aberrations.

Nonetheless, the assumption that the reprogramming process is inversely correlated to the number of reprogramming barriers advocate for cell populations more amenable for nuclear reprogramming than the tumor bulk, either due to distinct mutations or specific epigenetic states. One potential barrier might exist in the energy metabolism. In contrast to adult somatic cells, PSCs obtain energy predominantly by glycolysis (Folmes et al. 2011; Kondoh et al. 2007). Therefore, it is likely that cells with a predominant glycolytic metabolism might be favored to become pluripotent. The identification of dynamic melanoma subpopulations differing in their metabolic energy supply (Roesch et al. 2013) might endorse the assumption

of privileged tumor cells. Taken together, the data from this study do not support previous results, following the hypothesis of a subpopulation with less chromosomal aberrations privileged to acquire pluripotency.

Evidence that reprogramming barriers in tumor cells might limit the induction of pluripotency complicates the work with cells from primary material. During the isolation of tumor cells from patient samples fibroblasts can contaminate the cell culture. Therefore, reprogramming of contaminated tumor cells might enrich for iPSCs derived from somatic cells. In order to get rid of potential contaminations in the primary melanoma line Ma-1, cells were passaged for more than six times prior the establishment of clones. During passage four to eight large senescent fibroblasts emerged but were diluted with continuous passage numbers. Based on the morphological appearance, established clones showed no fibroblast-like cells. Additionally, drug tests indicated that generated cell lines harbored the same $BRAF^{V600E}$ mutation like the patient (Bernhardt et al. 2014). HT-144-iPCCs were additionally validated by a cell authentication service analyzing 24 single-nucleotide polymorphisms. Interestingly, no differences in the reprogramming efficiency between BRAF (HT-144, WM266-4, Ma-1) or NRAS (SKMEL147) mutated melanoma cells compared to wild-type (Mewo) tumor cells (table 4) were noticed. The incidence of iPCC clones was independent of the mutational status, leading to the hypothesis that the mutational status of BRAF or NRAS does not play a decisive role in the reprogramming process.

In order to verify that barriers of reprogramming were overcome, it was shown that iPCCs fulfill *in vitro* criteria of pluripotency. Reactivation of loci from members of the core circuitry of pluripotency (Boyer et al. 2005) were observed. *NANOG* and *SOX2* in clones of varying reprogrammed tumor cells reached expression levels similar to NHiPSCs. Members of the pluripotency circuitry induce a network of transcription factors, regulating the maintenance of the pluripotent state and persistence of self-renewal. Successful activation of the core circuitry is indicated by the up-regulation of the extended pluripotency network (Buganim et al. 2012) like *SALL4* and *TET1*. The up-regulation of these genes, which are located downstream of the core network, to levels observed in NHiPSCs might suggest that the reprogrammed tumor cells acquire a similar pluripotent potential. This notion is supported by the detection of the glycosylation epitope Tra-1-60 (figure 5). The stem cell marker is widely used in stem cell research to identify pluripotent stem cells (Badcock et al. 1999; reviewed in Draper et al. 2002). Taken together, the data indicate that tumor cells can be successfully reprogrammed into an iPSC-like state. This is supported by the observation that the transcriptional profile of iPCCs is more similar to iPSCs than to their parental cells (figure 6A).

X.1.2. Mesenchymal to epithelial transition of melanoma cells is reverted during reprogramming

In the human skin, melanocytes and keratinocytes form a homeostatic balance, mediated by the cell adhesion molecule E-Cadherin and P-Cadherin (Hirai et al. 1989). Loss of the growth regulatory function is a consequence of an epithelial to mesenchymal transition-like process during melanomagenesis (Tang et al. 1994). Thereby, a cadherin switch results in loss of epithelial markers and cell adhesion molecules (Danen et al. 1996; Poser et al. 2001) in favor of a mesenchymal phenotype (Caramel et al. 2013; Hao et al. 2012). Forced up-regulation of E-cadherin restores the keratinocytic growth regulation (Hsu et al. 2000) inhibiting their invasiveness (Molina-Ortiz et al. 2009). Accordingly, melanoma cells used here were mainly negative for E-Cadherin expression (figure 6C). Previous studies demonstrated that even though Mewo cells have significantly down-regulated E-Cadherin levels in comparison with NHM (Matsuyoshi et al. 1997; Suyama et al. 2002), they still express detectable levels of the cell adhesion molecule (Fenouille et al. 2012; Tsutsumida et al. 2004). However, levels of membrane-bound E-Cadherin are tightly regulated and can be decreased by proteolytic cleavage of the extracellular domain, a process called ectodomain shedding (Billion et al. 2006; Shirahama et al. 1996). Therefore, the gene expression levels of the adhesion molecule do not necessarily correlate with its protein amount in melanoma cells. Besides that, the expression levels were compared to NHiPSCs, which might express E-Cadherin at lower levels than melanocytes.

During reprogramming, somatic cells undergo a MET in the early phases of the process, induced by Sox2/Oct4-mediated suppression of the E-Cadherin repressor Snail and resulting in a cadherin switch towards E-Cadherin expression (Li, R. et al. 2010; Samavarchi-Tehrani et al. 2010). Furthermore, E-Cadherin loss prevents nuclear factor-based reprogramming but can be compensated by replacement with N-Cadherin (Bedzhov et al. 2013; Redmer et al. 2011). Similarly abrogation of E-Cadherin function in ESCs can be partly replaced by other cadherins *in vitro* (Hawkins et al. 2012), but results in the inhibition of trophectoderm formation *in vivo (Larue et al. 1994)*. In the acquisition and maintenance of pluripotency the transmembrane protein plays multiple functions. In mouse ESCs, E-Cadherin is involved in the stabilization of the LIF receptor-GP130 complex required for proper activation of STAT3 signaling (del Valle et al. 2013; Hawkins et al. 2012). Furthermore the formation of cell junctions and cell adhesions is a prerequisite to generate pluripotent stem cell colonies (Bedzhov et al. 2013). Likewise E-cadherin is essential for the pluripotent state in human cells by activating LIF signaling in naïve and primed human PSCs (Li, D. et al. 2010; Li, L. et al. 2010). Taken together, E-Cadherin plays a central role during reprogramming and is up-regulated during reprogramming. When human PSCs start to differentiate, the cadherin switch is reverted from an epithelial-to-mesenchymal phenotype sharing many features with the EMT process in melanoma cells

