`

Investigation of a novel experimental therapeutic involving transcriptional reprogramming of invasive cancer cells with "stem-cell like" characteristics

-

Henry Guanghao Yu, MSc Candidate

-Department of Experimental Medicine

McGill University, Montreal

April 2015

Abstract

Metastatic breast cancer is currently an incurable disease with no "gold standard" therapy. There is mounting evidence supporting that a primary tumors contain subpopulations of stem-like cancer cells, expressing stem cell markers and gene signatures. These cell variants have been hypothesized to drive metastatic progression due to their higher plasticity and invasive capacity. The aim of this work is to explore the therapeutic potential of small molecules interfering with stem cell signaling to reprogram stem-like cancer cells into non-stemness.

The thesis is organized into two chapters: Chapter 1 addresses a review on cancer stem cell hypothesis and small molecule-induced cell reprograming. For the thesis, chapter 1 is also intended serve as general background.

Chapter 2 is a research paper exploring the anti-metastatic potential of SLLN06, a novel small molecule multi-kinase inhibitor of Aurora A, Aurora B, Jak2, and Ret kinases. This molecule was identified through phenotypic screening of a chemical library synthesized in my host laboratory based on the compound capacity to reverse the expression status of stem cell markers implicated in breast cancer stem cells, namely $CD44^{high}/CD24^{low}/ALDH^{high}$. SLLN06 at nM range was able to reprogram stem-like cancer cells to lose their stem-cell characteristics, including a shift from $CD44^{high}/CD24^{low}/ALDH^{high}$ to $CD44^{high}/CD24^{high}/ALDH^{low}$ phenotype, as well as inhibition of the cells' capacity to form mammospheres. SLLN06 also prevented metastasis formation induced in vivo by stem-like cancer cells. These results lay the foundation for further investigation of reprogramming mechanisms for this class of molecules.

Résumé

Le cancer du sein avancé ou métastatique demeure une maladie incurable avec les modalités de traitements actuels. La littérature montre qu'une sous-population de cellules cancéreuses, ressemblant aux cellules souches, sont enrichies dans les types de cancers agressifs. Ces variantes de cellules peuvent jouer le rôle de cellules précurseurs pour la formation de métastases. Le but de cette étude est d'explorer le potentiel thérapeutique de molécules chimiques pour reprogrammer ce type de cellules vers des formes non-invasives.

Cette thèse organisée en deux chapitres: Le premier est une revue des connaissances scientifiques actuelles dans le domaine de cellules souches et leurs programmation dans le context des maladies cancéreuses. Le deuxième chapitre résume mon travail de recherche consacré aux études des mecanismes d'action et de l'activité anti-métastatique de SLLN06, une nouvelle molécule qui inhibe les kinases Aurora A/B, Jak2 et Ret et induit une reprogrammation des cellules cancéreuses deriveés de cancer de sein et ayant des charactéristiques de cellules souches. En particulier, SLLN06 est capable d'induire une transition de ces cellules du phenotype CD44+/CD24-/ALDH1+ vers un phenotype CD44+/CD24+/ALDH1-. Enfin, nous avons demontré que ce SLLN06 réduit l'incidence de métastases chez les animaux de laboratoires. Ces résultats ouvrent la voie à des études plus approfondie pour mieux comprendre les implications des mécanismes de reprogrammation des cellules cancéreuses.

Table of Contents

Abstract **2**

Résumé **3**

Table of Contents **4**

List of Figures and Tables **6**

List of Abbreviations **7**

Acknowledgments **10**

Chapter 1: The cancer stem cell concept and its potential implications for cancer therapeutics **11**

1.1 Abstract **12**

1.2. Cell differentiation programming: a process essential for development and maintenance of tissue homeostasis **12**

1.2.1 Transcriptional programming: A signaling network essential for the regulation of cell pluripotency and self-renewal 14

1.2.2 Cooperative intrinsic and extrinsic signals direct cell differentiation 16

1.3 Transcriptional reprograming as a potential cancer therapeutics **17**

1.3.1 Evidence of a tumor as an entity with tissue-like cellular hierarchy 17

1.3.2 Cancer cell transdifferentiation to acquire invasive phenotype 21

1.4 The differentiation program is a multi-level integration of cellular signaling that can be amenable for modulation by cellular factors and small molecules **25**

1.4.1 Transcription factors 27

1.4.2 Epigenetics 29

1.4.3 Cytokine and growth factor receptor signaling 33

1.4.4 MicroRNA network 36

1.4.5 Metabolic reprogramming 38
1.5 Conclusion 40

Chapter 2: Identification of a small molecule kinase inhibitor selectively targeting cancer cells expressing stem-cell markers 43
2.1 Abstract 45
2.2 Introduction 45
2.3 Results 47
2.3.1 SLLN06 reduces the expression of cancer stem cell markers 47
2.3.2 SLLN06 is *not* enriching non stem-like cells through selective cytotoxicity 49
2.3.3 SLLN06 inhibit *in vitro* organoid differentiation and tumorsphere formation 50
2.3.4 High-throughput kinase assay identifies potential targets of SLLN06 51
2.3.5 SLLN06 has distinct or superior effect compared to other molecules reported to target cancer stem cell or cell differentiation signaling 51
2.3.6 SLLN06 induces polyploidy 52
2.3.7 SLLN06 reduces *in vivo* distant metastasis and primary tumor growth of orthotopically transplanted tumor cells 53
2.4 Discussion 72
2.5 Materials and methods 76

Summary and Discussion 82

Supplemental data 86

Chapter 1 References 91

Chapter 2 References 104

List of Figures and Tables

Chapter 1

Figure 1. Stem cell differentiation and dedifferentiation ……..……15

Figure 2. Cancer cell transdifferentiation ……..……..22

Table 1. Small molecule modulators of cell differentiation features ...…….……..26

Chapter 2

Figure 1. SLLN06 reduces the % of CD24-/CD44+breast cancer cells ……..……...……..55

Figure 2. SLLN06 impacts on the expression profile of stem cell markers ……..……57

Figure 3. SLLN06 inhibits cell proliferation via cytostatic effects ……...…….…..60

Figure 4. SLLN06 offers no proliferative advantage between stem-like and non-stem like cancer cells……...…62

Figure 5. SLLN06 inhibits spheres formation ……64

Figure 6. SLLN06 is a multikinase inhibitor ……..……66

Figure 7. SLLN06 induces polyploidy ……68

Figure 8. SLLN06 inhibits cancer metastasis *in vivo* ……..71

Table 1. SLLN06's impact on stem cell markers in alternative cancer models ……..……58

Table 2. SLLN06 has a unique feature compared to commercially available kinase inhibitors ……...…….…….……69

Supplemental data

Figure S1: Quantification of E-cadherin and N-cadherin by flow cytometry ….……...…….. 86

Figure S2: EMT in PMC42-LA ……..…….....87

Figure S3: Impact of SLLN06 on EMT markers ……88

Figure S4: Wound healing assay in MDA-MB-231 cells ……..……..……..……...…89

Figure S5: qPCR and FACS analysis of CD24 using SLLN06-treated SUM-149 cells ……90

List of Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
ALDH	Aldehyde dehydrogenase
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APL	Acute promyelocytic leukemia
ATP	Adenosine Triphosphate
ATRA	All-trans retinoic acid
Bmi-1	B cell-specific Moloney murine leukemia virus integration site 1
CD	Cluster of differentiation
C-myc	Avian myelocytomatosis virus oncogene cellular homolog
CSC	Cancer stem cells
DNA	Deoxyribonudeic acid
DNMT	DNA methyltransferase
EMT	Epithelial mesenchyal transition
ER	Estrogen receptor
ESc	Embryonic stem cell
EZH2	Enhancer of zeste homologue 2
FAK	Focal adhesion kinase
Gsk3	Glycogen synthase kinase-3
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HGFR	Hepatocyte growth factor receptor
HIF	Hypoxia inducible factor

IL	Interleukin
iPSC	Induced pluripotent stem cell
Lox	Lysyl oxidase
Klf4	Kruppel-like factor 4
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal epithelial transition
MiR	MicroRNA
MRP	Multidrug resistance protein
MyoD	Myogenic determination factor
NMDA	N-methyl-D-aspartate
NMMII	Nonmuscle Myosin II
Oct4	Octamer-binding transcription factor 4
OLIG2	Oligodendrocyte transcription factor 2
PcG	Polycomb-group
PPARGC1A	Peroxisome proliferator-activated receptor-γ co-activator 1α
PPAR-γ	Peroxisome proliferator-activated receptor-γ
ROCK	Rho-associated, coiled-coil-containing protein kinase
SALL2	Spalt-like transcription factor 2
siRNA	Small interfering RNA
Smad	Contraction of Sma and Mad
Sox2	Sry-box transcription factors 2
Stat3	Signal transducer and activator of transcription 3
TF	Transcription Factor

TGF	Transforming growth factor
TMA	Tissue microarray
TMZ	Temozolomide
Zeb1	Zinc finger E-box-binding homeobox 1

Acknowledgments

I would like to foremost offer my deepest gratitude to my supervisor Dr. Moulay Alaoui-Jamali for inspiring me to undertake an exhilarating scientific journey. His constant support, patience, and guidance throughout my project gave me the space to improve in science and in life. His breadth of knowledge and vision drives me to always expand my scope and fuels my passion for science.

I would also like to thank my co-supervisor Dr. Krikor Bijian for being a constant source of mental support, for assisting me with experimental design, and for maintaining an impeccable work environment for my colleagues and me.

I would also like to thank my academic advisor Dr. Lorraine Chalifour and my thesis committee members Dr. Jianhui Wu and Dr. Rongtuan Lin for offering me timeless advice and encouragement along the way.

Also innumerable thanks to all my current and former lab mates, YingJie, Xu Bin, Jasper, Sabrina, Amine, DingHong, Su Jie, HuiJie, Grégoire, YuanHang, Hind, Maisa, and Dominik for accompanying me through my journey and gifting me courage and motivation. The *in vivo* studies for my project was the work of Dr.Su Jie, and the live cell locomotion assay was conducted by Dr.Xu Bin.

Special thanks to the Quebec Breast Cancer Foundation for their support to this work.

Finally, I would like to thank my parents for blessing me with years of unconditional love and support, without whom I would never be the happy person I am today.

Chapter 1: The cancer stem cell concept and its potential implications for cancer therapeutics

1.1 Abstract

Cell differentiation is a change of state where the cell adopts new, more specialized properties essential for normal physiological functions. Cells within an organism are hierarchically organized by their capacity to differentiate from pluripotent to unipotent cells. This process is determined by cooperation of transcription and epigenetic factors that dictate lineage-definitive genes and cell fate. In pathological conditions and particularly cancer, aberrant differentiation programs are common in advanced cancer with poorly differentiated features often predictive of poor prognosis. Mounting evidence supports the presence of precursor cancer cells capable of recapitulating the hierarchy of somatic cells; these cells are referred to as cancer "stem" or "stem-like" cells based on the expression of many characteristics reminiscent of normal stem cells. Landmark studies have shown that the cell differentiation status is amenable to experimental modulation, both in normal and cancer contexts, and this approach has open-up exciting opportunities for discovering novel differentiation and de-differentiation therapies. In this background section, I am providing a comprehensive overview of molecular circuitries that regulate cell differentiation/reprogramming, their implication to cancer development and progression, and the potential of targeting the differentiation pathway as a therapeutic approach.

1.2. Cell differentiation programming: a process essential for development and maintenance of tissue homeostasis

Complex mammalian organisms develop from a single zygote. From a single cell, an entire body of interacting specialized cells can be generated. In the context of organ development, the differentiation program occurs in sequential stages, from "totipotent" zygote, to "pluripotent" embryonic cells, to terminally differentiated somatic cells. A totipotent cell is defined as a cell which has the potential to differentiate into any and all cells within an organism. A pluripotent cell is defined as a cell with the potential to differentiate into any of the three germ layers: the ectoderm, endoderm, and mesoderm. This potency continuum extends to multipotent cells with limited differentiation potential, finally to unipotent cells with no differentiation potential. This hierarchy of differentiation potential is maintained throughout adulthood to maintain tissue specificity and homeostasis such as the neural network [1], muscle fiber [2] and mesenchyme[3].

Upon fertilization, the fusion of the male and female gamete generates a totipotent zygote. The zygote harbors all the genetic information necessary to reconstruct an entire organism, but it is dependent on exogenous maternal factors to initiate early cell division to form a blastocyst[4]. Cells of the blastocyst diverge into the pluripotent embryoblast (also known as inner cell mass) that forms the fetus, and the non-pluripotent trophoblast that forms the placenta. Cells within the inner cell mass (ICM) are stimulated by growth factors to form a heterogeneous "salt and pepper" population of cells that will develop into any of the primary germ layers[5]. These early stages of development highlight a key feature of the differentiation paradigm where cells possess inherent signaling network to promote morphological changes that are tightly under the control of specific environmental factors.

Our understanding of the cell differentiation is largely derived from studying two types of stem cells: the embryonic stem cells and the somatic stem cells. Pluripotent embryonic stem cells derived from the inner mass of a blastocyst during early development can be cultured and maintained in a pluripotent state *in vitro*. On the other hand, multipotent somatic stem cells, which include hematopoietic stem cells, intestinal stem cells, neural stem cells and mesenchymal stem, reside within their specific organs to serve as progenitor cells for tissue regeneration [6]. One major aspect of stem cell biology is establishing efficient method for reprogramming somatic stem cells to a fully pluripotent state, termed "induced pluripotent stem cells" (iPSC)[7].

IPSCs maintain "epigenetic memory" of their previous lineage [8], where some of their DNA and chromatin modification patterns more closely resemble their pre-reprogrammed state, and when allowed to differentiate, iPSCs would preferentially revert to their original lineage. Moreover, environmental cues such as growth factors can supersede inherent lineage-bias [9]. Taken together, these findings depicted a model of tissue development that is dynamically regulated at two levels.

In the first, pluripotent stem cells are primed by their epigenetic state to preferentially follow one of many internal differentiation program while in the second, environmental signals contribute to ensure that differentiation pattern occurs in a spatially and temporally specific manner.

Many lines of evidence suggest that tissue-specific somatic stem cells reside within a niche compartment where they must carefully balance self-renewal and differentiation to maintain tissue homeostasis [10]. This balance is tightly controlled by intrinsic molecular pathways and microenvironment signals [11]. However, while many major pathways controlling stem cell activity has been identified, ongoing efforts tackle the daunting task of pinpointing precise molecular cause-and-effect of an increasing number of factors in lineage specification. Early developmental program is internally regulated by "fate-determining" networks consists of multiple antagonistic pathways that either activate or repress lineage-specific transcription factors, which induces global gene pattern changes through epigenetic remodeling [12]. These complex molecular interactions are slowly being unraveled as we gain the tools and knowledge to experimentally modulate differentiation pathways.

1.2.1 Transcriptional programming: A signaling network essential for the regulation of cell pluripotency and self-renewal

Historically, the earliest proofs that mammalian cells can be experimentally reprogramed was in 1987, when the Davis group showed that embryonic fibroblasts can be differentiated into myoblasts by viral transfection of the transcription factor MyoD1 [13]. On the other hand, experimentally-induced *de*-differentiation proved to be a much more challenging task, but was accomplished only in 2006 when the Yamanaka group reprogrammed mouse fibroblasts into an embryonic stem cell-like state by viral transfection of four transcription factors: Oct4, Sox2, c-

Myc, and Klf4 [7]. Later, a similar approach was used to reprogram human somatic stem cells using a slightly different TF combination of Oct4, Sox2, Nanog, and Lin28 [14]. These transgenic factors are stem cell regulatory elements that each play a key role in maintaining stem cell pluripotency and self-renewal [15]. Moreover, studies have shown that the transcription factor Nanog serves as a gatekeeper of cellular reprograming towards a pluripotent state, and is activated later than Oct4/Sox2/Klf4 during somatic reprograming by enabling the transition from a partially reprogramed intermediates to a complete pluripotent state (Summarized in Figure 1)[16].

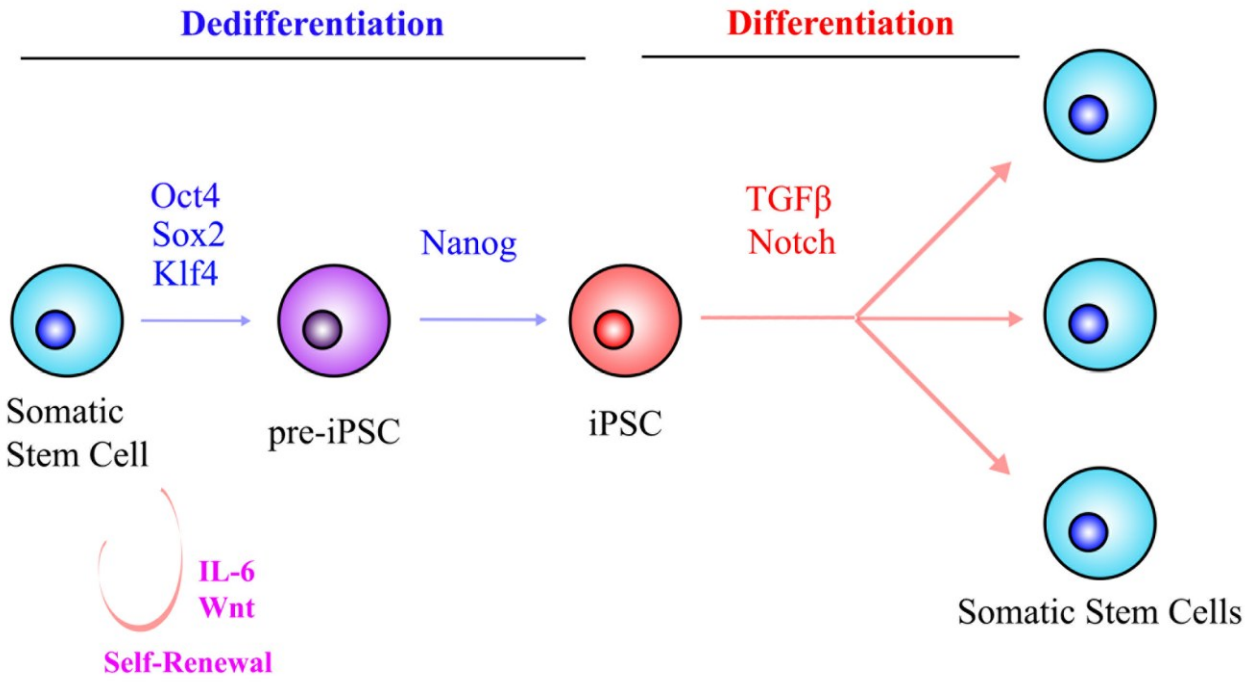


Figure 1: A simplified schematic of key factors regulating stem cell differentiation, dedifferentiation, and self-renewal.

1.2.2 Cooperative intrinsic and extrinsic signals direct cell differentiation

Integration of soluble factor signaling is essential for proper development of multiple tissues [17] [18] through signaling network that's highly conserved across all vertebrates [19] [20]. Integration of environmental cues from key signaling pathways, including receptor tyrosine kinases TGFß, Wnt, integrin, Hedgehog and Notch receptors, form the basis for controlling tissue differentiation patterns through cell proliferation, migration, differentiation and death. During the early stages of embryogenesis, fibroblast growth factors (FGF), epidermal growth factors (EGF), vascular endothelial growth factors (VEGF) and other differentiation factors orchestrate the developmental processes of gastrulation, vasculogenesis, limb development, neural patterning and placentation through receptor tyrosine kinase (RTK) signaling [21]. Spatially and temporally controlled activation of RTK regulates proper proliferation patterns through downstream activation of RAS-MAPK-ERK axis [22]. Activation of RTK signaling is required for lineage segregation of epiblastic and hypoblastic cells within the inner cell mass [23].

The cell differentiation program is highly conserved. As the cell acquires new differentiation features during development, their molecular wiring can adapt to respond differently to selective environmental stimuli, enabling the sequential construct of complex organs from relatively few directing factors. Moreover, there is redundancy within differentiation signaling. For instance, the presence of leukemia inhibitory factor (LIF) is sufficient to maintain ES pluripotency through activation of JAK/STAT signaling [24] but can also be replaced by other growth factor[25]. This indicates that the complex molecular circuitries that enable orchestrated differentiation patterns ultimately converge towards a few master regulators, and suggest that experimental modulation of cell differentiation can distill much of the complexity by targeting key nodes.

1.3 Transcriptional reprograming as a potential cancer therapeutics

Despite recent advances in cancer treatment, most types of metastatic cancer are currently incurable. Traditional cytotoxic chemotherapy is insufficient for eradicating advanced cancer due to the lack of selectivity and frequent relapses. The introduction of targeted agents, given alone or in combination with chemotherapy, greatly improved overall survival in patients with advanced cancer but their efficacy, like chemotherapy, has been limited by response heterogeneity and frequent recurrences. Cell differentiation and dedifferentiation are fundamental biological programs essential for growth, development, and maintenance of all multicellular organisms. Mounting evidence support that cellular differentiation is highly plastic and susceptible to experimental modulation. This paves the way for an alternative strategy of cancer therapy where the goal is subverting cancer cells towards a non-malignant state, thereby bypassing pro-survival adaptations. An example of a classical differentiation therapy is all-trans retinoic acid (ATRA), which has been used to treat acute promyelocytic leukemia (APL) by forcibly promoting terminal differentiation of cancer cells[26]. Since then, great progress has been made in understanding the regulatory networks that govern differentiation of solid tumors, opening-up opportunities for the discovery of innovative therapeutic approaches. In the next section, I am reviewing the relevance of cancer differentiation program to cancer progression to metastasis.

1.3.1 Evidence of a tumor as an entity with tissue-like cellular hierarchy

Studies over the past decade have provided strong support for the cancer stem cell hypothesis, suggesting that both tumor progression and tumor recurrence are driven by a minor subpopulation of progenitor cells expressing stem cell-like characteristics. The cancer stem cell

hypothesis stipulate an alternative to the classical "clonal evolution" model of cancer development which suggest that tumor cells thrive through constant selective pressure to gain growth advantage [27]. Under the clonal evolution model, all cancer cells are equipotent to an extent, and tumor heterogeneity is driven by a high proliferation and mutation rate [27]. The cancer stem cell hypothesis was initially based on observation in leukemia that cancer cells are hierarchically organized similarly to hematopoietic cells [28]. From this observation it was proposed that perhaps a minor subset of cancer cells is driving tumorigenesis in the same way that somatic stem cells renews tissue. Under this model, cancer heterogeneity would be established by the pluripotent nature of those cancer stem cells[29]. Moreover, it is now apparent that conversion between stem and non-stem cells is a bidirectional process, thus allowing for *de novo* generation of cancer stem cells from non-stem transformed cells [30]. In recent years, multiple studies have reconciled cancer stem cells and clonal evolution since evidence show that these "progenitor" cancer cell themselves are susceptible to genetic and epigenetic evolution over time[31]. Nonetheless, the significance, characteristics, and even the existence of cancer stem cells remain a highly controversial topic.

Key experiments have shown that solid tumors share similar hierarchical structure as normal tissues. Lineage tracing studies in papilloma and squamous cell carcinoma (SCC) showed that tumor growth is sustained by a rare population that expanded and differentiated following a stochastic pattern of fate [32], reflecting the same differentiation pattern of normal epidermis [33]. Notably, the study showed that at a certain point, the SCC differentiating pattern led to the generation of a minority of mesenchymal variants, a pattern that was not observed in the benign papilloma[32]. Furthermore, the aggressive SCC contained few cells that have undergone terminal differentiation compared to benign papilloma [32]. In brain cancer, following treatment with the drug

Temozolomide, lineage tracing identified the origin of tumor reoccurrence to be a quiescent subpopulation of endogenous glioma cells resembling neuronal stem cells [34].

Cancer stem cells were originally identified by the expression of surface stem cell markers. When these cells were sorted by these surface markers, one group displayed significantly greater self-renewal and tumorigenicity in xenograft studies [35]. Using this method, putative cancer stem cells was identified and isolated in a wide range of cancers including breast [36], brain [37], pancreatic [38], small intestine and colon [39], and prostate [40]. While these studies have clearly demonstrated that cancer cells are highly diverse in their tumorigenic potential, the validity of the environmental factors seen in humans. Moreover, the reliability of cancer stem cell markers for highlighting tumorigenic cells is highly contested [41]. Nevertheless, there are several lines of evidence that show that the differentiation status of cancer cells is an important determinant of their capacity for self-renewal. Aggressive, poorly differentiated cancer subtypes often overexpress genes involved in embryonic stem cell signaling such as Nanog, Oct4, Sox2 and c-Myc [42]. As well, cell subpopulations expressing stem cell markers possess enhanced self-renewal, which is an inherent property of stem cells allowing them to maintain persistent dedifferentiated status. Self-renewal can be distinguished from somatic cell proliferation by asymmetric cell division to maintain the core stem cell number and lack of senescence. These properties are believed to derive from conserved signaling programs that regulate mammalian development [43]. It is therefore reasonable to speculate that to reprogram cancer cells toward non-malignant or non-invasive cells, manipulating the highly-plastic progenitor cells is a logical approach. Though cancer stem cells do not necessarily derive from normal stem cells, there are many parallels functions and properties between stem cells and cancer stem cells [44]. The glycoprotein CD133 is a stem cell marker found on hematopoetic stem cells [45], endothelial stem cells [46], and neural stem cells [47]. The function of

CD133 is not fully understood [48], but its prominence as a stem cell marker implies a vital role in stem cell biology. In the context of cancer, CD133 is an important prognostic factor for glioma patients, and the proportion of $CD133^+$ cells correlates with tumor relapse and the rate of tumor progression[49]. Both brain and colon cancer-initiating cells can be identified by the expression of CD133 marker [37]. In glioblastoma, $CD133^+$ cells -express high levels of neural precursor genes and resistance to chemotherapy [50]. In lung cancer, $CD133^+$ cells isolated from patient tissues displayed higher levels of the pluripotent marker Oct-4 and the multidrug resistance marker ABCG2; knockdown of Oct-4 in $CD133^+$ lung cancer cells ablated their regenerative capacity [51]. In addition to sharing common markers, stem-cells and experimentally isolated cancer stem cells can thrive under similar culture conditions, including a preference for fibroblast growth factor (FGF) [52].

Modern cancer therapy is constantly struggling with the inherent adaptability of cancer cells to develop eventual resistance to treatment. Enhanced drug resistance is a commonly suggested characteristic of cancer stem cells, because residual cells that remain after extended drug treatment often become enriched in cancer stem cell markers. In general, their mechanisms of drug resistance include high expressions of ATP-binding cassette (ABCG) transporter protein[53], their high level of aldehyde dehydrogenase (ALDH) activity[54], expression of anti-apoptotic factors, enhanced DNA damage repair, loss of death receptors [55], and activation of pro-survival signaling[56]. Importantly, many of these represent hallmarks of normal stem cells[57]. For instance, the expression of ABCG2 can identify stem cells within the adult heart [58], liver [59], and cornea [60], whereas ALDH activity can identify neural stem cells [61], colonic stem cells [62], and mammary stem cells [63]. As such, targeted therapies against cancer stem cells have potentially higher negative impact on the function of normal somatic stem cells.

1.3.2 Cancer cell transdifferentiation to acquire invasive phenotype

The metastatic potential of a cancer cell is dependent on two major factors – the capacity to invade neighboring tissues and the capacity to survive circulation and re-establish secondary colonies. A key cellular program called epithelial mesenchymal transition (EMT), and its reverse process (MET), are believed to be important enablers of cancer cell invasion. Cells undergoing EMT loses cell junction proteins, loses apical-basal polarity and adopt a skeletal shape that enhances cell mobility [64]. The epithelial mesenchymal transition program occurs in three distinct settings: during early development stages, during tissue or organ repair, and during cancer progression [65], and in all three cases, EMT activation also is believed to be integrated with cell differentiation.

The dynamic switch between epithelial and mesenchymal state through EMT/MET is an overt physiological example of somatic reprograming. The EMT program can be activated through an increasing list of signaling pathways including βcatenin, PDGF, Ras, and TGFβ, and the activation of EMT in turn regulate pluripotent gene regulatory factors [66]. Therefore, there are many overlapping mechanisms that enable EMT while also governing cell differentiation. Indeed, a landmark study demonstrated that the induction of EMT can enrich for CSC characteristics, including expression of $CD44^{+}/CD24^{low}$ cell surface markers and the ability to form mammospheres [43]. This phenomenon was further demonstrated *in vivo* where EMT induced by immune cells also enriched tumor in cancer cells expressing stem cell markers[67]. Notably, the same CSC markers, such as CD44 and CD133, are frequently associated with cancer stem cells from multiple origins, which further advances the notion that cancer stem cells could be generated from dedifferentiation. For instance, $CD44^{+}$ prostate cancer cells are highly enriched in metastatic and

stem-like characteristics, including a higher mRNA level of progenitor-associated genes and the capacity for differentiation or asymmetric division, relative to their $CD44^-$ counterparts [68]. Furthermore, $CD44^+$ prostate cancer cells are exclusively capable of undergoing EMT to invade Matrigel membranes, concurrent with an acquisition of a cancer stem-like genomic profile.[69].

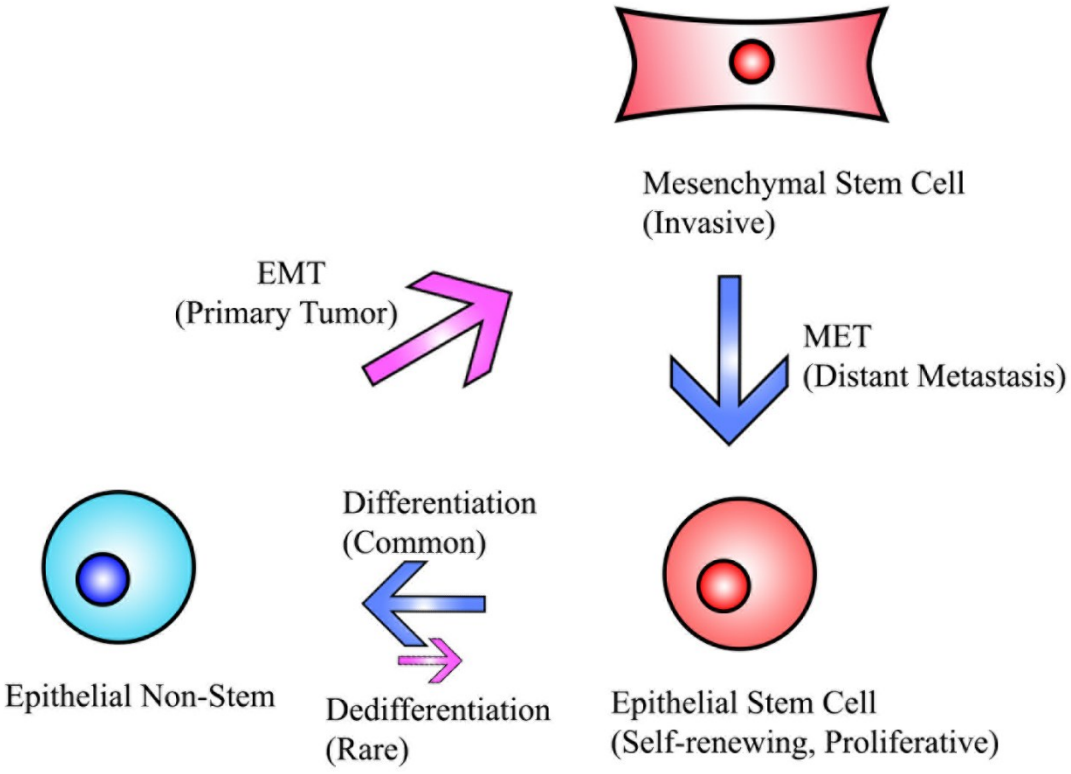


Figure 2. A simplified model of the trans-differentiation events during cell transition between epithelial and mesenchymal state, and between differentiated (blue) and stem-like state (red), during cancer progression to metastasis.

However, though EMT had been shown to enrich cancer stem cell population, a supposedly highly tumorigenic subpopulation, there were several contradictory studies reporting that silencing EMT can actually promote cancer dissemination [70]. Further studies have reported that cancer cells

negative for canonical stem-markers (such as CD44) maintain tumor-initiating capacity but at a lower frequency[71]. Lineage tracing of co-transplanted marker-positive and marker-negative cells showed a comparable contribution towards tumor growth from each subgroup [71]. Together with the repeated observation that cells isolated from distant metastasis have an epithelial rather than mesenchymal molecular signature [72], the direct relationship between EMT and cancer stem cells became highly contested.

This apparent contradiction may be resolved by recognizing that much like EMT, the reverse MET plays an essential role during early development. MET is essential during organogenesis of the adult kidney to generate the highly specialized epithelial cells of the nephron [73]. The interaction between MET and EMT is also prominent during blastocyst implantation, where the stromal cells of the endometrium expresses E-cadherin [74] to create a permeability barrier against the invading trophoblast which concurrently loses E-cadherin[75]. Therefore, cellular plasticity, the capacity to undergo transdifferentiation via EMT/MET, is an essential attribute for normal cell development.

In cancer, studies have shown that the epigenetic silencing of E-cadherin by EMT factors is highly unstable within an evolving population, indicating that EMT in cancer is spontaneously reversible [76] [77]. MET has been highly implicated in metastatic cancer homing and tumor growth at secondary sites [78]. Furthermore, many recent studies explored the apparent synergy between epithelial and mesenchymal cancer cells. For instance, one study remarked that within a solid tumor, cancer "stem-like" cells actually exist in two variations – an epithelial proliferative population and a mesenchymal invasive population [79]. Another study showed that in non-tumorigenic basal breast cancer cells, a minor "stem-like" epithelial subpopulation that is high in regenerative potential and ALDH activity can undergo EMT to spontaneously generate invasive

mesenchymal-like cells, with enhanced mammosphere-formation and invasive behavior[80]. Two variants of somatic stem cells –active and quiescent– was even identified in normal tissues where they reside within separate yet adjoining niches[81]. Captured circulating tumor cells (CTC) from microfluidic herribone chips showed that epithelial-like CTCs are enriched during chemotherapy, but transition into a mesenchymal state during relapse[82]. Via tissue microarray (TMA), it has been also been shown that primary breast cancer tumors, particularly the ones originally derived from less differentiated tissue, contained cells that co-expressed both epithelial and mesenchymal markers[82]. Another similar study in squamous cell carcinoma showed there were two separate populations of cancer stem cells – one epithelial and one mesenchymal - that could spontaneously transition from one form to the other and notably, switching from mesenchymal to epithelial phenotype was more restrictive and was only accessible to cells expressing high levels of ALDH [83].

One interesting study reported that isolated subsets of breast cancer cells from a heterogeneous populations can rapidly recapitulate the initial heterogeneity[84]. The study suggests that there's an inherent capacity for breast cancer cells to convert from any one state to another, with the probability of frequent transitions (stem to non-stem) and rare transitions (non-stem to stem) dictating the final proportion at equilibrium[30]. In this model, a knockdown of pluripotent genes *Oct4* and *Nanog* reduced cell proliferation and differentiation potential, but conversely increased the occurrence of spontaneous differentiation [85]. The loss of cell plasticity from knocking down pluripotent transcription factors demonstrate an obvious link between the overarching concept of cell plasticity and cell differentiation in cancer. Together, these studies show that the defining characteristics of "cancer stem cells", including regenerative potential, resilience, and invasiveness could be shared among interconverting cancer cells.

1.4 The differentiation program is a multi-level integration of cellular signaling that can be amenable for modulation by cellular factors and small molecules

Reprograming of cellular "state" is accomplished via a multi-layered regulatory network cumulating in a global shift in the epigenetic and transcriptome landscape. Transcription factors and epigenetic modifiers cooperatively activate entire set of genes corresponding to a differentiation state. This broad regulatory mechanism implies that during differentiation, cells transition from state to state in a stochastic manner [86], enabling differentiation-status reprograming without micromanaging individual genes. An expanding number of internal and environmental factors, including cytokines, microRNAs, and nutrient availability is found to coordinate cell fate determination, providing novel avenues for fine-tuning reprograming efficiency.

Much ongoing effort is focused on developing small molecules that can experimentally redirect cell differentiation. This was proven to be possible using small molecules such as a purine-derived small molecule called Reversine, which was able to induce dedifferentiation of lineage-committed myoblasts into a pluripotent state [87] . In the context of cancer, reversine was reported to exert anti-cancer activity although the relationship to cancer cell differentiation was not proven [88]. Though the mechanism of Reversine-induced dedifferentiation is not fully understood, Reversine has been shown to target non-muscle myosin II [89], Aurora B, MEK1, MPS1, and FAK[90]. It has been proposed that Reversine-induced changes impact on chromatin remodeling to induce a shift in global gene expression. Here I will discuss the key factors that can be manipulated for experimental cancer cell reprograming and discuss the therapeutic potential using small molecules in modulating these factors (summarized in table 1).

Factor of Differentiation	Mechanism of action	Examples
Transcription Factors	STAT3 inhibitor	Stattic[91]
	Snail inhibitor	GN-25 [92]
	Substitute for transgenic c-Myc and Sox2	BIX-01294 and BayK8644[93]
	Substitute for transgenic c-Myc	Valporic acid[94]
Epigenetic Regulation	DNMT1 inhibitor	Azacitidine[95], Decitabine[96], Zebularine[97], RG108[98]
	EZH2 inhibitor	DZNep[99], EPZ-6438[100]
	LSD1 inhibitor	Tranylcypromine[101]
Kinase Signaling	c-MET inhibitor	PF-2341066[102] and PHA665752[103]
	GSK3 inhibitor	6-Bromoindirubin-3′-oxim[104], CHIR99021[105]
	Wnt signaling inhibitor	Tankyrase[106], Pyrvinium[107]
	MEK inhibitor	PD0325901[105]
	EGFR inhibitor	Afatinib[108]
	TGF-β kinase activity	SB341542, Noggin[109], RepSox[110]
	RasGAP/Erk1 inhibitor	Pluripotin[111]
MicroRNA Regulation	Enhancing microRNA processing	Enoxacin[112]
	Inhibition of siRNA unwinding	Dihydropteridinones [113]
	Inhibition of miRNA pathway	Poly-L-lysine hydrobromide; 3,6-diamino-10-methylacridinium chloride [114]
Metabolic Regulation	HIF-1α inhibitor	103D5R, echinomycin, NSC-134754, 2-methoxyestradiol[115]
	PPAR-γ agonist	Troglitazone[116]
	PPAR-γ antagonist	T0070907[117], GW9662[118]

Table 1: A non-exhaustive list of small molecules with reported potential in cell reprograming through targeting of diverse factors controlling cell differentiation. The list of examples is extracted from the main text.

1.4.1 Transcription factors

Genetic and epigenetic regulations are central for cell's differentiation potential. The landmark study by the Yamanaka group demonstrated that it's possible to reprogram somatic stem cells into fully pluripotent iPSCs by forced expression of only four defined transcription factors: Oct4, Sox2, Klf4, and c-Myc. The "Yamanaka factors" demonstrated that the impact of gene regulatory mechanisms can be equally profound outside of ordinary developmental context. Therefore, those defined factors unsurprisingly have implicated roles in cancer development.

Oct4 is valuable diagnostic marker for immunohistochemical identification of testicular germ cell tumors [119]. Oct-4 is also frequently overexpressed in cancer stem-like cells in lung cancer and ovarian cancer [51]. Sox2 is frequently overexpressed in esophageal [120], lung [120], and ovarian cancer [121]. Furthermore, Sox2 expression was reported to generate cancer stem-like cells in Ewing sarcoma [122]. Klf4's nuclear localization is frequently detected during cancer progression and is a prognostic factor for aggressive phenotype in early breast [123]. c-Myc is a proto-oncogene often amplified in multiple types of cancer, and a recent study indicate that c-Myc is part of a separate transcriptional module separate from Oct4/Sox2/Nanog and accounts for much similarity between embryonic stem cell and cancer gene signature [124]. Overall, transcriptional reprogramming of somatic stem cells involves multiple regulatory factors that are inherently involved in cancer progression.

Furthermore, Suvà et al. showed that transgenic transcription factors approach used for reprogramming normal stem cells was also applicable for cancer cells when they used a core set of neurodevelopmental factors: Oct4, Sox2, SALL2, and OLIG2 to dedifferentiate glioblastoma cells [125]. Another study showed that gastrointestinal cancer cells are sensitized to differentiation

therapy when they are forced to express Yamanaka factors (Oct4,Sox2,Klf4,c-Myc)[126]. Taken together, it appears that the knowledge gained from transcriptional reprograming of mouse embryonic fibroblasts could also be applied to reprograming of cancer cells. Moreover, a deeper implication for the frequent overexpression of Yamanaka factors in cancer is that cancer progression could already be inherently driven, to some degree, by transcriptional reprogramming.

The investigation of small molecules as an alternative approach to genetic manipulation has been explored [127]. Small molecule mimics of transcription factors have been designed with an amphipathic helix domain tethered to a DNA-recognition domain to directly bind and activate specific genes, but the clinical efficacy of these molecules are still unproven [128]. Alternatively, small molecule kinase inhibitors can modulate cell signaling to reduce the transgenic requirement for successful reprogramming. For instance, a combination of mitogen-activated protein kinase (MAPK) inhibitor and glycogen synthase kinase-3 (GSK3) inhibitor is commonly used to maintain embryonic stem cell pluripotency, and can also enhance somatic reprograming by Yamanaka factors[129]. Moreover, small molecule inhibitors of TGF-β signaling including RepSox and SB341542 can replace Sox2 and c-Myc during fibroblast dedifferentiation by inducing the expression of Nanog [110]. A combination of BIX-01294 and BayK8644, a small molecule methyltransferase inhibitor and calcium agonist respectively, can induce dedifferentiation in mouse fibroblasts with only Oct4 and Klf4 [93]. Similarly, the histone deacetylase inhibitor valporic acid can enhance reprogramming and substitute for c-Myc [94]. Even more impressively, a combination of 7 small molecules can induce pluripotent stem cells without any exogenous transgenes, demonstrating that in theory, the broad regulatory functions of transcription factors can be chemically replicated [130].

In addition, expressing of EMT-associated transcriptional factors including Snail, Twist and Zeb1 are another potential avenue for transcriptional reprograming [131]. These transcription factors have associated roles in the differentiation of multiple lineages including bone and muscle cells [132]. One study showed that in epithelial-ovarian cancer stem cells, constitutive degradation of TWIST-1 maintains the cell in an undifferentiated state, whereas re-expression of TWIST-1 triggers EMT[133]. Snail, another EMT-inducing factor, acts by directly inhibiting E-cadherin promoter [134]. A Snail inhibitor GN-25 was reported to reverse this transcriptional reprograming and re-activating MET, offering proof of concept for chemical reprograming of cancer cells via targeting of EMT/MET factors[92]. In summary, these findings show that EMT and MET-related transcription factors are also integral regulators of cell differentiation, and represents an alternative set of reprograming factors distinct from pluripotent factors.