(Eastham et al. 2007; Evseenko et al. 2010; Jung et al. 2012). The switch from E- to N-cadherin is accompanied by increased cellular motility, up-regulation of E-cadherin repressors and enhanced expression of matrix metallo-proteases (Eastham et al. 2007; Liao et al. 2013; Richter et al. 2014). Taken together, there is evidence that melanoma cells were able to complete MET to acquire pluripotency similarly to somatic cells. Furthermore, it is likely that E-cadherin-mediated cellular junctions are responsible for the formation of the tightly packed colonies, mimicking the morphological appearance of murine ESCs. Due to the observation that ectopic E-cadherin expression in melanoma cells restores their growth regulation by keratinocytes, the malignant phenotype of the cells might be altered. In addition, the data suggest that differentiation of melanoma-iPCCs into a melanocytic-lineage requires a complete EMT, thereby mimicking the process from a melanoma *in situ* to malignant cells. This might raise the question whether cancer-derived PSCs are suitable to investigate early events in cancer development. Kim, J. et al. (2013) demonstrated that pancreatic adenocarcinoma-derived iPCCs gave rise to neoplasia precursors progressing to the invasive state. Thereby, they discovered novel networks activated only in the early stage of pancreatic cancer progression. Consequently, cancer-derived pluripotent stem cells might serve as a tool to study early events in tumor development.

X.1.3. Induction of the endogenous pluripotency network during reprogramming

Fully reprogrammed cells and ESCs are characterized by the activation of the pluripotency network. Members of the core network are Oct4, Sox2, and Nanog. To our surprise, endogenous Oct4 was hardly up-regulated in most iPCCs compared to the parental tumor line. This rise the question whether Oct4-deficient or -low cells can be pluripotent. $Oct4^{-/-}$ embryos fail to generate pluripotent stem cells during the development (Nichols et al. 1998) and Oct4 repression in ESCs comes along with the differentiation into trophectoderm (Niwa et al. 2000). Recent studies showed that oocytes from Oct4-null mice were still able to initiate the reprogramming machinery in somatic cells after NT (Wu et al. 2013), and ICM cells of these mice were still characterized by active Nanog expression albeit the cells were not pluripotent (Le Bin et al. 2014; Wu et al. 2013). Taken together, Oct4 is not essential for the foundation but for the maintenance of pluripotency. Thereby, the expression level of the pluripotency factor plays an ambivalent role. Control of the pluripotent state by transcription factors provides heterogeneity within the ICM or ESCs, resulting in individual cells naturally more prone to differentiation-inducing stimuli. Therefore, stem cells residing under the same environmental conditions can respond differently to external stimuli. This strategy enables the proper development of embryos but does not necessarily support extended self-renewal. Oct4-heterozygous ESCs are characterized by reduced expression levels of the transcription factor,

although the pluripotent state is stabilized by enhanced binding of pluripotency-associated enhancers and increased self-renewal capacities (Karwacki-Neisius et al. 2013). Consequently, Oct4 expression either induces differentiation (Niwa et al. 2000) or stabilization of the pluripotent state in a dose-dependent manner. Moreover, ESCs of the naïve state balance Oct4 and Nanog expression at defined ratios, while variances in the expression ratio are observed in epiblast-like PSCs or further differentiated cells (Munoz Descalzo et al. 2012). The observation that HT-144-iPCCs formed tumors containing cells of different lineages conflicts with previous results of Oct4-low reprogrammed cells. Based on these data, it is doubtful whether endogenous reactivation of Oct4 is high enough to be biological relevant. However, fully reprogrammed iPSCs derived from somatic cells were generated using the STEMMCA vector (previously demonstrated to be pluripotent, http://www.ub.uni-heidelberg.de/archiv/17870), suggesting that ectopic Oct4 expression by the transgene is sufficient to trigger activation of other pluripotency markers. Supported by *in vitro* and *in vivo* characteristics, it was suggested that human melanomas from cell lines and from patient material are susceptible to nuclear factor-based reprogramming. Consequently, the same reprogramming factors used for somatic cell reprogramming, are able to induce pluripotency in human cancer cells under equivalent conditions.

Tumor cell lines express single pluripotency factors at levels nearly equivalent to those in iPSCs. Here, melanoma cells endogenously express c-Myc (figure 3B), and also Sox2 (figure 4A). Previous studies demonstrated that cells with baseline expression of single reprogramming factors require fewer genes to induce pluripotency (Kim, J. B. et al. 2009a; Kim, J. B. et al. 2009b; Utikal et al. 2009a). Therefore it's tempting to speculate that introducing fewer factors would be sufficient to reprogram cancer cells. Accordingly, it was observed that iPCCs generation using a STEMCCA cassette in which c-Myc is replaced by a puromycin resistance for selection is equally efficient.

X.1.4. Mediators of pluripotency in tumor cells

Expression of pluripotency markers and their function in tumor cells is controversially discussed (reviewed in Bernhardt et al. 2012). On the one hand many reports suggest a role for Oct4, Sox2, and Nanog in driving cancer progression and regulating self-renewal of cancer cells (Atlasi et al. 2007; Ling et al. 2012; Monk & Holding 2001; Schoenhals et al. 2009; Wang et al. 2014), on the other hand other studies did not detect any expression or criticize the lack of functional evidence (Cantz et al. 2008). Here, it was demonstrated that the majority of parental tumor cell lines express mRNA of one or more pluripotency factors at detectable levels although significantly below the level of iPSCs in most cases. Several reasons might be responsible for this observation. According to the model of melanoma subpopulations, a low number of privileged tumor cells might express pluripotency markers but the expression is

diluted by the tumor bulk. Therefore, qPCR experiments showing mean expression over the whole populations might give false negative results. Another reasonable possibility might be that tumor cells express low amounts of pluripotency factors as regulators of self-renewal. Nevertheless, most of the investigated pluripotency factors were more than 100-fold down-regulated compared to iPSCs.