1.4.2 Epigenetics

Epigenetics is broadly defined as any hereditary pattern of gene expression not associated with inherent DNA sequence. It was long established that epigenetic mechanisms are essential regulators of cell differentiation [135], and from one perspective, a differentiated cell is one whose lineage has been firmly established by epigenetic markers [136]. Epigenetic reprogramming through DNA modification and histone remodeling coordinate global gene expression alongside transcription factors through reciprocal regulation and through the formation of transcription factor-epigenetic factor complexes [137]. Since large gene sets are under the control of the same epigenetic "on/off" switches, many epigenetic regulators controlling cell differentiation are also potential targets for cancer therapy [138].

At the DNA level, the most common epigenetic marker is methylation of cytosine-phosphate-guanine (CpG), a dinucleotide sequence highly enriched within gene promoter sequences. iPSCs derived from different origins shows a significant variability in genome-wide DNA methylation patterns, and the analysis CpG-methylation can help distinguish between iPSC, embryonic stem cells, and fibroblasts as well as identify the cell of origin[139]. This suggests that generation of iPSC from transgenic factors alone induces an incomplete cellular reprograming, whereas compete reversion of lineage traces requires a full reset of epigenetic markers [140].

DNA methylation is carried out by two main groups of DNA methyltransferases (Dnmt). Dnmt1 targeting of hemi-methylated DNA is responsible for duplicating epigenetic markers onto newly replicated DNA strands [141]. As such, Dnmt1 is essential for maintaining lineage identify as well as maintaining stem-like signatures in somatic stem cells, serving as an important mechanism for long term self-renewal [142]. In contrast, it is recognized that the DNMT3 family is responsible for *de novo* DNA methylation[143]. Studies report dnmt's and transcription factors as mutual regulators. In mesenchymal stem cells, pluripotency factors Oct4 and Nanog directly up-regulate Dnmt1 through its promoter sequence[85]. During embryonic development Dnmt3a and Dnmt3b mediates *de novo* methylation (silencing) of pluripotent genes including *Oct4* and *Nanog* [144]. Multiple studies have investigated the potential of epigenetic regulators as cancer therapy. Two DNMT1 inhibitors azacitidine [95] and decitabine [96] have been tested in phase III clinical trials for myelodysplastic syndrome, a disease of impaired differentiation in precursor blood cells. Zebularine is another dnmt1 inhibitor that offers better stability in aqueous solution and has reported good preclinical effects against bladder and ovarian cancer [97]. RG108, a newer class of dnmt1 inhibitors, was shown to reactivate tumor suppressor genes and dedifferentiate myoblasts into cardiac stem cells [98].

At the chromatin level, key differentiation genes are regulated by "bivalent" domains that can be alternatingly activated or repressed by acetylation or methylation at Lysine 4 and 27 [145]. This inherent plasticity facilitates cancer-induced epigenetic reprogramming[146], and studies suggest that many tumor suppressor genes can be particularly sensitive to mutational gene silencing due to having a bivalent chromatin pattern[147]. Histone modifications by a growing list of enzymes including histone deacetylases (HDAC) and histone methyltransferases (HAT) serve as directors of chromatin modelling and present attractive targets against epigenetically-driven cancers.

The polycomb-group (PcG) is a family of proteins involved in methylation of H3K27 and is critical for transcriptional repression of many genes involved in development and differentiation [148]. PcG members mediate gene silencing via a repressor complex and are involved in early mouse development [149] as well as cancer progression [150] and cancer stem cell maintenance. The PcG component, enhancer of zeste homologue 2 (EZH2), directly controls DNA methylation by serving as a recruitment platform for Dnmt's [151]. In particular, EZH2-targeted genes are strongly associated with differential methylation patterns of prostate cancers [152]. A small molecule inhibitor "DZNep" selectively induced apoptosis in AML cells and prostate cancer cells by reactivating PcG-silenced genes [99] but has broad activities against all PcG activity. High-throughput screening have identified several small molecules with pyridone amide motif, such as EPZ-6438, that can selectively target EZH2. EPZ-6438 has entered phase I/II clinical trial for treatment of refractory B-cell lymphoma or advanced solid tumors with elevated H3K27 methylation [100].

The discovery of histone demethylases changed the notion that histone modifications were permanent. Certain histone demethylases such as lysine specific demethylase 1 (LSD1) was found to be strongly expressed in poorly differentiated cancer including breast and neuroblastoma [153],

and is required for the maintenance of leukemia stem cells [154]. Tranylcypromine, a small molecule inhibitor of LSD1 previously used as anti-depressant, has shown promise as a differentiation therapy in acute myeloid leukemia (AML) by reactivating the all-trans-retinoic acid differentiation pathway [101], and represents an effective therapeutic target previously restricted to acute promyelocytic leukemia (APL).

Anther PcG member, the B cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1), is highly involved in the cell cycle, cell immortalization, and senescence [155]. Notably, Bmi1 was found to be essential for long-term self-renewal of neural stem cells[156], haematopoietic stem cells [157], and leukemic stem cells [158] by inhibiting senescence pathways. Bmi-1 have been shown to play an essential role in self-renewal in both embryonic stem cells [159] and cancer including leukemia [160], breast cancer [161] and prostate cancer [162]. In a recent study, a small molecule inhibitor of BMI-1 PTC-209 was reported to inhibit tumorigenicity in colorectal cancer by impairing tumor-initiating cells self-renewal[163].

Cancer aside, inhibitors of DNA and chromatin epigenetic modification have been investigated in the context of experimental reprograming of normal stem cells. Small molecule HDAC inhibitor trichostatin A (TSA) and Dnmt inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) individually and synergistically improved transcriptional reprograming by defined TFs[94], and directly up regulated pluripotency gene Oct-4 in tropoblast stem cells [164]. Reprograming of somatic cells by transfer of zygotic nucleus can be enhanced by TSA but impaired by 5-aza-dC [165]. Loss of Dnmt3a and Dnmt3b does not inhibit nuclear reprograming, but does instill lineage-restrictions on reprogramed cells [166]. Overall, while it is evident that large-scale epigenetic changes can occur during cellular differentiation, the limited understanding of gene-specific epigenetic

regulatory factors means that chemical modulation of epigenetic control is restricted to global mechanisms.

1.4.3 Cytokine and growth factor receptor signaling

Receptor signaling through soluble growth factors and chemokine such as interleukins can instigate specific differentiation signals. One best investigated example is the TGFβ superfamily of differentiation factors, where response to TGFβ is highly cell-type specific [167]. In response to tissue injury, TGF-β stalls endothelial cell proliferation and induces expansion and differentiation of smooth muscle cell precursors to form new blood vessels [168]. TGFβ inhibits myogenic differentiation *in vitro* [169] but promotes myogenic differentiation into fibrotic cells in response to injury *in vivo* [170]. In a clinical context, TGFβ also promotes the transdifferentiation of bone marrow mesenchymal stem-cells into carcinoma associated fibroblasts. [171] Dual inhibition of TGF-β signaling using small molecules Noggin and SB431542 efficiently induces embryonic stem cell differentiation towards the neural lineage [109]. Likewise, TGFβ's cancer progression is also context sensitive [172]. TGFβ-targeted therapies can instigate both oncogenic and oncosuppressive effects, suggesting the need for extra care in specifying proper cellular context [173].

The ErbB family of tyrosine kinase receptors, including EGFR and Her-2, are established oncogenic proteins involved in cancer development and progression [174]. These receptors have emerged as targets for targeted therapies (including monoclonal therapeutic antibodies and small molecule kinase inhibitors , TKIs) in several cancer types, including breast cancer and lung cancers [175] [176]. One example is Afatinib, a TKI currently approved for treatment of EGFR-mutant lung cancer [108] and is also under investigation for treatment of solid tumors including colorectal [177], and head and neck cancer [178]. Afatinib showed clinical benefits in a phase II trial for Her2-positive breast cancer that become resistant to Her2 targeted therapy [179]. Interestingly, A study of Afatinib

mechanism showed that it selectively eliminated cancer stem-like cells by inhibiting ABCG2, which re-sensitizes them to conventional chemotherapy [180]. As side effect, Afatinib treatment also induced an increase in differentiation of epidermal keratinocytes [181]. Blocking of EGFR signaling was also found to induce neural cell differentiation [182]. Other small molecule RTK inhibitors such as Gefitinib and Erlotinib have been approved for clinical treatment of advanced lung or pancreatic cancers [183] [184]. Drug resistance to EGFR-inhibitors are frequently associated with activation of alternative receptors such as c-MET [185] [186]. c-MET, or hepatocyte growth factor receptor (HGFR), and its ligand HGF have been reported to have a role in the function and maintenance of stem cells [187] [188] and cancer stem cells [189] [190]. HGFR released from the limb mesenchyme induces migration of myogenic precursor cells towards the limbs, diaphragm, and tongue during embryonic development [191]. c-MET activity was found to be critical for liver cell and dendritic cell differentiation and maturation [192]. c-Met is also highly expressed in mammary luminal progenitor cells, where its activation was shown to induce a transdifferentiation towards a basal cell lineage [187]. In cancer, c-MET overexpression is associated with aggressive phenotype, and small molecule inhibitors of c-MET including PF-2341066 and PHA665752 are reporting positive preclinical results [102]. Blocking c-MET activity was also shown to inhibit melanoma differentiation [193]. Overall, the up regulation of c-MET as a mechanism of drug resistance does provide another perspective on how cell differentiation can indeed contribute to increased cancer malignancy.

Aside from growth factors, hormones and interleukins also have proven roles in cellular differentiation, [194] and current studies suggest this extends to cancer cells. The inflammatory response is often a promoting factor of cancer proliferation and metastasis [195]. Drug resistance to receptor-targeted molecular therapy in breast cancer has been linked to a dramatic increase in IL-6 secretion, leading to an inflammatory loop that expands the population of resistant “cancer stem

cells"[196]. Inhibition of IL-8 receptors CXCR1 and CXCR2 showed strong synergy with Her2-targeted therapy for reducing cancer stem cell activity[197], and in another study, dual inhibition of IL-6 and IL-8 was able to inhibit growth of the dedifferentiated triple negative breast cancer [198]. Furthermore, Stat3 is a canonical downstream effector of IL-6 and inhibition of Stat3 activation using a small molecule "Stattic" has shown strong chemo-sensitization in multiple types of cancer [91].

Wnt and Notch receptors are highly conserved cell-fate controls that are critical for stem-cell maintenance [199]. Soluble Wnt ligands can promote somatic reprograming in generating iPSC [200]. Small molecule activator and inhibitors of Wnt signaling have been investigated in the context of regenerative medicine and cancer therapy [201]. In the context of stem cell reprograming, activation of Wnt signaling was shown to promote the generation of iPSC [200]. Small molecule activation of Wnt signaling can be accomplished using inhibitors of glycogen synthase kinase-3 (GSK3) such as 6-bromoindirubin-3′-oxime [104], or inhibition of endogenous Wnt-receptor antagonists [202]. Conversely, inhibition of Wnt signaling has been accomplished using small molecules tankyrase or pyrvinium to stabilize the proteosomal complex, leading to the degradation of Wnt signaling components [106]. Wnt signaling inhibition promotes ES differentiation and preferentially induces mesodermal progenitors towards a cardiomyocyte lineage [203]. In cancer, inhibition of Wnt-2-mediated signaling promotes apoptosis of non-small-cell lung cancer [204].

Notch signaling is mediated by intercellular contact. Notch signaling is highly involved in the differentiation of multiple cell lineages including T-cells [205], pancreatic cells [206], oligodendrocytes [207], and lymphoid cells [208]. Recently, it was shown that Notch inhibition enabled iPSC generation without transgenic Klf4 or c-Myc [209]. Notch signaling can also rapidly reprogram pancreatic acrinar cells to ductal intraepithelial neoplasia [210]. Aberrant activation of Notch

signaling has been associated with cancer survival, angiogenesis, and resistance, and as a result, inhibitors of Notch signaling has been heavily explored as potential cancer therapy [211]. In glioblastoma, inhibition of Notch signaling using gamma secretase inhibitors drastically reduces the percentage of CD133+ stem-like cells[212]. Notably, cells positive for the multi-lineage markers Nestin were 10-folds more sensitive to notch inhibition. [213] Notch inhibitors in clinical settings are restricted to gamma-secretase inhibitors, but other forms of Notch regulation are theoretically feasible [211].

While the role of multiple receptors are being defined in cell differentiation, it is worth bearing in mind that in normal physiological context, cell-fate is ultimately directed by integration of all differentiation signals. In a study where naïve mesenchymal stem cells were grown in tissue-mimicking matrices, cells can be reprogrammed towards either neurogenic, myogenic, osteogenic lineages purely depending on matrix elasticity, independent of any other soluble induction factors[89]. This intriguing study shows that *in vitro* studies may potentially overestimate the effect of certain differentiation factors vis-à-vis physiological conditions.

1.4.4 MicroRNA network

MicroRNAs are single-stranded non-coding RNAs that regulate gene expression at the post-transcriptional level. MicroRNA (miR) are endogenously coded and functions by targeting select mRNA through RNA interference.

Compounding evidence suggest that the microRNA network is highly intertwined in cell fate reprograming. Stem cell-specific microRNAs, miR-291-3p, miR-294, and miR-295, are shown to be enhancers of somatic reprograming, and are suggested to act as downstream effects of transcription factor c-Myc [214]. In another study, a pair of stem-cell specific microRNA miR-302 and miR-372 promoted human fibroblast dedifferentiation through activation of multiple cell cycle,

epigenetics and EMT-related targets [215]. Moreover, a wide series of microRNAs are found to interact with TGF- β signaling, including miR-181, miR-17-92 and miR-21[216].

Mounting evidence also demonstrate that microRNA can initiate malignant reprograming of cancer. In breast cancer, prolonged culture of the breast cancer cell line MCF-7 can induce EMT through microRNA-targeting of the estrogen receptor [217]. In pancreatic cancer, Notch induces EMT and the acquisition of cancer stem cell phenotypes through activation of miR-21 and repression of miR-200, let-7a, let-7b, and let-7c[218]. In particular, miR-200, which antagonizes Zeb1 and the polycomb complexes, was found to be down-regulated in putative cancer stem cells[219]. In another case, direct repression of CD44 via microRNA-43a inhibits prostate cancer metastasis and reduces stem-cell properties [220]. Finally, the aforementioned CSC-promoting inflammatory feedback loop associated with IL-6/STAT3 signaling axis was confirmed to be mediated by miR-21, miR-181b, and let7 [221].

MicroRNA enhances cell plasticity by allowing flexible regulation of key signaling pathways required for homeostasis, development and EMT. Presently, there are small molecules that can either target specific microRNAs, or the RNA interference pathway [222]. The small molecule enoxacin can bind to miRNA biosynthesis protein TRBP to enhance the production of tumor-suppressing miRNAs [112]. An analogue panel of substituted dihydropteridinones can impair microRNA function by inhibiting siRNA unwinding. From another high-throughput screening, two more compounds poly-L-lysine hydrobromide and 3,6-diamino-10-methylacridinium chloride were identified as small molecules that can inhibit RNA interference activity [114]. So far, the Deiters group have identified two small molecules specifically targeting miR-21 [223] or miR-112 activity, respectively [224].

1.4.5 Metabolic reprogramming

Aberrant activation of the glycolytic pathway in cancer, known as the "Warburg effect", is now proposed to be a defining hallmark of cancer [225]. Cancer cells adopt glycolytic metabolism that, although initially counterintuitive, allows them to gain metabolic advantage to fuel cell proliferation. Several lines of evidence directly link the induction of metabolic reprogramming with mechanisms that regulate cell differentiation.

A central initiator of metabolic reprogramming is the hypoxia-inducible factor (HIF) family of oxygen-sensing transcription factor. As a result of excessive cellular proliferation, oxygen supplies in tumors become limited and induce up regulation of HIF which, together with c-Myc, activate or repress metabolic pathways in concert with the fluctuating oxygen levels [226]. HIF has been shown to be an activator of EMT and promotes cancer metastasis, by directly regulating the EMT activator Twist or Snail [227]. Hypoxia-induced EMT also appears to share overlapping molecular components with Notch signaling[228]. In at least breast cancer, the induction of EMT is associated with a Snail-G9a-Dnmt complex to promote glycolysis and macromolecule synthesis and thus confer a metabolic advantage to post-EMT cells[229].

In addition to promoting EMT, hypoxic conditions have been shown to promote long-term self-renewal of hematopoietic stem cells [230], mesenchymal stem cells [231], and neural stem cells[232]. Since oxygen levels are variable within a physiological system, it was hypothesized that somatic stem cells may reside specifically within low oxygen "niches". Through similar mechanisms, injury-induced hypoxia could promote formation of temporary hypoxic niches for recruitment of progenitor cells [233]. Stem-cell renewal within these niches may be partially driven by an induction of reactive oxidative species (ROS), which was shown to inhibit GSK3 and activate Wnt signaling [234]. Furthermore, expression of HIF can directly promote activation of the pluripotent factor Oct-

4 [235]. These studies suggest that hypoxia not only provides an environment favoring stem cell self-renewal, but can induce *de novo* activation of stem-cell related genes.

Unsurprisingly, hypoxia has been implicated in cancer and cancer stem cells. A hypoxic environment was reported to promote expansion and maintenance of glioma and glioblastoma stem cells [236]. Small molecules targeting the hypoxia-inducible factors (HIFs) have been discovered including 103D5R, echinomycin, and NSC-134754[237]. Notably, 2-methoxyestradiol (2MO), an orally active small molecule inhibitor of HIF-1α has undergone phase II clinical studies for treatment of advanced prostate cancer [115],glioblastoma [238] and ovarian cancer [239]. Although most attention have been given to the ubiquitously-expressed HIF-1α, a recent study in glioblastoma has shown that targeting of the cell-type specific HIF-2α [240] could be more selective for cancer stem cells over normal stem cells [241]. HIF-2α but not HIF-1α is notable for promoting Oct4 expression, suggesting that it may have the exclusive role of mediating hypoxia-induced cell reprograming. Therapeutic benefits of small molecule inhibitors of HIF-2α may be worth exploring.

Aside from reduced oxygen dependence, hypoxia-stimulated responses also mediate an increase in glucose import via upregulation of the transporter Glut1 [242]. Glucose level plays a vital role in regulating differentiation in cell lineages in which glucose level is vital to their function such as in T-cells and renal proximal tubules [243]. Glucose-triggered EMT in renal proximal tubules can be reversed by a peroxisome proliferator-activated receptor-γ (PPAR-γ) ligand Troglitazone. PPAR-γ is a nuclear receptor that regulates adipocyte differentiation [244] and glucose/lipid metabolism [116]. In cancer, Troglitazone also inhibits *in vivo* TGFβ-mediated metastasis [245] and has antitumor activity against breast cancer *in vitro* [246] and prostate cancer both *in vitro* and *in vivo* [247]. Troglitazone and another PPAR-γ agonist Efatutazone inhibited EMT by blocking TGF- β

signaling in lung cancer [245]. Paradoxically, the PPAR-γ antagonist T0070907 inhibited cell mobility and proliferation in pancreatic cancer [117]. Another PPAR-γ inhibitor GW9662 inhibited breast cancer growth synergistically with PPAR-γ ligand. These appears contradictory, but more recent studies suggest that antimetastatic effects from PPAR-γ ligands are not actually due to direct activation of PPAR-γ, since Troglitazone inhibits EMT even in cells where PPARγ-activity is abolished via an irreversible antagonist or dominant negative mutation [248].

PPAR-γ is also involved in the metabolism of polyamines, organic compounds with multiple amine groups. Though the biological function of polyamines is not fully clear, polyamine synthesis is highly critical for cell growth [249], and its emphasis in rapid proliferating cells makes it an attractive chemotherapy target. Polyamine analogues are currently being tested as a potential anti-cancer drug. These small molecules acts by accumulation within cancer cells to inhibit the synthesis of natural polyamines via a negative back loop [250]. Studies have shown that polyamines are important modulators of ion channels including NMDA channels and AMPA receptors [251], and preliminary studies have shown that this class of drugs have the potential reduce cancer stem cell population[252].

In summary, metabolic reprogramming is intertwined with mechanisms of classical differentiation and cellular plasticity but molecular mechanisms and implications remain to be established.

1.5 Conclusion

The process of cell differentiation and dedifferentiation is driven by complex cellular programs involving multiple signaling networks that greatly impact on cell plasticity. At present,

knowledge gained from stem cells has provided breakthrough information regarding normal stem cell behavior and potential implications to pathological conditions, in particular cancer.

The identification of cancer stem-cell selective small molecules can reveal unexpected CSC markers and provide new insight into unique facets of CSC biology. Salinomycin was originally identified in a high throughput screening for small molecules that are highly selective against breast cancer stem cells [253]. Salinomycin has been shown to inhibit the maintenance of breast cancer stem cells by disrupting the autophagy cycle[254], and overcome multidrug resistance in human leukemia [255] possibly by reducing drug efflux [256]. In addition, Wnt signaling has also been proposed as a potential target of salinomycin[257]. Salinomycin's function as a potassium ion transporter is well established [258], but much like with the polyamines, there is insufficient reconciliation of salinomycin's anti-CSC effects with its biological function as an ion modulator.