In the parental cell lines elevated Nanog mRNA levels were exclusively observed in HT-144 cells. Anyhow, Nanog expression was not detected in any tumor cell line by immunofluorescence. Previously, several tumor types were demonstrated to contain individual cells with high Nanog expression promoting a stem-cell like phenotype with enhanced tumorigenicity and increased metastatic potential (Jeter et al. 2009; Lee et al. 2011; Lu et al. 2014; Shan et al. 2012; Xie et al. 2014). Two possibilities might account for the controversial observation regarding Nanog expression in HT-144 cells. Either expression levels for the antibody staining were below the detection limit or primer pairs used for qPCR analysis recognized a potentially expressed Nanog pseudogene. These arise either by the integration of mRNA copies or by gene duplications. Eleven human Nanog pseudogenes exist (Booth & Holland 2004), although only Nanog-P1, demonstrated to be expressed in human leukemia and colon cancer cells (Eberle et al. 2010; Ishiguro et al. 2012), shares the identical intron-exon structure. In prostate cancer the expression of Nanog-P8 increases clonogenicity and tumor regenerative capacity (Jeter et al. 2011). Due to the structural identity, the expression of Nanog or its pseudogene Nanog-P1 cannot be discriminated, leaving the possibility that the detected expression is based on pseudogenes. Nevertheless, most of the potential pseudogenes are not translated and additionally there is no report that pseudogenes expression reaches expression levels similar to PSCs. Therefore, it seems unlikely that pseudogenes might be responsible for endogenous reactivation of pluripotency markers. Based on these results, the possibility was excluded that under the described cell culture conditions individual tumor cells evolve, following the theory of a cancer-initiating subpopulation with high expression of the pluripotency factor Nanog.

The pluripotency factor Sox2 has been observed in three of five melanoma cell lines and was absent in HeLa cells. Previously, Sox2 was identified as driving force of melanoma metastasis and invasion (Girouard et al. 2012; Justilien et al. 2014; Santini et al. 2014b). Additionally, successful reprogramming of melanoma cells in the absence of ectopic Sox2 indicates its functional expression (Utikal et al. 2009a). The transcription factor is an early marker of neural progenitors (Rogers et al. 2009) and is involved in proliferation and differentiation of migratory and postmigratory neural crest cells (Wakamatsu et al. 2004). Its down-regulation in melanocytic progenitors is required for functional differentiation into melanocytes (Adameyko et al. 2012). In various tumor entities Sox2 promotes a stem cell-like phenotype with a highly aggressive tumorigenic potential (Boumahdi et al. 2014; Favaro et al. 2014; Gangemi et al.

2009; Leis et al. 2012). The function of Sox2 in melanoma is not fully understood. It is conceivable that Sox2 plays a similar role as its family member Sox10. Sox10 is a neural crest marker and active during melanocyte differentiation (Britsch et al. 2001; Wong et al. 2006). Its detection in melanoma and requirement for proliferation and cell survival suggests an incomplete silencing during melanocyte differentiation, resulting in melanoma development (Shakhova et al. 2012).

X.1.5. Loss of the melanoma expression signature

Signaling pathways controlling melanocyte development and differentiation are commonly deregulated in melanoma cells (Ordonez 2014; Vance & Goding 2004). Thus, a large number of melanocytic markers are used to distinguish melanoma from other tumors entities. In this study, melanocytic markers were investigated in the melanoma cell line HT-144 and in melanoma cells derived from a trametinib-resistant metastasis (Ma-1) as well as their reprogrammed iPCCs. Members of the MITF signaling pathway were highly expressed in HT-144 but not in Ma-1 cells. This is in accordance to clinical observations. Trametinib-resistant metastases lost pigmentation compared to the tumor before treatment. Accordingly, the key enzymes of melanogenesis, namely MITF, TYR, TRP1 and DCT were low expressed in the parental Ma-1 cells (figure 7A&C).

Comparing the melanocytic markers to NHM revealed that MITF, TYRP1, and DCT were expressed in Ma-1 at comparable levels. Furthermore, this would indicate that HT-144-iPCCs, although significant down-regulated compared to their parental cells, still express TYRP1 and DCT at similar levels as NHM (figure 7A). This is conflictive to the assumption that the reprogrammed cells lost all terminal markers and the melanoma transcription profile. As NHM were isolated from the foreskin of newborn babies, it might be, that the resulting melanocyte population was immature and expressed low levels of terminal differentiation markers. Therefore, normalization to these NHM might over-estimate the expression levels of melanocytic markers in the parental and reprogrammed melanoma cells.

In contrast to the results obtained from microarray experiments, qPCR analysis demonstrated a significant down-regulation of MITF in Ma-1-iPCCs compared to the parental cells. This might be due to the higher sensitivity of the qPCR technique, which is superior to microarray analysis. As reprogrammed Ma-1-iPCCs showed MITF levels below the detection limit in qPCR analysis, a technical significant down-regulation was observed (figure 7). However, the data indicate that the melanoma cell lines HT-144 and Ma-1 clearly differ in their MITF signaling pathway activity. Genes of this pathway are already low expressed in Ma-1, and therefore a semi-quantitative comparison of these genes in Ma-1-iPCCs does not indicate a down-regulation due to low gene expression levels already observed in the parental cell line.

Similar to terminal melanoma markers also genes highly expressed in neural crest cells were down-regulated in reprogrammed melanoma cells (figure 7D). This is in agreement with results obtained by the reprogramming of murine melanocytes decreasing melanin production and melanocytic markers (Utikal et al. 2009a). In contrast to nuclear factor-based reprogramming Kulesa et al. (2006) showed the reversion of melanoma cells towards a neural crest-like phenotype in an embryonic environment demonstrating high plasticity of melanoma cells and their ability to respond to molecules provided by the stem cell microenvironment. This indicates that active signaling pathways of ESCs can similarly stimulate melanoma cells (Postovit et al. 2006; Topczewska et al. 2006). These data suggest that nuclear factor-based reprogramming, in contrast to dedifferentiation by external stimuli of melanoma or melanocytes, result in the loss of melanocytic lineages markers through a dedifferentiation beyond an intermediate stage of multipotent neural crest cells.