Another high throughput study screened for drugs that displayed differential efficacy between normal human pluripotent stem cell and a neoplastic derivative. Here, a dopamine receptor antagonist thioridazine, normally an antipsychotic drug, was found to selectively induce differentiation only in the neoplastic derivatives [259]. Prior to this study, dopamine-antagonism was only mildly linked with an *increase* in breast cancer risk [260]. Although here the model for distinguishing normal and neoplastic stem cells are artificial, it still suggests that there are aspects of cancer stem cell biology that are virtually unexplored.

The extent of somatic and cancer cell plasticity continue to impress. It is evident cancer progress can involve developmental programs are induced by multiple layers of interconnected environmental and cellular stimuli, and effected through reversible changes in the transcriptome and epigenetic landscape. Given all the evidence of a cell's high innate plasticity, it is highly likely that the most efficient steps towards directing cellular reprograming involves exploitation of

inherent cellular mechanisms already present in the cell. Future paradigms for generating iPSC and cancer therapy may involve faithful recapitulation of normal differentiation and dedifferentiation process. A multi-faceted approach to experimental reprograming integrating all our knowledge of cellular differentiation program will likely prove to be the most effective way to progress.

Chapter 2: Identification of a small molecule kinase inhibitor selectively targeting cancer cells expressing stem-cell markers

Identification of a small molecule kinase inhibitor selectively targeting cancer cells expressing stem-cell markers

Henry Yu, Krikor Bijian, Dominik Wernik, Su Jie, Hind Amzil, DingHong Qiu and Moulay A. Alaoui-Jamali*

Segal Cancer Centre and Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, Departments of Medicine, Oncology, and Pharmacology and Therapeutics, Faculty of Medicine, McGill University, Canada

Acknowledgement

This study was supported by the Quebec Breast Cancer Foundation

Running title: Targeting breast cancer stem-like cells

***Correspondence author:**

Moulay A Alaoui-Jamali, PhD

e-mail: moulay.alaoui-jamali@mcgill.ca

2.1 Abstract

Mounting evidence support that cancer progression to metastasis is driven by a subset of cancer cells with stem-cell like properties. In this study we investigated the therapeutic potential of a novel small molecule SLLN06 capable of selectively reprogramming breast cancer cells expressing low CD24 receptor ($CD24^{low}$) or high ALDH activity ($ALDH^{high}$) into cells with $CD24^{high}$ or $ALDH^{low}$. This reprogramming effect was seen in vitro in a dose-dependent manner and was associated with inhibition of cells' ability to form tumorsphere and to differentiate into organoids. Moreover, this reprogramming effect is not due to differential toxicity since equal antiproliferative activity was seen across a panel of cell lines regardless of the CD24 or ALDH status, as well between cells sorted into either $CD24^{high}$ or $CD24^{low}$ from the same parental cell population. A comparison of equimolar concentrations of SLLN06 and other molecules reported to target stem cell signaling revealed that SLLN06 has a disctinct mechanism and induces the greatest impact on CD24 and ALDH cancer stem cell markers. A high-throughput in vitro kinase screening identified major potential targets as aurora kinase A and B, JAK2, and Ret tyrosine kinase. Finally, SLLN06 was found to strongly inhibit metastasis induced by cells enriched in CD24 low and ALDH high at non-toxic dosing. These results support the potential of this class of molecule to modulate cell differentiation in invasive cancer cells with stem-cell like characteristics and to serve as an antimetastatic agent.

2.2 Introduction

At present, no “gold standard” therapeutics are available for advanced metastatic breast cancer. Cytotoxic chemotherapy remains the cornerstone therapeutics for this disease but this therapeutic modality has yielded marginal benefits and with high frequency of relapses. Search for alternative effective therapeutics targeting metastasis mechanisms and tailored to specific metastatic breast cancer subtypes is at the forefront of drug discovery research.

Progression of breast cancer to metastasis is governed by a complex microenvironment where multiple paracrine and autocrine regulatory signaling loops occur between cancer cells, and their surrounding stromal cells and infiltrating inflammatory and immune cells. Furthermore, genome-wide analyses further revealed additional intrinsic cell heterogeneity within breast cancer resulting in diverse molecular subtypes with distinct biological and clinical manifestations, in

particular in relation to metastasis incidence [1] [2]. Several studies inextricably linked cancer development and progression to the presence of cell variants expressing stem cell markers, referred to as cancer stem cells, and possibly acting as cell precursors for metastasis [3] [4]. Selection of these cancer stem cell-like cells (CSC) involves a broad range of signaling mechanisms and can also be dictated by the cells ability to switch back and forth between epithelial to mesenchymal states with epithelial-mesenchymal transition (EMT), also a key contributor for cancer heterogeneity and metastasis mechanisms, being implicated in general in enrichment in CSC [5, 6].

The identification of these cancer stem cell variants has been based on the expression of cell surface receptors that characterize stem cells, and their ability to self-renew indefinitely in culture, to differentiate into heterogeneous lineages of cancer cells, and to recapitulate traits required for the initiation and progression of transplantable tumors in mice [3] [7]. Of clinical relevance, these cancer-stem cells have been shown to not only they can drive progression to metastasis [8] but also to contribute to recurrence in part because their possible broad resistance to chemotherapy and ionizing radiation therapy [5] [9]. Furthermore, several studies identified stem cell gene signature or a high incidence of cancer cell population expressing stem cell markers in specific breast cancer subtypes, in particular those with high incidence of metastasis [10] [7].

The surface-presentation of CD24 has currently contradictory prognostic significance in different types of cancer. Low levels of CD24 identified progenitor cells in breast and prostate cancer[11], whereas high CD24 identified progenitor cells in human liver [12], gastric [13], pancreatic [14] and ovarian cancer [15] and in mouse breast cancer [16]. The functional role of CD24 in cancer is still highly obscure despite its prominence as a biomarker in multiple types of cancer [17]. Recent studies linked CD24 and downstream effector p38MAPK to being negative regulators of Notch1 [18]. Notch has been characterized as an inducer of EMT, where Notch induces the expression of key EMT

transcription factors Snail and Slug [19], as well as cross-talk with EMT-inducing growth factors[20]. In contrast, the $ALDH^{high}$ phenotype has been a consistent marker for identifying both normal and cancer stem cells [21]. A reduction in ALDH activity was seen to reduce radiation and chemoresistance in human breast cancer cells [22]. Importantly, the $CD24^{low}$ and $ALDH^{high}$ phenotypes have been associated with aggressive molecular subtypes of breast cancer such as basal and Her2-overexpressing [23]. Interestingly, the two set of markers appear to identify two non-overlapping "types" of cancer stem cells with distinguishable set of tumorigenic properties [24], suggesting the need to find a therapy that could target both populations.

Based on this remarkable knowledge, it is conceivable to hypothesize that terminally differentiated cells can be manipulated to regain or lose stem cell traits [25, 26], and this can theoretically impact the metastatic phenotype, e.g. rendering cells with metastatic trait non-invasive "indolent" and possibly more susceptible to chemotherapeutics. In this context, we report herein a novel class of small molecules capable of selective targeting of breast cancer cells enriched in stem-cell markers and of preventing metastasic development.

2.3 Results

2.3.1 SLLN06 reduces the expression of cancer stem cell markers

Our goal in initiating this project was the discovery of novel insights into targeting putative precursor cells that are suspected to drive cancer metastasis. In breast cancer, many prominent studies have confirmed that two sets of cell surface markers - $CD24^{low}$/CD44+ and $ALDH^{high}$ are linked with enrichment of stem-like properties [3, 21]. During early screening, we used two cell lines, MDA-MB-231and BT-20 which has, which represent tumorigenic cells that were found to express

$CD24^{low}$/CD44+ and ALDH activity respectively [27] [23]. Both cell lines are tumorigenic, with MDA-MB-231 also long-recognized as possessing potent metastatic potential [28].

Using flow cytometry, we screened a library of novel small molecules for their ability to modify expression of stem cell markers $CD24^{low}$/$CD44^{high}$ and $ALDH^{high}$. Our library was initially inspired by previous studies in our group reporting that a small molecule dedifferentiation agent Reversine displayed potent antimetastatic properties. While there is little empirical reconciliation of Reversine's dedifferentiation capabilities with its anti-metastatic potential, previous studies did see therapeutic benefits to inducing cancer dedifferentiation, which not only reduced their proliferation rate but also enabled their re-differentiation towards less malignant forms [29].

We identified a lead compound, SLLN06, which was found to dramatically reduce the % of $CD24^{low}$ and $ALDH^{high}$ expressing cells (Figure 1), while marginally reducing the expression of CD44. Notably, treatment with SLLN06 appeared to increase the expression of CD24 in MDA-MB-231, a cell line with very low endogenous expression of CD24.

We then investigated the impact of SLLN06 on the other putative stem cell markers including the surface receptor CD133 and the activity of ALDH. We found that SLLN06 at under 500nM has no impact on CD133, but dramatically reduces the detectable ALDH activity (Figure 2). In stark contrast, the parental molecule Reversine induced a dramatic increase in CD133 and ALDH. Because the enrichment of cancer stem cell markers CD133 by drug treatment had previously been lined to activation of Akt signaling, we conducted a western blot analysis to compare Akt activation between SLLN06 and Reversine. We treated MDA-MB-231 cells with 500nM of either SLLN06 or Reversine for 24H, and found that only the reversine treated cells displayed increased Akt phosphorylation.

We also characterized the impact of SLLN06 on a panel of alternative human and mouse cancer cell lines (Table 1). Notably, the human inflammatory breast cancer cell line SUM-149, the mouse cancer cell line 4T1, the prostate metastatic cancer line PC3 (also enriched in $CD24^{low}$), and the advanced thyroid cancer cell line 8505c, showed potent responses to SLLN06 at sub 1 micro molar concentration.

2.3.2 SLLN06 is *not* enriching non stem-like cells through selective cytotoxicity

To rule out the possibility of selective cytotoxicity against $CD24^{low}$ and $ALDH^{high}$ cells, we investigated the impact of SLLN06 on cell proliferation using the MTT assay. We observed that SLLN06 reduced cell proliferation rate at higher doses with similar potency across multiple breast cancer cell lines regardless of their endogenous CD24/CD44 or ALDH status (Figure 3 A/B and Table 1). Notably, no cell death was visibly apparent during cell culture even at inhibitory doses, and staining the cells with 7-aminoactinomycin D, a fluorescent DNA marker confirmed that cells treated with higher concentrations SLLN06 were non-necrotic (Figure 3 C/D). Therefore it appeared that SLLN06 did not induce severe toxicity, and suggested that the reduced proliferation rate was a cytostatic effect.

Next, we wanted to determine whether SLLN06's cytostatic effect conferred any proliferative disadvantage to $CD24^{-}$ cells. We used fluorescence-activated cell sorted (FACS) to sort MDA-MB-231 cells into either high or low expression levels of CD24 and then re-quantified the proliferation rate of each sorted populations (Figure 4). Our results show that the $CD24^{high}$ and $CD24^{low}$ sorted populations had near-identical proliferation rates after SLLN06 treatment. Together with the observation that the treated MDA-MB-231 cells re-expressed CD24 at higher

levels than what could be observed in the natural population, our results indicated that that SLLN06 is directly inducing cells to express CD24.

2.3.3 SLLN06 inhibit *in vitro* organoid differentiation and tumorsphere formation

A defining characteristic of cancer stem-like cells is their capacity to propagate as tumorspheres when cultured in suspension, as represented by figure 5A [30]. We investigated the effect of SLLN06 on differentiation capability of PMC42-LA. Among breast cancer cell lines, PMC42 are remarkable for having a normal mRNA and microRNA transcriptome and possessing multilineage potential [31]. In the absence of drug treatment, PMC42-LA cells undergo well-characterized differentiation into spheroids when cultured on matrigel [32]. Our results show that exposure to SLLN06 did not reduce the number of overall colonies but visibly prevented the differentiation and expansion of these colonies into larger organoid structures (Figure 5B).

We also investigated the tumorsphere forming capacity of SLLN06-treated cells in MDA-MB-231, BT-20, and PC3-M by administering the drug for 3-5days, then reseeding both treated and non-treated cells in low-attachment plates in renewal media, as described in the section methods. Consistent with previous characterization of these cells, in the absence of SLLN06, both MDA-MB-231 and BT-20 are capable of propagating as small individual "grape-like" tumorspheres that survive passaging [30]. Figure 5C shows that SLLN06 induced a dramatic reduction in viable tumorspheres in both cell lines, consistent with their apparent loss of cancer stem cell marker. Furthermore, we also observed dramatic reduction in sphere formation in the prostate cancer cell line PC3-M (not shown).

2.3.4 High-throughput kinase assay identifies potential targets of SLLN06

To determine the molecular targets of SLLN06, we submitted an aliquot of SLLN06 for the SelectScreen Kinase Profiling Service from Invitrogen. The screening indicate that SLLN06 is a multikinase inhibitor with strong inhibitory activity against Ret, Aurora Kinase A and B, and Jak2 (Figure 5A).

To confirm the inhibitory activities of SLLN06, we used western blot analysis to quantify phosphorylation of the key kinase targets. In order to detect inhibition of de novo phosphorylation, we first starved MDA-MB-231 cells in serum-free media for 24H to deplete endogenous phosphorylation. We then treated the cells with increasing doses of SLLN06 for 1 hour, followed by stimulation with 20ng/mL EGF for 30 minutes. We found that SLLN06 can inhibit phosphorylation of multiple target kinases including Aurora A, Aurora B, FAK, Src, Ret, and Jak2 (Figure 6B). Notably, we could not detect Jak2 phosphorylation in serum starved MDA-MB-231 cells even after EGF stimulation, but 1H SLLN06 treatment was able to inhibit endogenous Jak2 phosphorylation when the cells were placed in complete media (Figure 6C). Furthermore, the lack of effect of SLLN06 on Akt or Erk signaling further suggests that it does not target cell proliferation nor cell survival.

2.3.5 SLLN06 has distinct or superior effect compared to other molecules reported to target cancer stem cell or cell differentiation signaling

Currently, there are many small molecules, commercially available, that targets putative cancer stem cells or modulate the cell differentiation pathway, but so far there were no comparative study to evaluate their impact on the $CD24^{low}/CD44^{high}$ or $ALDH^{high}$ markers. To that end, we

treated equimolar concentration of a panel of small molecules in parallel with SLLN06 in order to compare their effectiveness against stem-like markers.

Table 2 shows that SLLN06 had a greater suppressive effect on the $CD24^{low}/CD44^{high}$ and $ALDH^{high}$ markers in comparison to other tested compounds, including a larger reduction in both markers compared to salinomycin, a compound known for its selective targeting of cancer stem cells through a currently unresolved mechanism[33]. SLLN06 also showed greater potency than reversine, a known de-differentiation agent that shares a common scaffold (Chen et al., 2007). As expected, Retreversine, an inactive isomer of Reversine, displayed no activity [34*68]. We also used selective inhibitors of Wnt and Notch signaling, two prominent regulatory pathways of cell differentiation, and found no effect on CD24 expression, but an interesting increase in the expression of ALDH[35]. We also used selective inhibitors of FAK and MEK1, two putative targets Reversine, and found no impact on CD24 expression but a decrease in ALDH expression from MEK1 inhibition[36]. Finally, we used a pan-aurora kinase inhibitor, a Ret inhibitor, and a Jak2 inhibitor. Aurora kinase inhibition induced a similar but less potent increase in CD24 with no apparent increase in ALDH, whereas Ret and Jak2 inhibition displayed no impact on either marker.

2.3.6 SLLN06 induces polyploidy

We observed during SLLN06 treatment obvious morphological changes resembling giant cells, a phenomenon linked in literature with cell polyploidization (Figure 6A), the accumulation of multiple DNA copies in a cell. This anomaly sometimes occur as part of cell differentiation and is often associated with disruption of normal cell cycle [37]. Therefore, we investigated the effect of SLLN06 on cell cycle using FACS analysis to measure propidium iodide staining. As shown in figure 6, our results confirmed that SLLN06 induces a dose-dependent accumulation of

polyploidic cells. This is consistent with the identification of the aurora kinases, regulators of chromosome biorientation, as a main target of SLLN06 [38]. Interestingly, polyploidy is only observed at doses above 500nM, suggesting that the mechanism of polyploidy may not be directly linked to the mechanisms that induce shifts in cancer stem cell markers, which could be observed under 300nM SLLN06 treatment.

2.3.7 SLLN06 reduces *in vivo* distant metastasis and primary tumor growth of orthotopically transplanted tumor cells

In order to evaluate the anti-tumorigenic effect of SLLN06 *in vivo*, we transplanted three aggressive breast cancer cell lines, 4T1, MDA-MB-231/M and PC3/M, into the mammary fat pad of SCID female mice as described in methods. The mice were treated intraperitoneally with non-toxic levels of SLLN06. After four treatment cycles, the number of lung surface metastasis and primary tumor weight were quantified (Figure 8A/B). The results show that all three cell lines responded to treatment with reduced primary tumor growth and less distant metastasis. Notably, the PC3/M cells had a ~20-fold reduction in distant metastasis with only ~2-fold reduction in primary tumor, suggesting that the inhibition of metastasis may not solely be due to a reduced primary tumor.

To further confirm SLLN06's anti-metastatic potential, we conducted a live cell locomotion assay to quantify cell movement speed. As shown in figure 8C, moderate doses of SLLN06 limits the rate of cell mobility by 2-3folds.

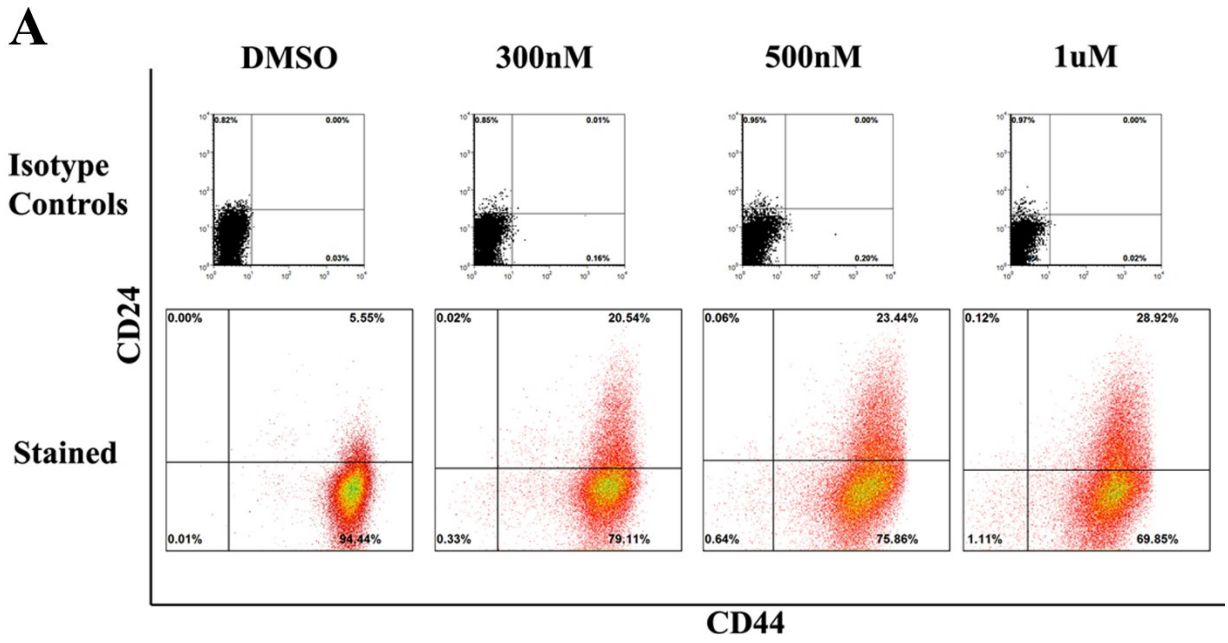
Figure 1: SLLN06 reduces the % of CD24-/CD44+breast cancer cells

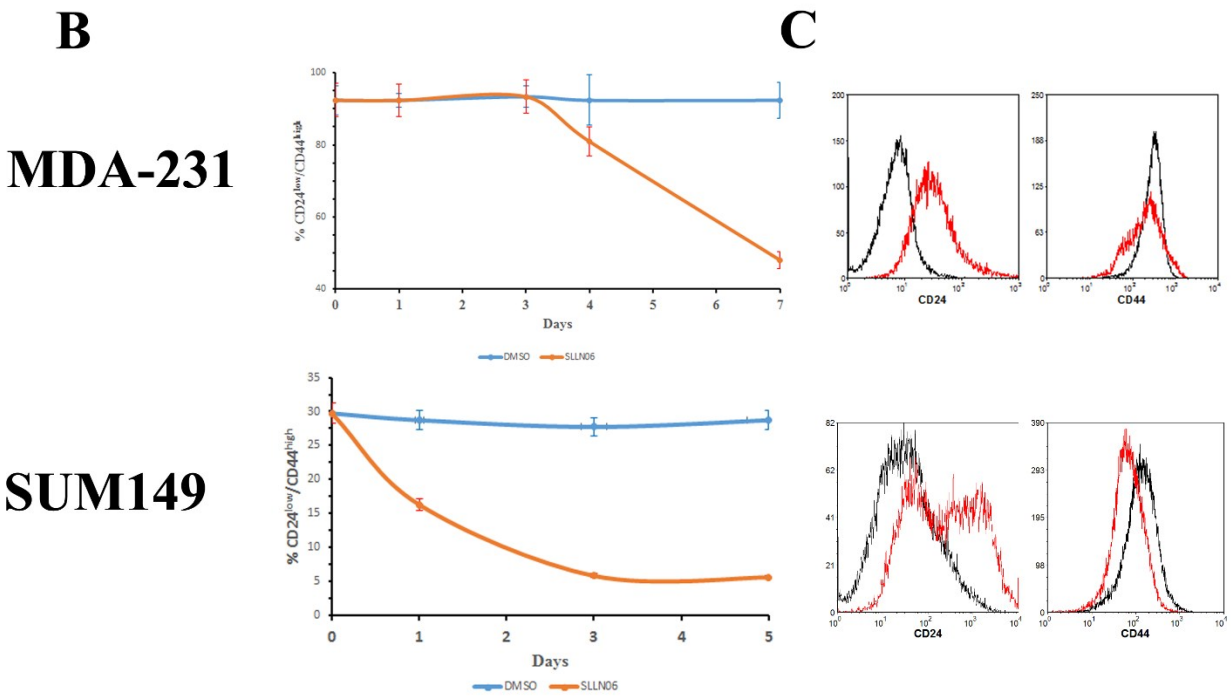
(A) MDA-MB-231 cells treated with SLLN06 show a dose-dependent reduction in cells expressing the combination of $CD24^{low}/CD44^{high}$ stem cell markers. The expression of cell surface marker was detected using flow cytometry. Non-specific isotype controls were used to establish background intensity. The threshold is established such that there is less than 1% positive signals in the isotype controls.

(B) MDA-MB-231 and SUM-149 cells treated with SLLN06 display a gradual reduction of cells expressing the combination of $CD24^{low}/CD44^{high}$ stem cell markers over a period of 5-7 days. The y-axis represents the percentage of cells whose CD24 expression is lower, and the CD44 expression is higher, than the background established by isotype controls.

(C) Histogram representation of CD24 and CD44 expression in MDA-MB-231 and SUM-149 cells.

(D) A western blot analysis of CD24-expression in both MDA-MB-231 and SUM-149 cell line after 5 day treatment with SLLN06.


A
DMSO
300nM
500nM
1uM
Isotype Controls
Stained
CD24
CD44
0.82%
0.00%
0.03%
0.85%
0.01%
0.16%
0.95%
0.00%
0.20%
0.97%
0.00%
0.02%
0.00%
5.55%
0.01%
94.44%
0.02%
20.54%
0.33%
79.11%
0.06%
23.44%
0.64%
75.86%
0.12%
28.92%
1.11%
69.85%


B
C
MDA-231
SUM149
% CD24low/CD44high
Days
DMSO
SLLN06
CD24
CD44

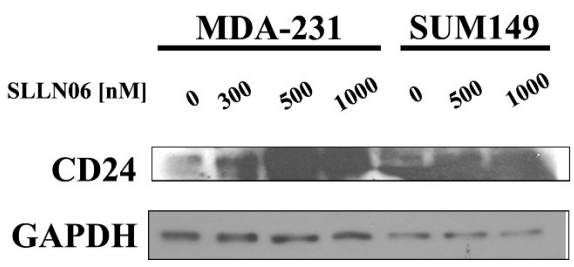

D
MDA-231
SUM149
SLLN06 [nM]
0
300
500
1000
0
500
1000
CD24
GAPDH

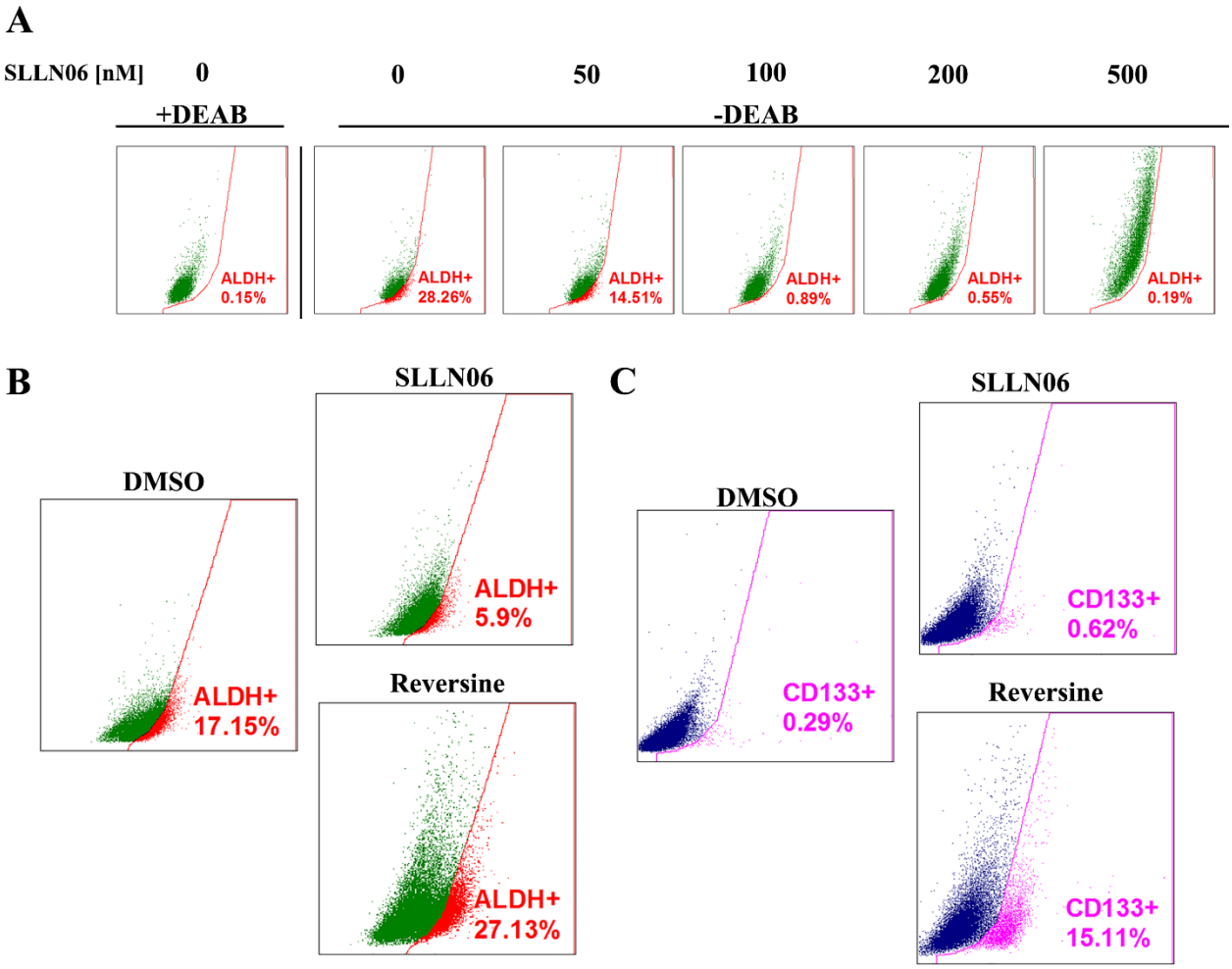
Figure 2: SLLN06 impacts on the expression profile of stem cell markers

(A) SLLN06 reduced the percentage of cells expressing high levels of aldehyde dehydrogenase activity in a dose-dependent manner. ALDH activity was quantified in BT-20 breast adenocarcinoma cells using the Aldefluor assay kit (StemCell).

(B) SLLN06 has superior effects against alternative stem cell markers in the thyroid cell line 8505c compared to Reversine. 8505c cells were exposed to SLLN06 and reversine were at 300nM for 72H. ALDH activity as quantified using flow cytometry.

(C) As with (B), the expression of the cell surface marker CD133 was also quantified using flow cytometry

(D) Western blow analysis of Akt and phosphorylated Akt (Serine 473) in MDA-MB-231 cells treated with 500nM SLLN06, Reversine, or the pan-aurora kinase inhibitor VX-680 for 24H.


A
SLLN06 [nM]
0
0
50
100
200
500
+DEAB
-DEAB
ALDH+ 0.15%
ALDH+ 28.26%
ALDH+ 14.51%
ALDH+ 0.89%
ALDH+ 0.55%
ALDH+ 0.19%
B
DMSO
ALDH+ 17.15%
SLLN06
ALDH+ 5.9%
Reversine
ALDH+ 27.13%
C
DMSO
CD133+ 0.29%
SLLN06
CD133+ 0.62%
Reversine
CD133+ 15.11%
D
DMSO
SLLN06
Reversine
VX680
p-Akt
Akt
GAPDH

	$CD24^{low}$ / $CD44^{high}$ (%)		$ALDH^{high}$ (%)	
	Control	**SLLN06**	**Control**	**SLLN06**
MDA-231	87.5 ± 7.5	34.43 ± 2.14	3.30 ± 0.80	2.00 ±1.40
BT-20	91.30 ±5.60	79.40 ±2.40	41.10 ± 4.90	4.10 ± 0.70
SUM-149	32.8 ± 2.1	3.2 ± 0.9	4.10 ±1.20	2.00 ± 0.40
MCF-7	0.04±0.01	0.05± 0.02	13.24 ±1.20	10.81 ±1.20
PMC42-LA	8.00 ±0.40	5.7 ± 2.4	9.04 ± 2.4	11.92 ± 6.5
4T1	ND	ND	33.60 ± 4.20	14.50 ± 0.20
PC3	85.2 ± 7.4	73.5 ± 5.2	ND	ND
PC3-M	66.4 ± 2.4	35.1 ± 4.2	ND	ND
8505c	98.4 ± 5.0	86.70 ± 7.5	42.1 ± 3.2	2.7 ± 0.21
HMLE	41.9% ± 6.4	46.9 ± 4.9	ND	ND
HMLE Her2	95.8% ± 2.40	92.2% ± 2.40	ND	ND

Table 1: SLLN06's impact on stem cell markers in alternative cancer models

Sub-confluent cells were exposed to 1μM SLLN06 for 72h and then cell surface markers were quantified using FACS analysis, as described in methods. Control cells were exposed to the vehicle alone. Shown are the average ± SD of at least 3 independent experiments each in duplicates.

Figure 3: SLLN06 inhibits cell proliferation via cytostatic effects

(A) The basal level of CD24 and CD44 three breast cancer cell lines was determined using flow cytometry, illustrating a difference in basal expression of both CD24 and CD44.

(B) Cells in (A) were treated with increasing dose of SLLN06. Treatment of several breast cancer cell lines with SLLN06 inhibits cell proliferation with comparable efficiency.

(C-D) The percentage of viable cells during treatment with MD82 was determined using 7-aminoactinomycin D (7-AAD) staining assay. 7-AAD is a fluorescent intercalator with high affinity for DNA, and is excluded in live cells but brightly stains necrotic cells. For comparison, cells treated with 5nM of the mitotic inhibitor Taxol display a much greater proportion of necrotic cells.

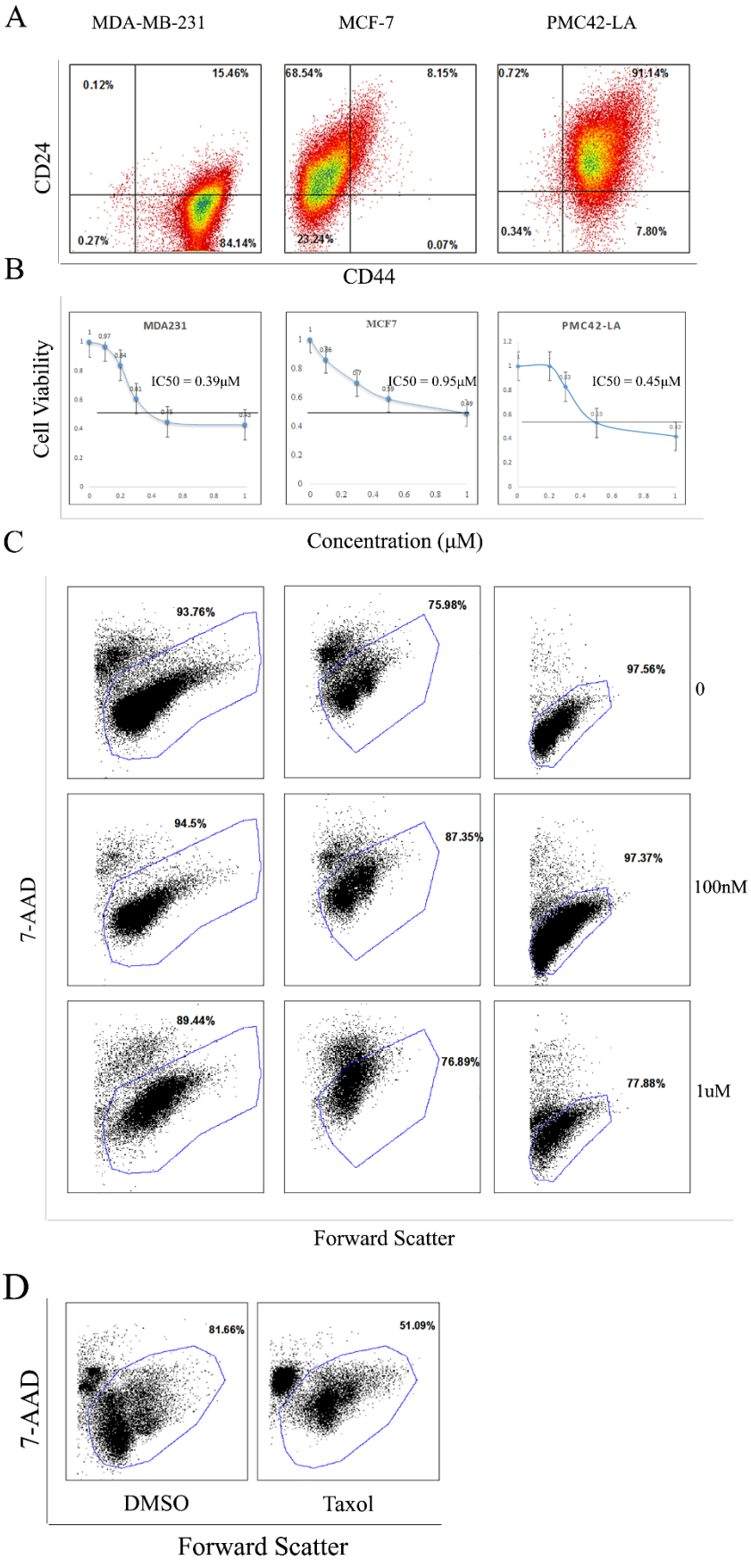
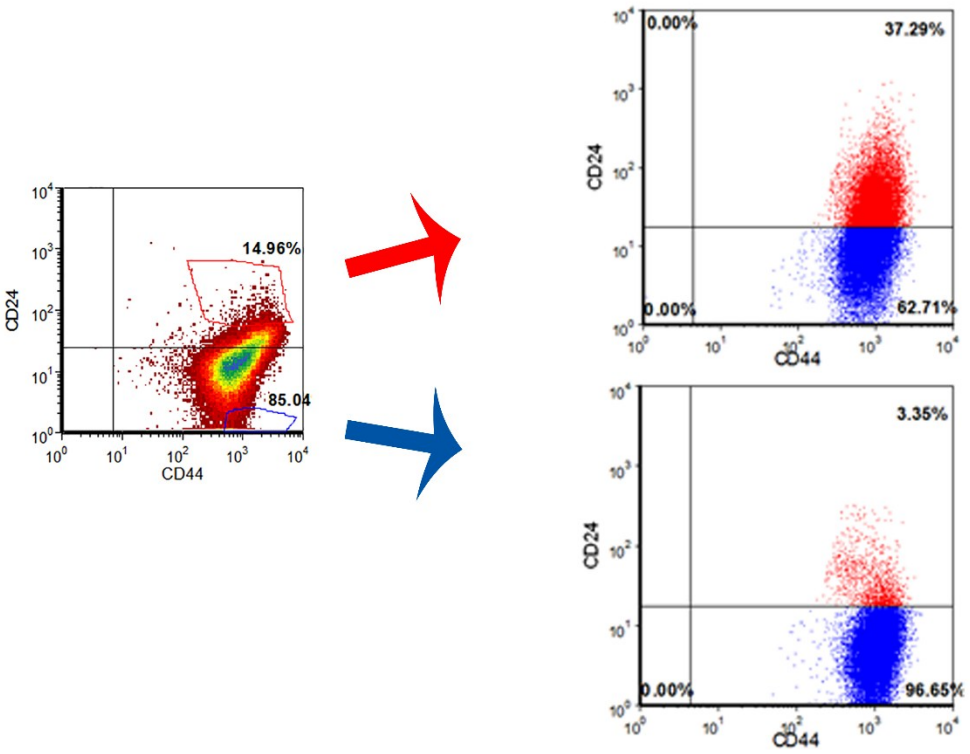

A
MDA-MB-231
MCF-7
PMC42-LA
CD24
CD44
0.12%
15.46%
0.27%
84.14%
68.54%
8.15%
23.24%
0.07%
0.72%
91.14%
0.34%
7.80%
B
Cell Viability
MDA231
IC50 = 0.39μM
MCF7
IC50 = 0.95μM
PMC42-LA
IC50 = 0.45μM
Concentration (μM)
C
7-AAD
93.76%
75.98%
97.56%
0
94.5%
87.35%
97.37%
100nM
89.44%
76.89%
77.88%
1uM
Forward Scatter
D
7-AAD
81.66%
51.09%
DMSO
Taxol
Forward Scatter

Figure 4: SLLN06 offers no proliferative advantage between stem-like and non-stem like cancer cells

(A) An unsorted sample of MDA-MB-231 were sorted using BD FACSfusion according to their expression of CD24 and then reanalyzed after two passages.

(B) The sorted cell population was treated with increasing concentrations of SLLN06 for 96H and the impact on cell survival was quantified using MTT assay. The inhibitory concentrations of SLLN06 against either CD24+ or CD24- sorted cells was presented in the manner of a bar graph.

A


14.96%
85.04
CD24
CD44
0.00%
37.29%
0.00%
62.71%
3.35%
0.00%
96.65%

B

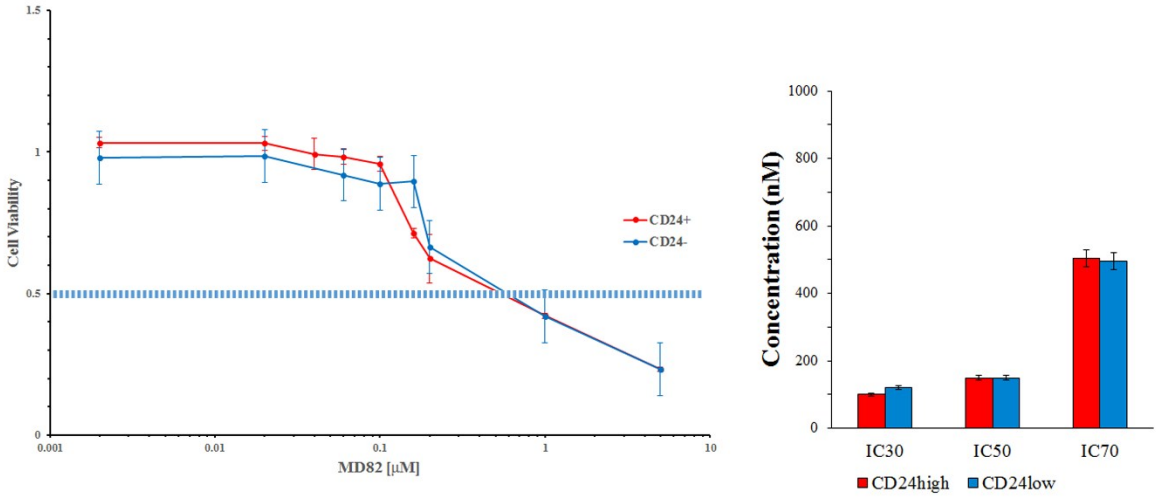

Cell Viability
MD82 [μM]
CD24+
CD24-
Concentration (nM)
IC30
IC50
IC70
CD24high
CD24low

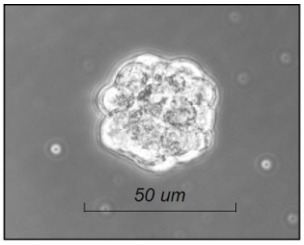
Figure 5: SLLN06 inhibits spheres formation

(A) Representation of mammosphere taken at 40x magnification

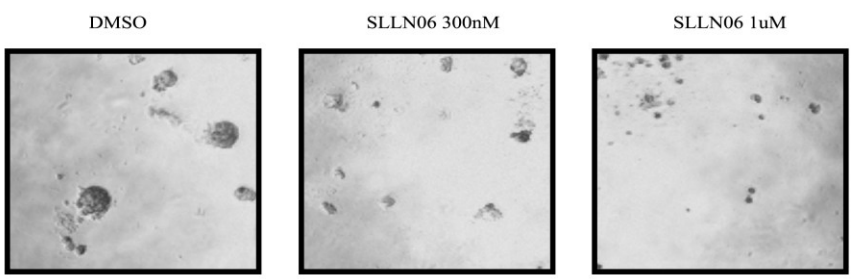
(B) SLLN06 inhibits PMC42-LA organoid formation. PMC42-LA cells grown on matrigel surface as described in methods, differentiates into sphere-like organoids. Upon treatment with SLLN06, the cells are unable to differentiate into organoid structures.