X.1.6. Acquisition of unstable pluripotency in tumor-derived reprogrammed cells

After transferring reprogramming tumor cells onto feeder cells, they formed small, tightly packed colonies, consisting of cells that share hallmarks with murine ESCs including dome-shaped colony morphology, resistance to trypsin-passaging and single cell cloning. In contrast to somatic iPSCs (Chen et al. 2011; Park et al. 2013) the iPCCs do not require bFGF to be maintained in an undifferentiated state but are dependent on the continuous expression of the transgene. Similarly, it was demonstrated that bFGF-dependent human iPSCs derived from healthy melanocytes can be forced into an alternative state in which they shares features of murine ESCs. It is likely that the continuous expression of Oct4, Sox2, and Klf4 is partly responsible for this switch. This is supported by the finding that overexpression of Nanog in addition to the Yamanaka factors reprograms human fibroblast in the presence of LIF directly into mouse-like iPSCs (Buecker et al. 2010), while overexpression of Klf4 or alternatively c-Myc pushes human ESCs to adopt a similar LIF-dependent state (Hanna et al. 2010a). Usually, the generation of iPSCs using retroviral vectors is split into two phases: a transgene-dependent phase where expressed transcription factors initiate epigenetic remodeling processes, and a transgene-independent phase in which the factors need to be silenced as they otherwise prevent normal differentiation and mediate enhanced tumorigenicity (Okita et al. 2007; Ramos-Mejia et al. 2012). To circumvent these problems a doxycycline-inducible system has been used for the derivation of iPCCs. Previous publications showed that the transgene activation results in the sequential activation of pluripotency markers according the hierarchical organization of the pluripotency network (Brambrink et al. 2008; Buganim et al. 2012; Polo et al. 2012). Accordingly, AP positive cells appeared during the reprogramming process before defined colonies were formed. Further reprogrammed iPCCs additionally expressed late stem

cell markers like Nanog and Tra-1-60 (figure 4A&5), supporting the hypothesis that tumor cell reprogramming is mediated by the same events as somatic cell reprogramming. Several reports demonstrate the existence of distinct pluripotent states. While stable ESCs from murine blastocysts can be isolated, PSCs derived from other species appeared to be stable exclusively under epiblast-like growth conditions (Brons et al. 2007; Thomson et al. 1998). Recent studies stepwise elucidated culture conditions stabilizing murine ESC-like pluripotent stem cells from human and other species (Buecker et al. 2010; Fang et al. 2014; Gafni et al. 2013; Hanna et al. 2009a; Hirai et al. 2012; Theunissen et al. 2014; Ware et al. 2014). Here, conditions reported to support the switch from an epiblast-like state to an alternative pluripotent state were used (Buecker et al. 2010; Hanna et al. 2010b). Therefore, cells were cultivated on dense feeder cells in the presence of doxycycline and human LIF.

Although previous publications already demonstrated successful reprogramming of various cancer entities, the generation of iPCCs-derived from human melanoma cells adopting this murine ESC-like state is shown for the first time. Although DPPA4, which is involved in stabilizing the pluripotent state, is expressed (figure 6D), attenuation of the transgene expression leads to the differentiation of iPCCs. It is demonstrated that withdrawal of doxycycline results in differentiation of the cells indicated by loss of Nanog expression and AP positive colonies. Accordingly, morphological changes are observed, leading to the disintegration of colonies (figure 8). Furthermore, addition of bFGF has no effect on the stabilization of the iPCCs, indicating that cells in the alternative pluripotent state differ in their requirements of culture conditions from epiblast-like iPSCs

Based on these results, it can be suggested that the epigenetic profile or genetic mutations of the melanoma genomes prevent the successful acquisition of a stable pluripotent state. Our hypothesis is that ectopic expression of reprogramming factors circumvents the inherent blocks, allowing to adopt a metastable pluripotent state which might explain the transcriptional differences between iPSCs and iPCCs cultivated under the same conditions (figure 6A). This is supported by the notion that even in previous studies reprogrammed tumor cells did not reach a stable pluripotent state. iPCC-derived from human pancreatic adenocarcinomas required the expression of Oct4, Sox2, Klf4 and c-Myc to give rise to teratomas and to prevent differentiation (Kim, J. et al. 2013). Zhang et al. (2013) reprogrammed sarcoma cells using lentiviral encoded pluripotency factors controlled by a constitutively active promoter. Although the group showed that the transgenes were silenced in PSCs, residual transgene expression influences the iPSCs phenotype (Sommer et al. 2012) and might maintain the iPCCs undifferentiated.

X.1.7. Alternative pluripotent states suitable for gene targeting

Genetic manipulation is a fundamental technique to study the implication of a single gene in its cellular context. Due to low resistance against single cell dissociation, the introduction of transgenes and reporter-constructs works poorly in human ESCs or iPSCs (Amit et al. 2000; Ohgushi et al. 2010; Thomson et al. 1998). Therefore, murine ESCs were crucial for the investigation of gene functions in the frame of mammalian development. To overcome the hurdle that human iPSCs or ESCs are intolerant to clonal selection, the conversion of NHiPSC into the alternative pluripotent state as demonstrated with melanocyte-derived iPSCs (figure 3) might represent an effective tool to study modified genetic elements in human pluripotent stem cells. As such fibroblasts reprogrammed directly in an alternative pluripotent state by overexpression of Nanog in addition to the Yamanaka factors allowed gene targeting with 4 kb large DNA fragments by electroporation (Buecker et al. 2010).