(C) SLLN06 inhibits mammosphere formation in MDA-MB-231 and BT-20. Cells were growth on low-attachment plates as described in the methods section. The number of mammosphere growing after 7 days in culture is presented as the number of counted mammosphere per 10,000 cells seeded. We defined mammospheres as cell clusters that are over 50 micrometers in diamters. The number of cell seeded was previously optimized to minimize cell aggregation.

A


50 um

B


DMSO
SLLN06 300nM
SLLN06 1uM

C

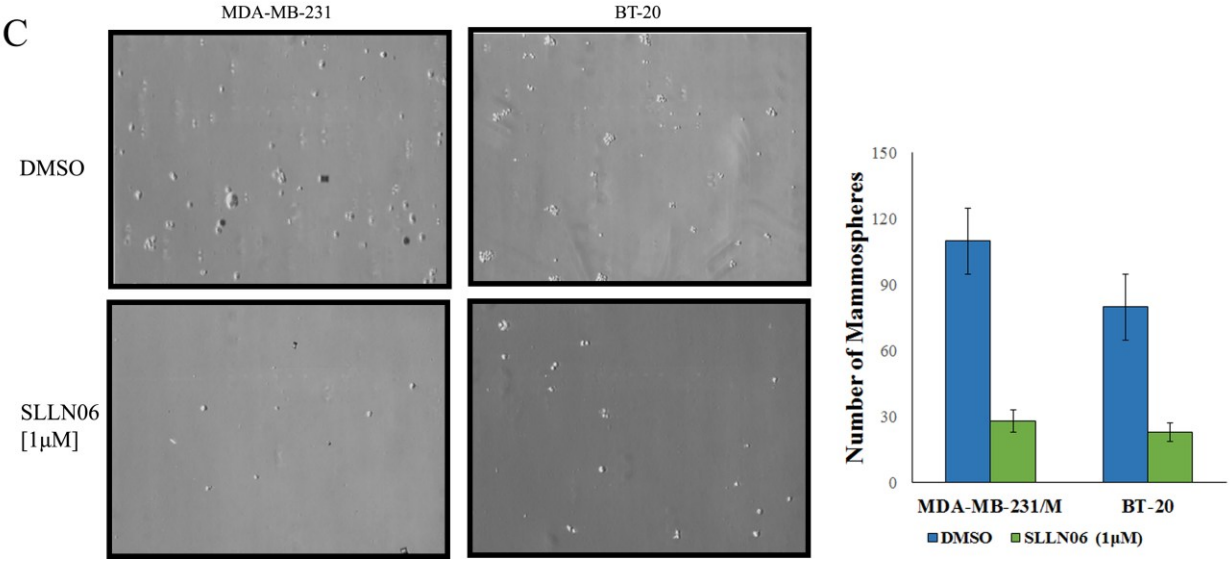
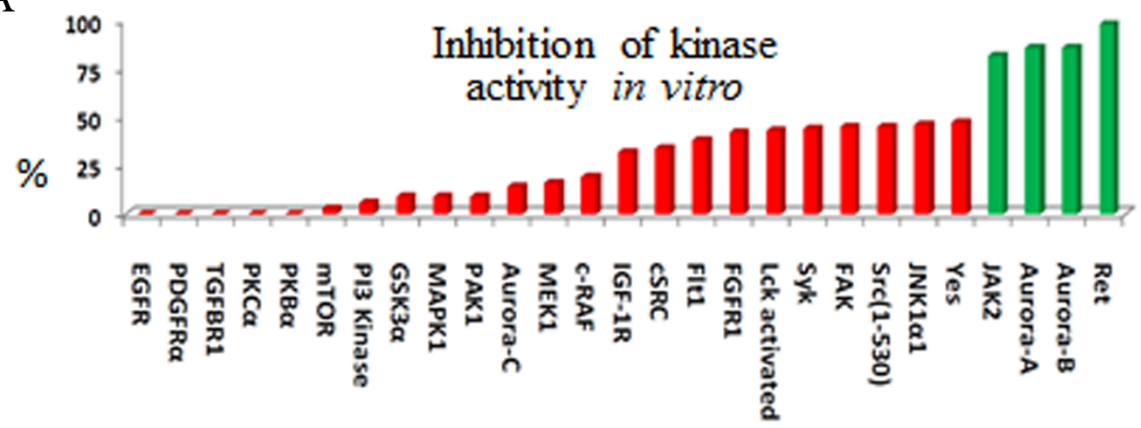

MDA-MB-231
BT-20
DMSO
SLLN06
[1μM]
Number of Mammospheres
150
120
90
60
30
0
MDA-MB-231/M
BT-20
DMSO
SLLN06 (1μM)

Figure 6: SLLN06 is a multikinase inhibitor

(A) High throughput screening shows the inhibitory impact of SLLN06 on a panel of recombinant kinases. Green bars represent the targets whose activity was significantly (>70%) reduced by SLLN06.

(B) Preliminary western blot analysis of total cell extract from MDA-MB-231 cells to evaluate the overall protein level and phosphorylation status of potential SLLN06 targets and other key oncogenes. Cells were treated with 1μM SLLN06 and then stimulated with 20ng/ml EGF to induce global phosphorylation.

A


Inhibition of kinase activity *in vitro*
%
100
75
50
25
0
EGFR
PDGFRα
TGFBR1
PKCα
PKBα
mTOR
PI3 Kinase
GSK3α
MAPK1
PAK1
Aurora-C
MEK1
c-RAF
IGF-1R
cSRC
Flt1
FGFR1
Lck activated
Syk
FAK
Src(1-530)
JNK1α1
Yes
JAK2
Aurora-A
Aurora-B
Ret

B


EGF - + + + + +
SLLN06 [nM] 0 0 100 300 500 1000
p-FAK Y397
FAK
p-Src Y416
Src
p-Ret Y1062
Ret
GAPDH
p-Aurora A T288
Aurora A
p-Aurora B T232
Aurora B
p-Akt S473
Akt
p-Erk1/2
Erk1/2
α-Tubulin

C

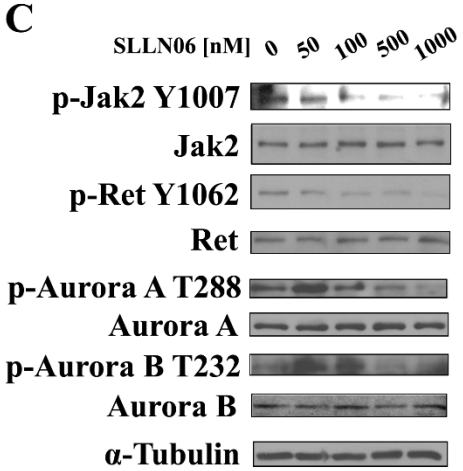

SLLN06 [nM] 0 50 100 500 1000
p-Jak2 Y1007
Jak2
p-Ret Y1062
Ret
p-Aurora A T288
Aurora A
p-Aurora B T232
Aurora B
α-Tubulin

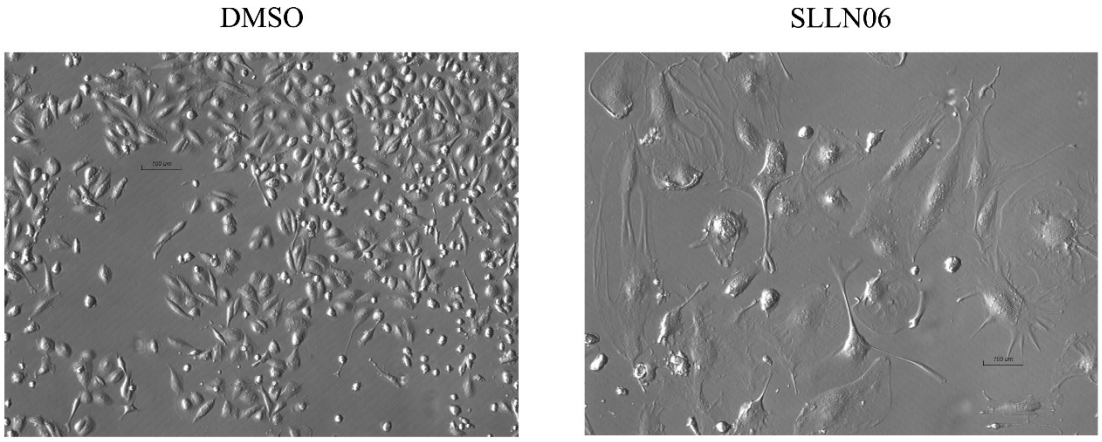
Figure 7: SLLN06 induces polyploidy

(A) Confocal images of MDA-MB-231 cells treated with either DMSO or 1μM SLLN06 after 72H, taken at the same magnification.

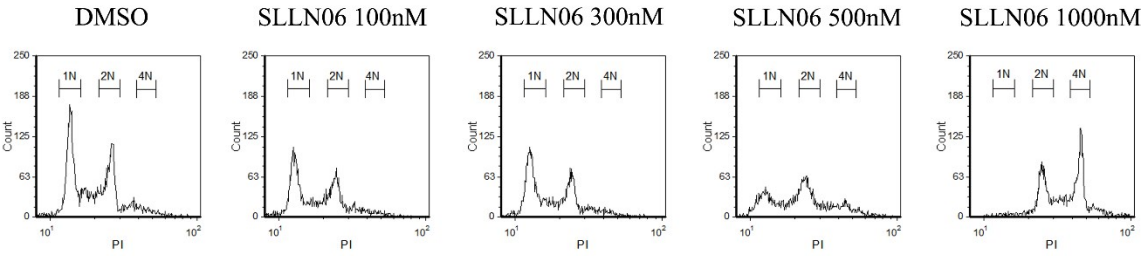
(B) Analysis of the cell cycle by flow cytometry shows that higher concentrations of SLLN06 induces polyploidy. MDA-MB-231 cells were treated with increasing concentrations of SLLN06 for 72H and then cultured for 2 more days. Cells were serum starved overnight to synchronize cell cycle, and treated with 100μg/ml DNase-free RNase prior to quantification. The level of DNA was quantified by PI staining and read using BD FACScalibur.

(C) Quantification of cell cycle analysis summarizing the percentage of total cell count which are either hapoid (1N), diploid (2N), or tetraploid.(4N) as determined by the peaks observed in (B)

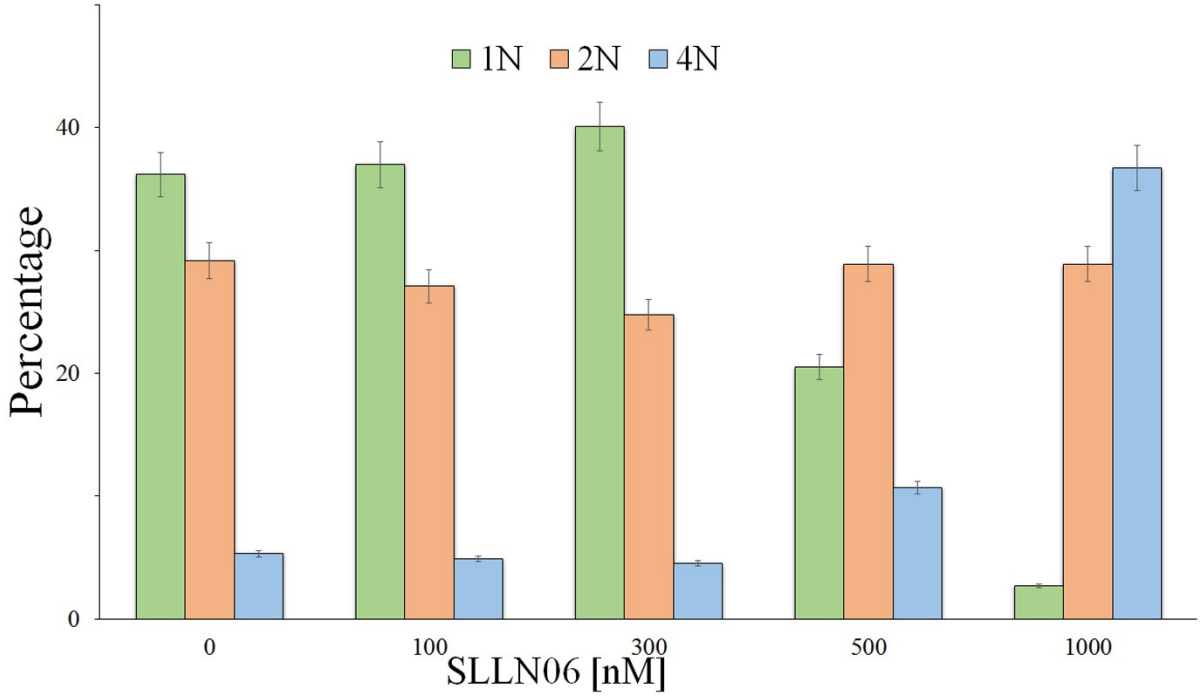
A


DMSO
SLLN06

B


DMSO
SLLN06 100nM
SLLN06 300nM
SLLN06 500nM
SLLN06 1000nM
1N
2N
4N
Count
PI

C


1N
2N
4N
Percentage
40
20
0
0
100
300
500
1000
SLLN06 [nM]

Compound	$CD24^{low}$/ $CD44^{high}$ in MDA-231 (%)	$ALDH^{high}$ in BT-20 (%)	Known primary target(s)
DMSO	87.5 ± 7.5	42.4 ± 9.6	-
SLLN06	34.4 ± 2.1	4.3 ± 1.2	Multi-kinase inhibitor
Reversine	53.2 ± 4.2	40.3 ± 7.2	Multi-kinase inhibitor [36]
Retreversine	86.5 ± 9.5	-	Inactive isomer of reversine [36]
Salinomycin	75.1 ±7.6	31.5 ± 6.5	Cancer stem cell signaling [33]
U1026	86.7 ± 5.4	18.3 ± 2.4	MEK1/2 inhibitor [39]
PF57	82.8 ±12.5	72.3 ±15.1	FAK inhibitor [40]
Tozasertib	42.2 ± 9.5	56.1 ± 8.2	Aurora kinases A-C inhibitor [41]
Vandetanib	72.4 ± 6.5	48.9 ± 9.3	VEGF, EGFR, RET [42]
RO4929097	80.0 ± 8.9	59.48 ± 5.9	y-secretase/Notch [43]
IPW-2	78.3 ± 6.7	67.3 ± 8.2	WNT [44]
Cabozantnib	79.3 ± 4.2	95.6 ± 8.5	MET, VEGFR2 [45]
XL-109	81.2 ± 4.2	58.9 ± 9.3	JAK2[46]

Table 2: SLLN06 has a unique feature compared to commercially available kinase inhibitors

Subconfluent cells were exposed to each molecule at a concentration of 100-500nM for 72h and then cell surface markers were Quantified using FACS analysis, as described in methods. Control cells were exposed to the vehicle alone. Shown are the average ± SD of at least 3 independent experiments each in duplicates.

Figure 8: SLLN06 inhibits cancer metastasis *in vivo*

(A) Table showing the number of distant metastasis and primary tumor weight resulting from xenograft of human tumor cell lines. SLLN06 was administered intraperitoneally 3 times a week for four cycles. The vehicle alone was administered in parallel as negative control. 4T1M, MDA-231/M and PC3/M are aggressive variants of their wild type counterparts.

(B) Bar graphs illustrating the impact of SLLN06 on distant metastasis and primary tumor weight respectively.

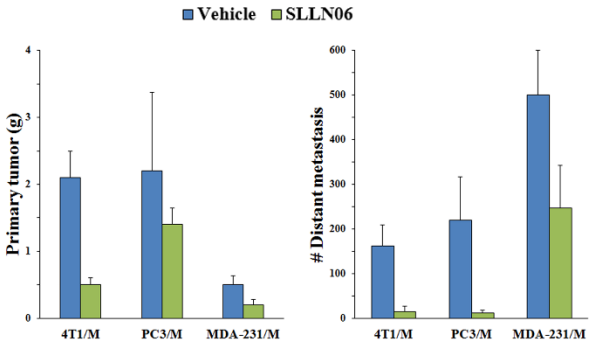
(C) Representative photos of lungs taken from vehicle and SLLN06 treated SCID mice injected with MDA231/M

(D) Snapshots of various timepoints from a live locomotion assay illustrating SLLN06's impact on in vitro cell mobility. The cell speed was quantified using Velocity software, and the results are summarized in a manner of box and whisker plot.

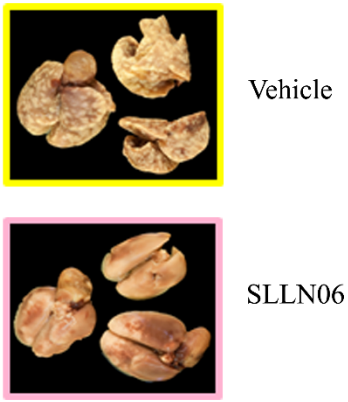
A

	# Distant metastasis		Primary tumor (g)	
	Vehicle	SLLN06	Vehicle	SLLN06
4T1	161±48	14±12	2.1±0.39	0.5±0.10
PC3/M	220±97	11±7	2.2±1.18	1.4±0.24
MDA-231/M	≥500	247±96	0.5±0.13	0.2±0.08

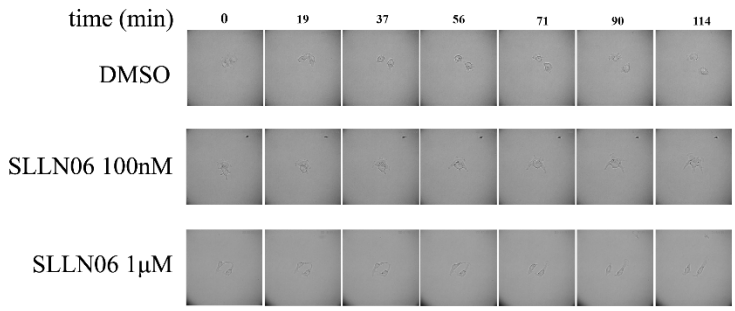
B

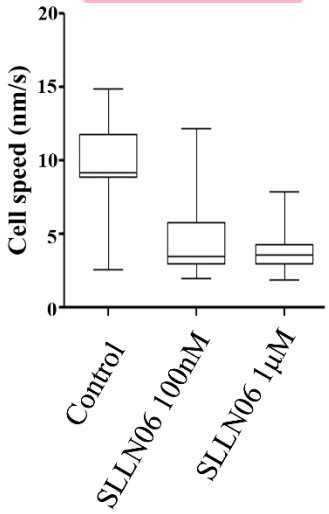


C



D





2.4 Discussion

We identified a small molecule that had potent anti-metastatic activity *in vivo,* and propose that the effect observed is at least partially attributable to cancer cell reprograming through its kinase inhibition activity. In particular, SLLN06 was shown to reduce the expression of canonical cancer stem cell markers $CD24^{low}/CD44^{high}$ and $ALDH^{high}$. While this is an interesting phenomenon in and of itself, our data further suggests that the reduction in stem cell markers is not solely due to reduced proliferation, since cells that are sorted for $CD24^{high}$ versus $CD24^{low}$ were independently treated with SLLN06 have equal cell viability under drug treatment. Moreover, the reduction in distant metastatic colonies were more much profound than the reduction in primary tumor size, and the live locomotion assay showed an evident reduction in cell mobility. The spheroid forming assays both in low-attachment plating and on matrigel showed a dramatic reduction in formation of spheres, with a lesser impact on the total number of floating clones, suggesting that the compound is specifically inhibiting the self-renewal capacity of the cells placed in spheroid growth rather than simply promoting cell death.

The main targets of our small molecule, Aurora Kinase, Jak2, and Ret all have previously reported oncogenic or proto-oncogenic activity [47] [48] [49]. Over the last few years, the aurora kinase family has emerged as highly attractive candidate for molecular therapy [49]. On study showed that Aurora kinase A maintains ES pluripotency through p53 inhibition [50]. In addition, aurora kinase A and B are frequently overexpressed in human cancers and are key regulators of genome stability [51]. Overexpression of aurora kinase A promotes distant metastasis in ER+ breast cancer[52], promotes cancer cell survival[53], and is suggested to drive tumorigenesis by inhibiting autophagy[54]. Inhibiting aurora A has reported therapeutic potential against ovarian cancer stem cells [55]. Functionally, aurora kinase inhibition induces polyploidy, the accumulation of more than 2 pairs

of chromosomes [56]. Polyploidy is essential to differentiation in select tissues during normal mammalian development [57]. A Sox2 mutation in mouse embryonic stem cells has previously shown to induce trophoectoderm differentiation through polyploidy[58], and in cancer therapy, the induction of polyploidy has been reported to reprogram leukemia cells towards terminal differentiation [37]. While the mechanics of polyploidy-induced differentiation is not clear, it is conceivable that the extra chromosomes would largely impact the epigenetic landscape, and we intend to explore whether the reprograming effects is perhaps conferred through selective global gene silencing of duplicate chromosomes.

Jak2 is a growth-hormone associated tyrosine kinase [59] that is crucial for cytokine signaling. Although Jak2/Stat5 signaling is highly involved in mammary gland development and breast cancer progression, the activation of its secondary substrate, Stat3, is perhaps even more relevant in metastatic cancer [60]. Stat3 was independently identified as an oncogene [61], and it's also an important factor for maintaining stemness in embryonic stem cells [62]. Notably, the IL-6/Jak2/Stat3 signaling axis is preferentially active in the aggressive basal subtype breast cancer [48]. Furthermore, an independent study confirmed that Stat3 signaling is crucial for maintenance of CD24-/CD44+ and ALDH+ breast cancer subpopulations [63]. Surprisingly, the Jak2-selective inhibitor XL-019 had no apparent effect on proliferation or biomarker expression of MDA-MB-231 or BT-20, both basal-type cell lines, and we intend to further investigate whether Jak2 inhibition was properly achieved using the commercially obtained compound. Interestingly, CD24 is also an activator of STAT3 through c-Src in an integrin-dependent fashion [64 65], and we suspect that SLLN06 mediated inhibition of Jak2 could concurrently increase CD24 as part of a feedback response.

Recently, autophagy has been shown to be a key regulator of the Jak2/Stat3 signaling pathway [66], and interestingly, the cancer stem-cell selective compound salinomycin was recently

reported to induce its selective activity by disrupting breast cancer autophagy [67]. Furthermore, autophagy inhibition was found to be particularly detrimental against triple-negative breast cancer due to their dependence on constitutive STAT3 activation [68]. Autophagic activation of Jak2/Stat3 promotes cancer cell survival through secretion of survival growth factors including IL-6 [69], very similar to the inflammatory feedback loop that was reported to expand cancer stem cells in Her2-targeted therapy [70]. Inhibition of autophagy enhanced the expression of CD24 in breast cancer stem cells while silencing the expression of mesenchymal markers by TGF-β stimulation[71]. Ret and Src, both activators of FAK, can be selectively degraded by autophagy to maintain cancer cell survival under cell adhesion failures[72], and it has been shown that activation of autophagy enhances the potency of Ret tyrosine kinase inhibitors in medullary thyroid cancer, and is possibly a contributing factor to our compound's efficacy against the 8505c thyroid cell line [73]. Furthermore, a small molecule inhibitor of autophagy, chloroquine, was reported to enable chemotherapeutic elimination of cancer stem cells by disregulating Jak2 and the epigenetic factor Dnmt1, illustrating an approach that combined targeting of transcriptional and epigenetic factors[74].

Currently there are very few effective to clinically reprogram cancer cells, where the only successful approach applied only to highly specific cancers subtype [75]. Many key developmental pathways, including Wnt and Notch are currently under investigation as potential targets for cancer therapy (reviewed in. [76]). However, while there are vast amount of literature regarding their role in normal cell differentiation and cancer progression, their relevance in specifically cancer differentiation is not as clear. This is because even though cancer differentiation status has long been accepted as a predictor of clinical outcome [77], the thought of cancer as a dynamic body of differentiating cells with a hierarchical organization is a relatively newer concept. Nevertheless, recent high throughput studies to identify CSC selective molecules did not identify any hallmark

differentiation or pluripotent pathways as cancer stem cell-specific targets, and instead identified "esoteric" targets such as dopamine receptor or potassium channels [33] [78]. During normal development, differentiation and dedifferentiation are highly context-specific and tightly controlled, and it's conceivable that cancer cells may be more resilient to the effects of differentiation signals due to taking on a differentiation state that's no longer contextually relevant to "traditional" differentiation signals (although those signals could still confer proliferative or anti-proliferative effects). Conversely, it is possible that cancer cells are more susceptible to reprograming from alternative, more functional approaches such as autophagy modulation or global epigenetic remodeling.

2.5 Materials and methods

Chemistry

The synthesis method of SLLN06 is described in details in the patent application: McGill University Report of Invention #14075 (*Molecules and methods for targeting metastatic and refractory cancer and use thereof*, 2014). Briefly, SLLN06 was identified from a high-throughput library we generated focusing around scaffolds of the de-differentiation agent reversine and of molecules we identified earlier to target focal adhesion signaling (*Benzisoselenazolone-derived molecules targeting the cell invasion protein complex, their pharmaceutical compositions and methods of use*. US application #61/377 504). MD32 was selected based on its superior potency to target cancer cells expressing stem cell markers. The structure/purity of SLLN06 was confirmed by ^{1}H NMR, ^{13}C NMR and HRMS.

Cell culture

Cancer cell lines: MDA-MB-231, BT-20, MCF-7, SUM-149, 4T1, and PC3 were obtained from ATCC. Epithelial breast adenocarcinoma cell line PMC42-LA were kindly provided by Dr. *Ackland*. The thyroid carcinoma cell line 8505c were provided courtesy of Dr. Trifiro. SUM-149 cell line was maintained in Ham's F-12 supplemented with 5% Fetal Bovine Serum, Insulin (5ug/ml) and Hydrocortisone (1ug/ml). All other cancer cell lines were cultured in RPMI1640x with 10% fetal bovine serum with 1% penicillin/streptomycin, and maintained at 37^{o}C in 5% atmosphere of CO2. In the case of growth factor stimulation, cells were serum starved for no longer than 12H, and then stimulated with 20ng/ml EGF for 40 minutes.

Antibodies and reagents

Primary antibodies: E-Cadherin rabbit polyclonal 24E10 from cell signaling; N-cadherin rabbit polyclonal antibody H-63 from Santa Cruz Biotechnology; p44/42 MAPK $_{\text{antibody}}$ from Cell Signalling; Erk2 from Santa Cruz Biotechnology;Mps1 antibody clone 4-112-3 from Millipore; c-Myc antibody from epitomics; Phospho-Akt antibody (Ser473) from Cell Signalling; Akt1 antibody from Cell Signalling; Phospho-Aurora A (Thr288)/Aurora B(Thr 232)/ Aurora C (Thr198) antibody from Cell Signalling; phospho-Ret (Tyr1062) from abcam; Ret (C31B4 clone) from Cell Signaling; phosphor-FAK (Tyr397) from Invitrogen; FAK (clone 4.47) from upstate cell signaling solutions; phosphor-JAK2 (Tyr1007/Tyr1008) from Santa Cruz Biotechnology; JAK2 (C-20 clone, goat) from Santa Cruz Biotechnology. Secondary antibodies: Goat anti-rat IgG conjugated to Alexa Fluor 647 from Cell- Signaling; Goat anti-rabbit IgG conjugated to Cy2 from Millipore. Conjugated primary antibodies: Alexa Fluor 647 anti-human CD24 antibody; FITC anti-human CD44 antibody from biolegend. Growth factors: Recombinant human EGF from Invitrogen. Small Molecules: Reversine, Retreversine, Salinomycin, IPW-2 and Vandetanib from Cayman Chemicals; Cabozantinib from Selleck Chem; RO4929097 from BioVision; U0126 from Cell Signaling; PF-573228 from Tocris Bioscience.

FACS analysis and sorting

The levels of CD24 and CD44 were co-analyzed using flow cytometry. Single suspended cells were collected after digestion with 0.25% trypsin. Live cells were suspended in blocking buffer (1% BSA and 10% FBS in PBS with 0.01% sodium azide) for 30 minutes at room temperature before staining with conjugated primary antibody for 1 hour, followed by 3 PBS washes. Staining was done in 100µL blocking buffer. 5 µl of CD24 conjugated to Alexa 647 and

CD44 conjugated to FITC were used per 10^6 cells. Non-viable cells were gated out by co-staining with 7-AAD. Cell fluorescence was measured using BD Bioscience FACScalibur flow cytometer, using the FL-1 and FL-4 channel for CD44 and CD24 respectively. The samples were analyzed by flow cytometry using BD FACScalibur and the data was analyzed using FCS Express software. For cell sorting, identical antibody staining conditions were applied but under sterile conditions. Stained cells were sorted separately into $CD24^{high}$ and $CD24^{low}$ using BD FACSaria Fusion cell sorter.

Cell cycle analysis

Cell cycles were synchronized prior to treatment through overnight serum deprivation. After treatment, cells were fixed and permeabilized using BD Cytofix/Cytoperm reagent (BD Bioscience), washed with PBS, and treated with 100µg/ml Bovine Pancreas RNase A (US Biological). Cells were then stained with 25µg/ml propidium iodide (Sigma) for 15 minutes and then immediately analyzed by flow cytometry using BD FACScalibur. The data was analyzed using FCS Express software.

Analysis of ALDH activity

The enzymatic activity of ALDH was detected using ALDEFLUOR staining kit (StemCell Technologies). Trypsinized single-cells were suspended in the ALDEFLUOR assay buffer and incubated with 1.5µM BODIPY™-aminoacetaldehyde (BAAA) per 1 x 10^6 cells for 30 minutes at 37ºC. As a negative control to establish background fluorescence level, a separate sample was treated with 15uM diethylaminobenzaldehyde, a selective ALDH inhibitor. In order to remove non-viable cells from analysis, the cells were stained with 7-AAD.

Mammosphere culture and organoid differentiation assay

The procedure for mammosphere culture assay was adapted from: [79]. After treatment with 1μM SLLN06 for 72H, MDA-MB-231 and BT-20 cells were digested with 0.25% trypsin and reseeded at 2.5 x 10^4 cells/ml in 60mm Ultralow Adherence plates (Corning) in DMEM/F12 supplemented with 5 ug/ml human insulin, 20ng/ml human epidermal growth factor, 0.5 ug/ml hydrocortisone and 2% B27 (Invitrogen). The procedure for cell culture on matrigel was adapted from: [32] with some modifications. 300 microliters of matrigel membrane matrix from BD bioscience was spread over a 24-well plate and then left to set in 37ºC. The appropriate concentrations of SLLN06 were premixed into the matrigel solution prior to plating. The solidified matrigel surface was washed twice with RPMI 1640, and then 100 microliters of RPMI containing 4000 PMC42-LA cells with the appropriate concentration of SLLN06 was then plated on top.

Cell proliferation (MTT) and cell necrosis assay

To quantify cell proliferation, exponentially growing cells were seeded in 96-well plates at a density of 5×10^3 cells per 200 μl well and left undisturbed for 24 h. Cell medium was removed at this time and replaced with medium containing the corresponding compound at the proper concentrations (dissolved in DMSO (<1% final concentration) and then incubated at 37 °C for 96 h. Cell proliferation was evaluated 96 h later using the 3-(4,5-dimethylthiazo-2-yl)-2,5-sdiphenyltetrazolium bromide (MTT) metabolic assay. Control samples received the vehicle (DMSO) alone. To quantify cell necrosis, live suspended cells were incubated with 7-aminoactinomycin D (7-AAD) in PBS with 5% FBS and 1% BSA to stain nuclear DNA. Nuclear staining was measured using BD Bioscience FACScalibur flow cytometer.

Western blot analysis

Western blotting analysis were carried out on total cell extracts from exponentially growing cells collected by scrapping into modified radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 1mM sodium orthovanadate, 1mM PMSF and Protease Inhibitor Cocktail (Roche). Blots were detected using appropriate antibodies and signal detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) detection system. When indicated, membranes were subsequently stripped for reprobing using stripping buffer from Gene Bio-Application Ltd.

Kinase activity profiling

An aliquot of SLLN06 was submitted for high throughput *in vitro* kinase profiling using the SelectScreen Kinase Profiling Service from Invitrogen.

Live cell locomotion assay

Cells were seeded at very low density on multi-well chambered coverglass (LabTek, Rochester, NY, USA). After starving, cells were stimulated with 10ng/mL EGF and plated on a heated humidified stage supplied with 5% CO_2. Phase contrast time-lapse images were captured every five minutes for three hours by optimized Nipkow spinning disk confocal microscope (WaveFx spinning disk, Quorum Technologies Inc, Guelph, ON, Canada). Cell motility was measured by tracing the cell periphery manually using Volocity software (Perkin Elmer, Waltham, MA, USA).

In vivo orthotopic model of breast cancer metastasis

In vivo studies were approved by McGill Animal Care Committee (protocol #4101). Cells growing in exponential phase of growth ($1x10^6$ cells/ mouse) were transplanted into the mammary fat pad of female SCID mice. When primary tumors become palpable mice were randomized and blindly assigned treatment groups. Treatment was given intraperitoneal for 4 cycles (3 times a week: day 1, day 3, day 5) (n=8-10 mice per condition). Control mice received the vehicle alone (1% DMSO in physiologic solution). A pilot toxicity study confirmed that this schedule using SLLN06 at doses even superior to 30mg/kg has no apparent toxicity and no body weight loss. Tumor size was measured using a calipter. At the study termination (60-90 days) primary tumors were excised and weighted. The lungs were fixed in 10% Bouin's fixative and lung surface metastases were counted using a stereomicroscope. Statistical analysis was performed as we described earlier [80, 81].

Summary and Discussion

The intent of this project is to lay the foundation for further investigation of chemical reprograming of cancer cells. Chapter 1 demonstrates the broadness of the cellular program regulating normal and cancer cell "state", and illustrate how there is at least proof of concept that these factors can be modulated by small molecule activity. Chapter 1 also explored the integration of the cell differentiation program into advanced cancer progression, which provided the rationale for exploring anti-metastatic effects of small molecule agents that can modulate normal cell differentiation status.

We identified SLLN06 largely through flow cytometry screening for compounds that can impact cancer stem cell markers. Flow cytometry is an experimental assay to quantify fluorescence intensity of individual cells. Its main advantage is the ability to not only detect very rare subpopulation of cells that would otherwise be undetectable by protein or RNA analysis, and to detect minor shifts the expression of markers (say 5% positive to 9% positive) that would be more difficult to quantify via immunofluorescence. Furthermore, the assay enables the detection of live cells and can be used to selectively analyse only membrane-bound target proteins, which reduces noise from cytoplasmic proteins. Conversely, the cells can also be fixed and permeabilized by reagents such as paraformaldehyde and methanol for the detection of cytoplastmic proteins. In our experiments, we used flow cytometry extensively to quantify the expression of cancer stem cell markers CD24 and CD44, as well as CD133, CD47, c-MET, and aldehyde dehydrogenase (ALDH) activity. Unlike the other cell surface markers, ALDH is a cytoplasmic enzyme and is detected via the addition of a substrate that converts to a fluorescent form by ALDH activity. On the other hand, large scale screening by flow cytometry is relatively expensive due to high usage of unrecyclable antibodies, and each experiments need to conducted in parallel with unstained samples and isotype

controls (antibodies that retain non-specific binding functions of the primary antibody), in order to establish the background fluorescence intensity. Furthermore, multiple isoforms of ALDH can contribute to the enzymatic activity, which can lead to confounding results.

We used an *in vitro* kinase assay using recombinant proteins to help narrow the molecular targets of SLLN06, and the screening identified a high level of inhibition against Ret, Jak2, Aurora A and Aurora B at 500nM and 1uM. From there, we used western blot analysis to investigate the impact of SLLN06 on signal transduction pathways, by measuring the level of protein phosphorylation of key kinases including the kinases identified by the kinase screening, and other central kinases such as MAPK, AKT, and PKC. The complete phosphorylation profile after compound treatment is still a work in progress, as we intend to investigate multiple time points and concentrations. Current data shows that SLLN06 heavily inhibits p-Aurora A and p-Aurora B, with some lesser impact on Ret and Jak2. It is possible that the discrepancy in inhibitory activity could be due to stronger autophosphorylation activity of the aurora kinases relative to Jak2 and Ret, and as a result we intend to further investigate downstream activity of Jak2 and Ret signaling including the phosphorylation of various STAT factors.

As discussed in chapter 1, the epithelial mesenchymal transition (EMT) program is believed to be an autonomous and reversible transdifferentiation process that enables cancer metastasis, and represents another facet of cell reprograming. While not shown in chapter 2, this project also consisted of building effective EMT models (select data shown in figure S1 and figure S2). We built and characterized multiple models for EMT, including EMT induced by TGFß in the normal murine mammary gland (NMuMG) cell line, EMT induced by EGF in the PMC42-LA cell line, and EMT induced by stable transfection of transcription factors Snail and Twist in an immortalized normal human epithelial cell line (HME1). We also determined the expression of

EMT markers in MDA-MB-231 and SUM-149 cell lines, following a 5-day treatment with SLLN06 at increasing doses. By western blot we have shown that while there's differences in the basal expression level of various EMT markers (high expression of cadherins and twist/snail in SUM149, but higher vimentin in MDA-231), there is little difference after treatment with SLLN06 (Figure S3). We have also characterized cancer stem cell markers and epithelial/mesenchymal markers of aggressive breast cancer cell lines that were re-isolated following metastatic dissemination in mice (Not shown).

Also not shown in chapter 2 are wound healing assays (Figure S4) illustrating the reduced migratory potential of MDA-231 cells following SLLN06 treatment, and qPCR quantification of mRNA transcript for CD24, CD44, and multiple ALDH isoforms including ALDH1A1, ALDH1A3, and ALDH2. We received some encouraging results, particularly in the SUM-149 inflammatory breast cell (Figure S5– qPCR figure is offered by Hind Azmil). However, the difference in expression was found to be not statistically significant, and we suspect that the change in gene expression may be diluted by the heterogeneity of the breast cancer cells where only a subset of cells is experiencing an increasing in CD24 gene expression; we intend to further investigate the gene expression of cancer stem cell markers but in sorted clones to reduce heterogeneity.

As discussed in chapter 2, the putative targets of SLLN06 have multiple implicated roles in cell differentiation program and maintenance of a cancer stem cell phenotype. Multiple pilot studies will need to be conducted to identify potential mechanisms involved, such as epigenetic remodelling and autophagy modulation. These mechanisms will be explored individually and in detail in order to fully elucidate the extent of reprograming capacity of small molecule inhibitors. Unraveling the complexity intracellular changes from multi-kinase inhibition is a necessary step

towards in-depth optimization of chemical structure and a closer step to fully understanding the complex molecular circuitries involved in metastatic cancer progression.

Supplemental data

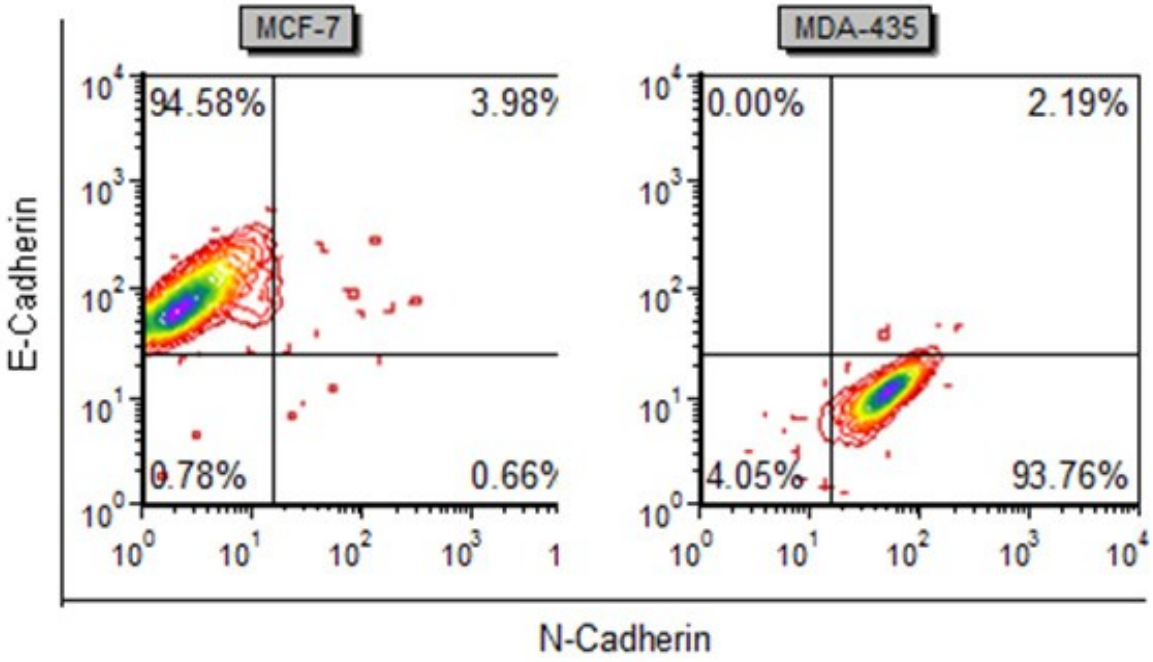
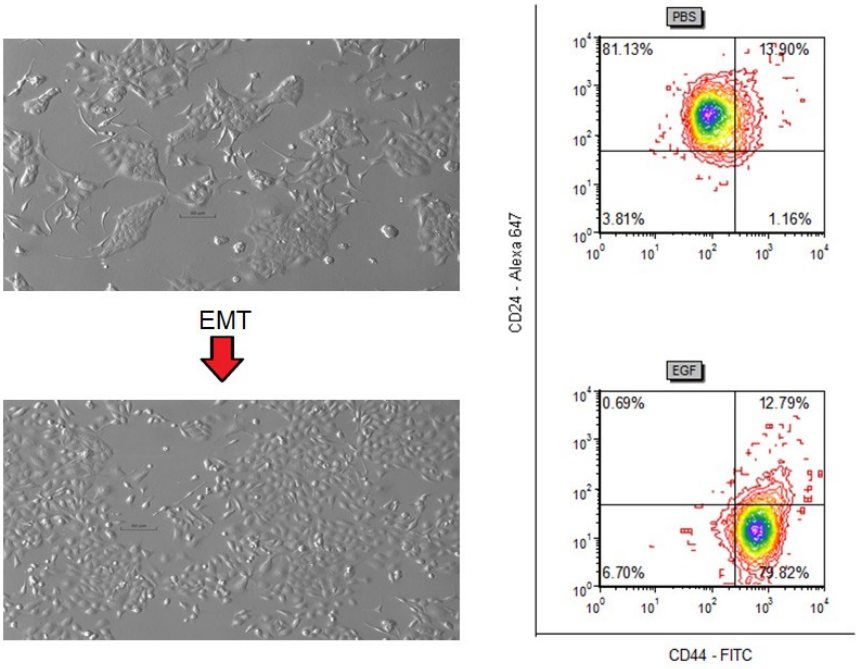


Figure S1: Quantification of E-cadherin and N-cadherin by flow cytometry

FACS analysis of epithelial (MCF-7) and mesenchymal (MDA-435) breast carcinoma cell lines for epithelial (E-cadherin) and mesenchymal (N-cadherin) surface marker



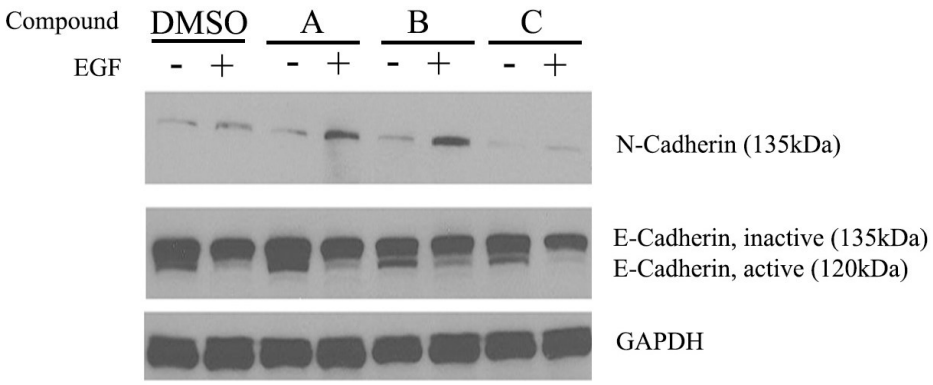


Figure S2: EMT in PMC42-LA

Top- Confocal images and FACS analysis of PMC42-LA cells, showing the morphology change and enrichment of $CD24^{-}/CD44^{+}$ cells following EMT induction by 20ng/mL EGF for 72H.