X.2. Reprogramming-induced epigenetic modifications controlling tumorigenicity

X.2.1. Melanoma-iPCCs re-acquire the potential to execute pathways of non-melanocytic differentiation

The analysis of the differentiation capacities of reprogrammed human cells is limited to the teratoma assay, while the gold standard, contribution to viable chimeric mice, is restricted to murine cells. Although cautiousness is appropriate when extrapolating the differentiation potential of a human cell from their capability to form tumors in mice, teratoma assays are a widely accepted technique. In this study parental tumor cell lines as well as their reprogrammed counterparts formed tumors after subcutaneous injection, albeit the histopathology of the samples differed markedly. iPCC-derived tumors exhibit a heterogeneous pattern of undifferentiated structures. HT-144-iPCCs generated tumors with the highest grade of heterogeneity, containing many undifferentiated structures compared to other iPCC-derived tumors. Nevertheless, from their morphological appearance iPCC-derived tumors showed the appearance of similar tissue structures. In contrast, melanoma cell lines formed homogenous tumors with clear distinction compared to the iPCC-derived tumors. Moreover, iPCC-derived tumors contained differentiated tissues of varying origin. Epithelial cells were detected as clusters localized in endodermal-like structures in HT-144-iPCC-derived tumors. Similarly, Sox2 as wells as BRAF are expressed in defined formations. Furthermore, staining of ERK was restricted to specific areas in teratoma-like tumors indicating that diverging parts originate from different lineages (figure 10). In accordance to the heterogeneous pattern found, also proliferating cells were located in specific areas. As expected, phosphorylated ERK was

restricted to areas of ERK staining although not all ERK positive areas activated the pathway. The majority of iPCC-derived tumors did not contain cells with a melanoma-like profile as only a few cells were positively stained for melanoma markers. In contrast to our results, other studies showed that reprogrammed pluripotent cells tend to differentiate into the cell type of their origin. For example, reprogrammed pancreatic cancer cells predominately differentiate into pancreatic tissue, recapitulating early and late events of the carcinoma development (Kim, J. et al. 2013). This phenomenon is imprinted by epigenetic anchored marks which together generate an epigenetic memory (Bar-Nur et al. 2011; Kim et al. 2011). Three hypotheses might account for the effect. One explanation might be that due to the mutational status of the melanoma cells, differentiations into lineages are favored which are less addicted to the pathways containing the mutated molecules. Nevertheless, this does not explain why Mewo cells harboring no known mutation in BRAF or NRAS did not differentiate back into a melanocytic lineage. Therefore, acquired DNA damage, leading to the transformation into malignant melanoma cells, might prevent differentiations according pathways of melanocyte development. Alternatively, specific culture conditions used for iPCCs, might influence their differentiation potential and generate cells restricted to differentiate into specific lineages. In line with this hypothesis, the relatively high amount of gland-like tissue found in teratoma assays might indicate that the conditions favor endodermal differentiations. Another option is that the iPCCs were only partially reprogrammed reaching a dedifferentiated state that prevents the equal differentiation into all three germ layers. Taken together, it was concluded that epigenetic or genetic barriers as well as specific environmental conditions impede the differentiation of melanoma-iPCCs back to their lineage. Furthermore, reprogrammed melanoma cells re-acquire the potential to accomplish differentiation pathways of non-melanocytic origin, which is supported by the fact that iPCCs efficiently differentiated into neurons and fibroblast-like cells *in vitro*.

Furthermore, the lack of distinct morphological structures between different teratoma-like tumors derived from cells with varying genetic backgrounds suggests that neither $BRAF^{V600E}$ mutations nor NRAS mutations affect the differentiation outcome under these conditions.

The parental cell lines used in this study are highly proliferative and tumorigenic, harboring innumerable amounts of genetic mutations. Nonetheless, it was observed that iPCC-derived tumors contained large parts of non-proliferating cells (figure 10). *In vitro*, cells converted to neuronal-like cells stopped proliferation and expressed markers of mature postmitotic neurons (figure 11). Previous studies showed that sarcoma cells can be reprogrammed leading to a reset of their epigenetic profile allowing the cells to terminally differentiate into connective tissue and blood cells (Zhang et al. 2013). The generation of a melanoma-iPCC-derived mice demonstrated that the murine melanoma cell R545 can differentiate into various cell types (Utikal et al. 2009a). Taken together, this might indicate that the reprogramming process allows

the cells to regain the ability to execute differentiation pathways towards postmitotic and non-tumorigenic functional cells.

X.2.2. Neuronal differentiation of melanoma-derived iPCCs

Independent of their complex karyotype solid tumors cells can be reprogrammed, gaining the ability to differentiate along different lineages. Directed differentiation into neuronal- and fibroblast-like cells were associated with down-regulation of pluripotency markers and in parallel up-regulation of specific lineage markers.

Spontaneously differentiating human PSCs often pursue the neural lineage. Also the culture of embryoid bodies, three-dimensional aggregates containing all three germ layers formed by human iPSCs or ESCs under non-adhesive conditions, enriches for neuronal cells (Boulting et al. 2011; Nat et al. 2007; Zhang & Zhang 2010). Guided differentiation using defined media and the supplementation with small molecule inhibitors greatly improves the efficiency of the process (Chambers et al. 2009; Kim, D. S. et al. 2010; Mak et al. 2012; Wattanapanitch et al. 2014), enables the differentiation into specific functional neuronal populations (Hester et al. 2011; Stanslowsky et al. 2014; Swistowski et al. 2010) and directs the conversion of fibroblasts into neurons (Ladewig et al. 2012; Thier et al. 2012). Therefore, these advances facilitate the study of human neuronal cells and provide great promises for the research of neurodegenerative disease, in spite of the existing complications due to the postmitotic nature of mature neurons (reviewed in Herrup & Yang 2007).

Neuronal cells and melanocytes share their origin in the neural crest raising the question whether the high plasticity of melanoma cells disburdens their transdifferentiation. Although melanomas can contain structures of differentiated non-melanocytic origin, hints for neuronal-like cells are rare (Iyengar & Singh 2010). Nonetheless, primary cutaneous melanoma but not metastatic melanoma express the neural marker MAP2 (Soltani et al. 2005) which is suggested to be induced by $BRAF^{V600E}$ (Maddodi et al. 2010). Our data demonstrate low expression levels of MAP2 in the malignant melanoma cell line HT-144 when compared to neuronal differentiated cells. The cell line was isolated from a metastatic site which might explain the low MAP2 expression. Nevertheless, MAP2 was the only detectable neuronal marker in the parental cell line. After accomplished differentiation into neuron-like cells the neuroectodermal marker Pax6 (Zhang et al. 2010) and RBFox3, which is restricted to late postmitotic neurons (Kim, K. K. et al. 2013), could be detected indicating the successful terminal differentiation of melanoma-iPCCs into neurons.

As Nanog is a repressor of neuroectoderm differentiation (Wang et al. 2012) and therefore consequently down-regulated during neuronal differentiation (Vallier et al. 2009; Wang et al. 2009), it was surprising to detect only a slight decrease of its expression. Assuming that some

cells remained undifferentiated, it might indicate that individual cells require longer to respond to differentiation-inducing stimuli.

Taken together, the presented data extend the current knowledge about the differentiation potential of reprogrammed cancer cells. So far, it was shown for the first time that the genome of human melanoma cells possesses the ability to differentiate in neuronal-like cells. Previous hints were gained from the genome of murine melanoma cells giving rise to viable animals (Hochedlinger et al. 2004; Utikal et al. 2009a). In agreement with previous iPCC studies, our data suggest that the terminal differentiation along re-engaged normal pathways modifies the epigenetic profile preventing uncontrolled proliferation and tumorigenicity (Zhang et al. 2013).

X.2.3. Fibroblast-like differentiations regain features of melanoma cells without their molecular profile

Differentiation towards non-melanocytic lineages of iPCCs provides a tool to study the influence of a melanoma genome in the context of varying epigenetic backgrounds. Besides neuronal-like cells, HT-144-iPCCs generated fibroblast-like cells able to be stably expanded in order to study melanoma oncogene dependence. As previously discussed, common chromosomal translocations identified in fibroblast-like cells confirmed their derivation from HT-144 melanoma cells. After a few passages the down-regulation of pluripotency markers as well as the lack of melanocytic markers demonstrates the differentiation into non-melanoma cells. Instead, their transcriptional gene signature provided evidence of their similarity with human fibroblasts (figure 12C). In line with a fibroblast-like phenotype expression of FSP1 and the mesenchymal cytoskeletal marker vimentin were detected. Expression of cell structure proteins is a prerequisite for cell migration and invasion, both features reacquired by the differentiated cells (Mendez et al. 2010). Although fibroblast-like clones were obtained by different protocols they showed a very homogenous expression profile for the investigated fibroblast markers. However, cells differentiated in the presence of FCS and cells differentiated in the presence of EGF and BMP-4 differed markedly in their migratory activity. Due to these data it was concluded that the iPCCs are able to activate differentiation pathways stimulated by the supplements of their respective media but also that different protocols can lead to distinct activation profiles of the generated cells.

The potential of iPSCs or ESCs to differentiate in stromal cells with the ability to integrate functionally into skin equivalents has previously been demonstrated (Hewitt et al. 2011). In agreement with these data fibroblast-like differentiated cells can be serially passaged over prolonged time periods. Depending on the cell type and age of the donor primary cells undergo a replicative exhaustion resulting in cellular senescence (reviewed in Kuilman et al. 2010), which indicates that the same mechanisms facilitating immortalization in the parental tumor cells are conserved in fibroblast-like cells but not in the neural differentiations. This might point

out that immortalization of HT-144 cells is at least partly epigenetically controlled and therefore revertible.

Deciphering the oncogene dependence of melanoma cells and their counterparts, effects of MAPK signaling inhibitors on melanoma cells in different epigenetic stages were tested.

The oncogene-specific drug PLX4032 affected only the $BRAF^{V600E}$-mutated cell lines HT-144 and Ma-1 while all cell lines were sensitive against GSK1120212 treatment. Surprisingly, even Ma-1 cells, that were isolated from reoccurring metastases after GSK1120212 treatment, showed decreased cell proliferation compared to the control cells. It is likely, that prolonged cell culture without selective pressure favored the reappearance and proliferation of a GSK1120212-sensitive subpopulation. Previous studies demonstrated that the cessation of drug application leads to the regression of established tumors, as drug-resistant cells also became drug-dependent (Das Thakur et al. 2013).

However, alterations of the epigenetic state within reprogrammed iPCCs or fibroblast-like cells significantly reduced the drug-based cytotoxic and cytostatic effects. Therefore, the present study extends the current knowledge about the link between epigenetic modifications and targeted therapy. Previously, it has been shown that BCR-ABL-dependent chronic myeloid leukemia cells lose their oncogene dependence through nuclear reprogramming indicated by their resistance against imatinib treatment (Carette et al. 2010; Kumano et al. 2012). Similarly, neuronal and fibroblast-like differentiated cells but not differentiations into hematological lineages acquired resistance against the BCR-ABL inhibitor (Carette et al. 2010) providing evidence that targeted therapy is dependent on specific genetic and epigenetic states. These findings lead to the conclusion that the resistance mechanism is not a compensatory function of the pluripotency network. Instead, terminally differentiated cells negative for the pluripotency factors lose their oncogene dependence suggesting that alternative signaling pathways missing in the parental lineage neutralize the drug effects. In our example this might be supported by the down-regulation of MAPK signaling on mRNA and protein level. Alternative pathways control proliferation and survival in iPCCs and in differentiated cells leading to the inactivation of the pathway. Nevertheless, more differentiations of different lineages might be necessary to gain further insights. Due to the cell cycle arrest of neurons drug sensitivity assays could not be performed in neuronal differentiated cells as drug-induced cytostatic effects might have been underestimated.

Nevertheless, epigenetic alterations can equally result in enhanced drug sensitivity. Reprogrammed hepatocellular carcinoma cells get more amenable to systemic chemotherapy using 5-fluorouracil than their parental cells (Koga et al. 2014) demonstrating that epigenetic remodeling mediated by reprogramming processes can also re-sensitize cells to drug treatments. Surprisingly, even embryoid body-mediated differentiation of reprogrammed gastrointestinal cancer cells enhanced their sensitivity to anti-cancer drugs (Miyoshi et al.

2010). Similar to the reprogramming approach, transformation of a mature somatic cell type into another mature somatic cell, a process called transdifferentiation results in epigenetic alterations affecting the drug response. Transdifferentiated squamous cell carcinomas were resistant to Lox inhibition in contrast to parental lung adenocarcinoma cells (Han et al. 2014). Endothelial cells transdifferentiated from tumor-initiating glioblastoma cells acquired resistance against anti-VEGF receptor treatment (Soda et al. 2011). Furthermore, the impact of the cancer-associated epigenetic profile is supported by numerous studies, investigating DNA- or histone modifying small molecules for cancer therapy (reviewed in Brown et al. 2014; reviewed in Yoo & Jones 2006). Tumor therapy with demethylating agents promote re-sensitization against apoptosis-inducing drugs in some cancer types (Al-Romaih et al. 2008; Fulda et al. 2001; Steinhart et al. 2013). Taken together, this study with melanoma-iPCCs extends the current knowledge of epigenetic modifications on therapy success.

According to our results, the activity of cancer-specific pathways depends on particular epigenetic states. Therefore, targeted monotherapy might be susceptible to fail in epigenetically dynamic cells similar as reprogramming-induced resistance might be exclusive for targeted therapy but not to systemic working drugs.

In sum, epigenetic modifications of tumor cells significantly influence their drug response leading to two conclusions. First, a high epigenetic plasticity in tumor cells might prevent an effective therapy explaining the high relapse rate of many cancer types. Second, a dual strategy considering the potential of differentiation therapies together with targeted therapy might reveal a powerful treatment option (Sung & Waxman 2007).

Furthermore, inhibition of MAPK signaling did not affect the integrity of iPCC colonies neither their pluripotent state indicated by AP expression. In human embryos MEK inhibition does not prevent epiblast or hypoblast formation (Kuijk et al. 2012; Roode et al. 2012) but favors ground state pluripotency in mice (Nichols et al. 2009). Additionally, simultaneous MAPK and GSK3β inhibition enhances reprogramming efficiency and efficacy (Lin et al. 2009). This is of interest as MAPK activity is required to maintain self-renewing pluripotent epiblast-like PSCs while its inhibition leads to rapid differentiation and cell death (Greber et al. 2010; Li et al. 2007). This controversy might be explained by the observation that epiblast-like iPSCs, reprogrammed by the expression of Nanog in addition to the Yamanaka factors, can be converted into mouse-like cells by a combination of LIF supplementation and MEK inhibition (Buecker et al. 2010). Furthermore, the isolation of human naïve ESCs as well as switching epiblast-like PSCs into a more naïve pluripotent state requires the supplementation by small molecules blocking MEK (Gafni et al. 2013; Takashima et al. 2014; Theunissen et al. 2014; Ware et al. 2014). These data suggest that reprogrammed tumor cells in the alternative state resemble a pluripotent state distinct from the epiblast-like bFGF-dependent phenotype. More likely, the alternative pluripotent state of the iPCCs reminds of murine the ESC-like state previously described

(Buecker et al. 2010; Hanna et al. 2010b) although significant differences in the cellular phenotypes and their requirements separate the studies. In contrast to these investigations the iPCCs did not require exogenous Nanog to adopt an alternative pluripotent state. It is likely that high endogenous reactivation of Nanog in the iPCCs circumvent the need for exogenous supplementation. In sum, the presented data provide for the first time evidence, that tumor cells are amenable to adopt alternative states of pluripotency. Besides, there is evidence that the development of resistance mechanisms against targeted signal inhibition is associated with dedifferentiation resulting in chromatin alterations (Sharma et al. 2010). Therefore nuclear factor-based reprogramming might represent an interesting tool to investigate epigenetic-mediated drug resistances.

X.2.4. Tumorigenicity as a consequence of epigenetic and genetic alterations

Characterizing the molecular signature of melanoma cells highlighted their phenotypic plasticity, switching between proliferating and invading, tumorigenic and non-tumorigenic phenotypes. Here, rapid tumor formation of the parental melanoma cell line HT-144 *in vivo*, teratoma-like formation of iPCCs and reduced tumorigenicity of iPCC-derived fibroblast-like cells were observed. In accordance with this phenotypic data, a deregulation of tumor suppressor genes and oncogenes compared to the parental tumor cell lines was found (figure 14&15). Previous studies demonstrated that ectopic expression of one or more Yamanaka factors is sufficient to dedifferentiate cancer cells, leading to reactivation of embryonic transcription factors. Forced dedifferentiation of a variety of tumor types by single pluripotency factors has been associated by an increased tumor-initiating potential and the acquisition of a stem cell-like phenotype (Chiou et al. 2010; Jeter et al. 2011; Leis et al. 2012; Santini et al. 2014b; Siu et al. 2013). Ectopic transcription factor expression represses anoikis and consequently increases tumorsphere formation (Leis et al. 2012). Additionally, forced expression of Oct4 or Sox2 in melanoma induces up-regulation of markers found in melanoma-initiating cells (Kumar et al. 2012; Santini et al. 2014b). These data demonstrate that the expression of single reprogramming factors enhances the tumorigenic phenotype of melanoma cells by a partial dedifferentiation. In contrast, complete reprogramming using a suitable mixture of pluripotency factors results in a widespread epigenetic remodeling process influencing the tumorigenic potential of cells. So far, the interplay of global epigenetic reconfigurations with a malignant phenotype is poorly investigated. Reversion of hypermethylated promoter regions of tumor suppressors as a consequence of reprogramming was first observed in immortalized fibroblasts (Ron-Bigger et al. 2010). In accordance, nuclear reprogramming of cells derived from several different tumor types showed that cancer-associated aberrant methylation sites are reverted upon successful nuclear reprogramming (Mahalingam et al. 2012; Stricker et al. 2013; Zhang et al. 2013). Tumor-associated

hypermethylated promoter regions comprise tumor suppressor genes and genes involved in development while oncogenes become hypomethylated. Abolishment of this cancer-specific methylome induced by global epigenetic modifications taking placing during reprogramming results in enhanced expression levels of tumor suppressors and in decreased levels of oncogenes (Mahalingam et al. 2012; Zhang et al. 2013). This seems to be contradictory to the studies demonstrating that iPSCs retain an epigenetic memory affecting the conversion into pluripotent cells as well as their differentiation potential (Chin et al. 2009; Polo et al. 2010). Therefore, incomplete reversion of methylation marks due to an epigenetic memory might be responsible for a tumorigenic phenotype. During the differentiations of stem cells the epigenetic memory favors the differentiation back into the lineage of their origin raising the question whether in iPCCs the tumorigenic methylome is reestablished with the loss of pluripotency. Against this idea argues the resetting of the epigenetic profile of sarcoma cells by reprogramming, which demonstrated that even partly reprogrammed cells regained the potential to execute differentiation pathways thus restoring tumor suppressor genes and silencing oncogenes. Accordingly, the induction of pluripotency enabled their terminal differentiation into osteogenic and adipogenic cells, thereby abrogating their tumorigenic potential (Zhang et al. 2013). These data encourage the hypothesis that cancer arise from deregulated epigenetic regulations causing abnormal growth (Ohnishi et al. 2014). In contrast, the malignant phenotype of glioblastoma-initiating cells is epigenetically imprinted but restricted to the neuronal lineage. Derived non-neuronal cells up-regulated expression of tumor suppressor genes resulting in the loss of tumorigenic behavior (Stricker et al. 2013). Nevertheless, it might be that observed differences are due to additionally acquired mutations reactivated in the appropriate epigenetic context. Similarly, BCR-ABL positive leukemia-iPCCs depend on the BCR-ABL signaling when differentiated into blood cells but lose their oncogene addiction in other cell types. Collectively, the data show that the tumor-initiating potential of a cell is epigenetically determined and therefore revertible. Erasing these marks either allows to execute novel differentiation pathways or to restore the potential to generate terminally differentiated cells without generating tumors. Development of a viable chimeric mouse derived from R545 melanoma cells supports the idea that a melanoma genome is able to adopt the cell fate of various functional cells without generating cancer (Utikal et al. 2009a). However, melanoma-iPCCs were not differentiated back into their melanocytic lineage. Therefore, the tumorigenic potential of iPCCs-derived melanocytic cells remains to be investigated.

Here, equal numbers of tumor suppressor genes were up- and down-regulated in melanoma-derived iPCCs, fibroblast-like and neuronal-like cells (figure 14A&15A). The same holds true for oncogenes. Furthermore, only a low number of tumor suppressor genes and oncogenes was commonly deregulated. This might indicate that individual tumor suppressor genes but also oncogenes are regulated in a cell type-specific manner. Only BIN1 was found to be

commonly up-regulated in all HT-144-derived cell types. BIN1 is the most important risk locus for Alzheimer's disease (Tan et al. 2013). It is a c-Myc-interacting adaptor protein and plays an important role as tumor suppressor in several cancer types (Cassimere et al. 2009; Ge et al. 2000; McKenna et al. 2012; Tajiri et al. 2003). Noteworthy, BIN1 is alternatively spliced in melanoma, resulting in a protein that is unable to suppress malignant transformation, thereby promoting melanomagenesis.

Similarly, only three oncogenes, MITF, GAS7 and NBL1 were commonly down-regulated in HT-144-derived iPCCs, fibroblast- and neuronal-like differentiated cells.

GAS7 is predominantely expressed in terminally differentiated brain cells and required for bone differentiation of mesenchymal stem cells (Chao et al. 2013; Hung et al. 2011). Furthermore, GAS7 plays a putative role as oncogene in childhood CNS tumors (Ebinger et al. 2006). Surprisingly, recent data from clinical trials demonstrated that melanoma patients showing tumor regression after receiving adoptively transferred autologous tumor-infiltrating lymphocytes had a common T cell subpopulation recognizing mutated GAS7 protein (Robbins et al. 2013; Zhou et al. 2005). Together, these data indicate GAS7 might represent an interesting target for melanoma therapy.

NBL1 is a relatively undefined gene and member of the DAN superfamily of BMP antagonists (Hung et al. 2012). Although NBL1 seems to be involved in prostate and pancreatic cancer (Hayashi et al. 2013; Olakowski et al. 2009), there is no study focusing on the role of NBL1 in human melanoma so far.

As previously described, MITF is a key molecule of melanogenesis and plays an important role in melanoma cells for transformation, cell invasion, cell survival and proliferation (VI.2.9). Microarray and qPCR data demonstrated significant down-regulation of MITF in iPCCs compared to the parental melanoma cells in a panel of cell lines (figure 7). Furthermore, MITF was also deregulated in HT-144-derived differentiations (figure 12). Taken together, it is coherent, that MITF appears as one of the most down-regulated oncogenes. However, it was demonstrated that melanoma cells like Ma-1 exist with a less active MITF signaling. Although previous data provided evidence that MITF inhibition might result in melanoma suppression (Yokoyama et al. 2008), it is questionable whether melanoma cells like Ma-1 would be affected.

The molecular analysis of human tumors reveals a growing amount of data demonstrating interconnections of tumor heterogeneity and its relevance for therapeutic treatment options. The basis of these intra-tumorigenic differences is encoded by genetic and epigenetic alterations. Here, the technique of nuclear reprogramming was applied on human tumors cells as a tool to induce epigenetic modifications and to investigate their impact on cellular phenotypes. The investigation demonstrated for the first time the successful reprogramming of solid human tumor cells into an early embryonic state sharing characteristics with murine ESCs allowing the differentiation into several lineages like mesoderm-derived fibroblast-like cells and

ectoderm-derived neuronal-like cells. Together with the endodermal *in vivo* differentiations it has been demonstrated that reprogrammed tumor cells are able to differentiate into all three germ lines, the hallmark of pluripotency.

Reprogramming resets cancer-specific DNA methylation marks allowing the redistribution of epigenetic modifications leading to non-tumorigenic cells after differentiation. Furthermore, the generation of iPCCs is depending on the reactivation of the same transcription factor network like somatic cells. Taken together, the study provides another piece in the puzzle of reprogramming and a novel tool to investigate phenotypic switches of tumor cells. In addition to that, it was shown that epigenetic modifications might conceal a tumorigenic genome harboring driver mutations of melanomagenesis.

Although the model presented here is artificial and cannot be directly translated into a clinical situation it highlights the problem of epigenetic-altered tumor cells, resistant against targeted therapy albeit being carrier of malignant mutations.

For the first time, it was shown that human melanoma cells can be converted into a metastable pluripotent state by consistent overexpression of the reprogramming factors acquiring a mouse ESC-like state. Chromatin modifications induced by reprogramming and subsequent differentiation provided resistance against targeted melanoma therapies in melanoma-derived cells of varying differentiation states. Furthermore, it was demonstrated that nuclear reprogramming in combination with directed differentiation is a powerful tool to identify reversibly regulated tumor suppressor genes for potential reactivation as anti-tumor strategies.

XI. References

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