Bottom – Western blot for epithelial (E-cadherin) and mesenchymal (N-cadherin) marker after 72H EGF stimulation in PMC42-LA cells, alone or in combination with select small molecule from our library.

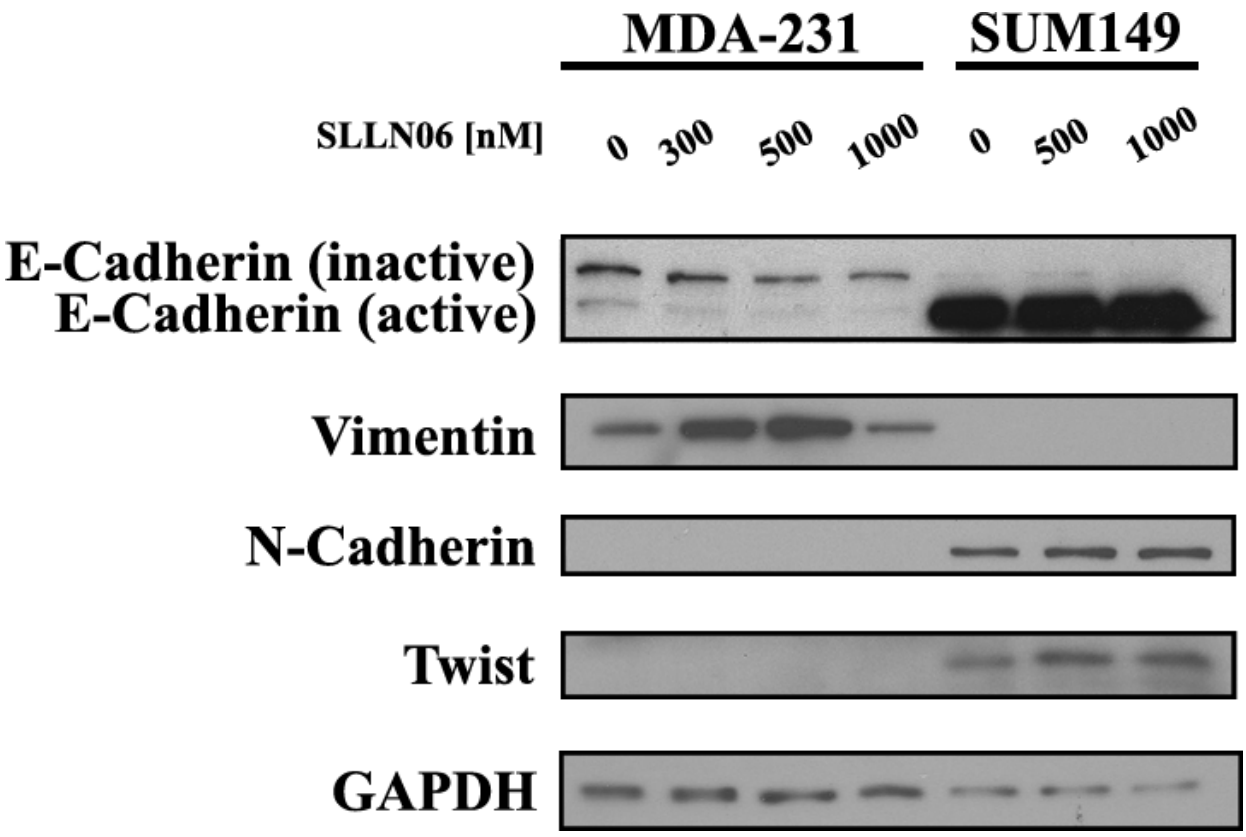


Figure S3: Impact of SLLN06 on EMT markers

Western blot analysis of EMT markers in MDA-MB-231 and SUM-149 after 5 day treatment with SLLN06. Cells were grown in complete RPMI media and the lysates were collected when the cells were at 90% confluence.

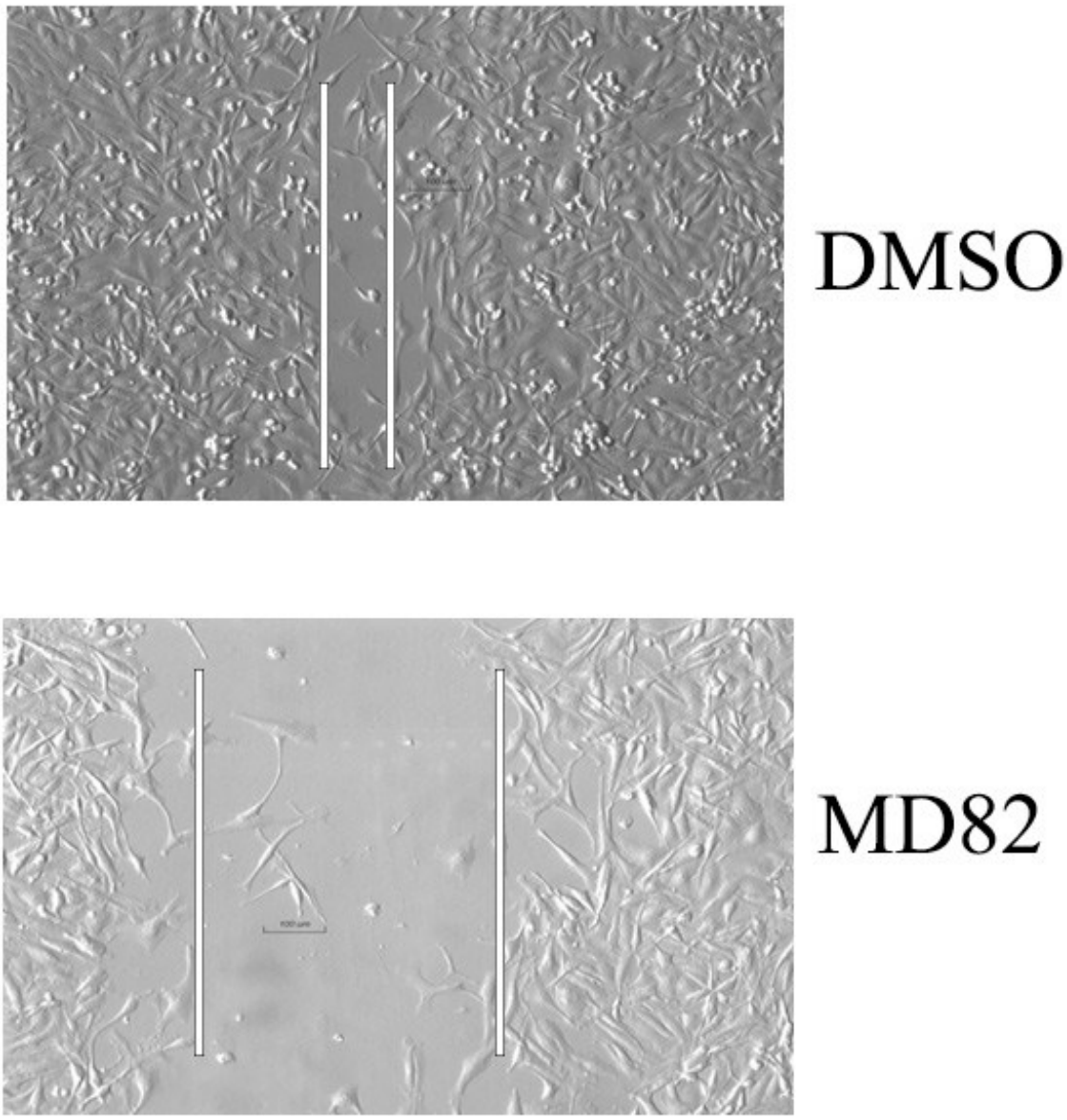


Figure S4: Wound healing assay in MDA-MB-231 cells

Wound healing assay represented by confocal images. MDA-MB-231 cells treated with either 1uM SLLN06 or DMSO were growth to full confluence before a scratch was made using a 100uL plastic pipette tip. Image of the wound was taken after 18H using a confocal microscope.

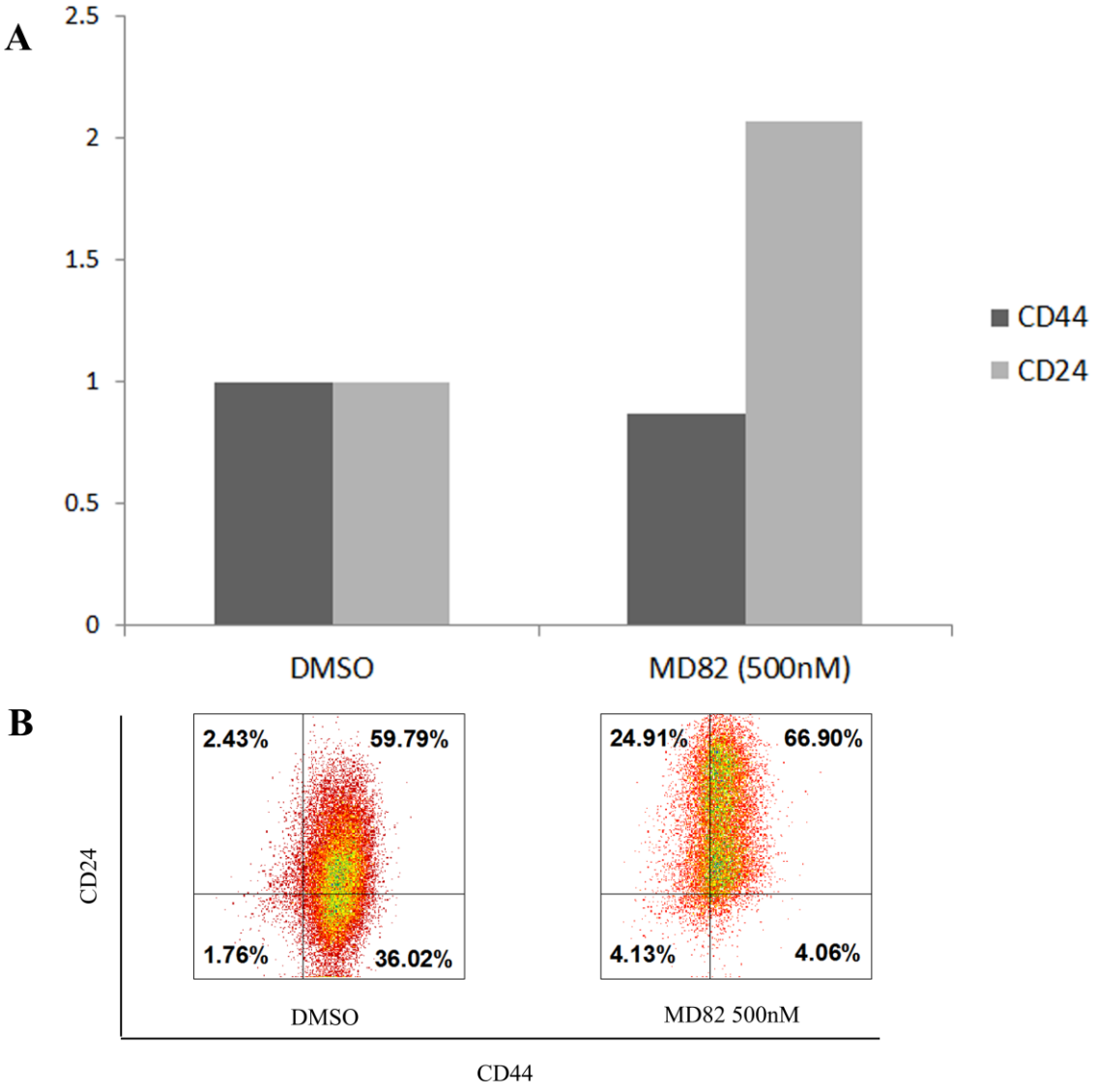


Figure S5: qPCR and FACS analysis of CD24 using SLLN06-treated SUM-149 cells

(A) preliminary qPCR data (provided by Hind Azmil) quantifying the mRNA transcript of CD24 and CD44 in SUM-149 inflammatory breast cancer cell line. However, the change in gene expression is not statistically significant.

(B) Flow cytometry data showing that SUM-149 expressed a higher level of cell-surface CD24 following SLLN06 treatment.

Chapter 1 References

1. Johansson, C.B. et al. Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* **96**, 25-34 (1999).
2. LaBarge, M.A. & Blau, H.M. Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* **111**, 589-601 (2002).
3. Pittenger, M.F. et al. Multilineage potential of adult human mesenchymal stem cells. *science* **284**, 143-147 (1999).
4. Solter, D. et al. in Cold Spring Harbor symposia on quantitative biology 11-18 (Cold Spring Harbor Laboratory Press, 2004).
5. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. *nature* **292**, 154-156 (1981).
6. Spangrude, G.J., Heimfeld, S. & Weissman, I.L. Purification and characterization of mouse hematopoietic stem cells. *Science* **241**, 58-62 (1988).
7. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *cell* **126**, 663-676 (2006).
8. Kim, K. et al. Epigenetic memory in induced pluripotent stem cells. *Nature* **467**, 285-90 (2010).
9. Boulting, G.L. et al. A functionally characterized test set of human induced pluripotent stem cells. *Nat Biotechnol* **29**, 279-86 (2011).
10. Fuchs, E., Tumbar, T. & Guasch, G. Socializing with the neighbors: stem cells and their niche. *Cell* **116**, 769-778 (2004).
11. Ito, K. & Suda, T. Metabolic requirements for the maintenance of self-renewing stem cells. *Nature Reviews Molecular Cell Biology* **15**, 243-256 (2014).
12. Osafune, K. et al. Marked differences in differentiation propensity among human embryonic stem cell lines. *Nature biotechnology* **26**, 313-315 (2008).
13. Tapscott, S.J. et al. MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science* **242**, 405-411 (1988).
14. Yu, J. et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-1920 (2007).
15. Schöler, H.R., Ruppert, S., Suzuki, N., Chowdhury, K. & Gruss, P. New type of POU domain in germ line-specific protein Oct-4. *Nature* **344**, 435-439 (1990).
16. Silva, J. et al. Nanog is the gateway to the pluripotent ground state. *Cell* **138**, 722-737 (2009).
17. Barron, M., Gao, M. & Lough, J. Requirement for BMP and FGF signaling during cardiogenic induction in non-precardiac mesoderm is specific, transient, and cooperative. *Developmental dynamics* **218**, 383-393 (2000).
18. Niswander, L. & Martin, G.R. FGF-4 and BMP-2 have opposite effects on limb growth. (1993).
19. Song, J., Kim, H.J., Gong, Z., Liu, N.-A. & Lin, S. < i> Vhnf1</i> acts downstream of Bmp, Fgf, and RA signals to regulate endocrine beta cell development in zebrafish. *Developmental biology* **303**, 561-575 (2007).
20. Aruga, J. & Mikoshiba, K. Role of BMP, FGF, calcium signaling, and Zic proteins in vertebrate neuroectodermal differentiation. *Neurochemical research* **36**, 1286-1292 (2011).

21. Marshall, C. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179-185 (1995).
22. Corson, L.B., Yamanaka, Y., Lai, K.-M.V. & Rossant, J. Spatial and temporal patterns of ERK signaling during mouse embryogenesis. *Development* **130**, 4527-4537 (2003).
23. Chazaud, C., Yamanaka, Y., Pawson, T. & Rossant, J. Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. *Developmental cell* **10**, 615-624 (2006).
24. Matsuda, T. et al. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *The EMBO journal* **18**, 4261-4269 (1999).
25. Reubinoff, B.E., Pera, M.F., Fong, C.-Y., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nature biotechnology* **18**, 399-404 (2000).
26. Degos, L. All-trans-retinoic acid treatment and retinoic acid receptor alpha gene rearrangement in acute promyelocytic leukemia: A model for differentiation therapy. *The International Journal of Cell Cloning* **10**, 63-69 (1992).
27. Nowell, P.C. The clonal evolution of tumor cell populations. *Science* **194**, 23-28 (1976).
28. Lapidot, T. et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. (1994).
29. Visvader, J.E. Cells of origin in cancer. *Nature* **469**, 314-322 (2011).
30. Chaffer, C.L. et al. Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. *Proc Natl Acad Sci U S A* **108**, 7950-5 (2011).
31. Notta, F. et al. Evolution of human BCR-ABL1 lymphoblastic leukaemia-initiating cells. *Nature* **469**, 362-367 (2011).
32. Driessens, G., Beck, B., Caauwe, A., Simons, B.D. & Blanpain, C. Defining the mode of tumour growth by clonal analysis. *Nature* **488**, 527-30 (2012).
33. Clayton, E. et al. A single type of progenitor cell maintains normal epidermis. *Nature* **446**, 185-189 (2007).
34. Chen, J. et al. A restricted cell population propagates glioblastoma growth after chemotherapy. *Nature* **488**, 522-526 (2012).
35. Bonnet, D. & Dick, J.E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine* **3**, 730-737 (1997).
36. Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J. & Clarke, M.F. Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences* **100**, 3983-3988 (2003).
37. Singh, S. et al. Identification of human brain tumour initiating cells. *Nature* **432**, 396 - 401 (2004).
38. Li, C. et al. Identification of pancreatic cancer stem cells. *Cancer research* **67**, 1030-1037 (2007).
39. Barker, N. et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**, 1003-1007 (2007).
40. Collins, A., Berry, P., Hyde, C., Stower, M. & Maitland, N. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* **65**, 10946 - 10951 (2005).
41. Muraro, M.G. et al. CD133+, CD166+ CD44+, and CD24+ CD44+ phenotypes fail to reliably identify cell populations with cancer stem cell functional features in established human colorectal cancer cell lines. *Stem cells translational medicine* **1**, 592-603 (2012).

42. Ben-Porath, I. et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* **40**, 499-507 (2008).
43. Mani, S.A. et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704-15 (2008).
44. Reya, T., Morrison, S.J., Clarke, M.F. & Weissman, I.L. Stem cells, cancer, and cancer stem cells. *nature* **414**, 105-111 (2001).
45. Horn, P.A. et al. Expression of AC133, a novel hematopoietic precursor antigen, on acute myeloid leukemia cells. *Blood* **93**, 1435-7 (1999).
46. Corbeil, D. et al. The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. *J Biol Chem* **275**, 5512-20 (2000).
47. Sanai, N., Alvarez-Buylla, A. & Berger, M.S. Neural stem cells and the origin of gliomas. *New England Journal of Medicine* **353**, 811-822 (2005).
48. Horst, D. et al. The cancer stem cell marker CD133 has high prognostic impact but unknown functional relevance for the metastasis of human colon cancer. *The Journal of pathology* **219**, 427-434 (2009).
49. Zeppernick, F. et al. Stem cell marker CD133 affects clinical outcome in glioma patients. *Clinical Cancer Research* **14**, 123-129 (2008).
50. Liu, G. et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer* **5**, 67 (2006).
51. Chen, Y.-C. et al. Oct-4 expression maintained cancer stem-like properties in lung cancer-derived CD133-positive cells. *PloS one* **3**, e2637 (2008).
52. Gotoh, N. Control of stemness by fibroblast growth factor signaling in stem cells and cancer stem cells. *Curr Stem Cell Res Ther* **4**, 9-15 (2009).
53. Dean, M. ABC transporters, drug resistance, and cancer stem cells. *Journal of mammary gland biology and neoplasia* **14**, 3-9 (2009).
54. Resetkova, E. et al. Prognostic impact of ALDH1 in breast cancer: a story of stem cells and tumor microenvironment. *Breast cancer research and treatment* **123**, 97-108 (2010).
55. Debatin, K.-M. & Krammer, P.H. Death receptors in chemotherapy and cancer. *Oncogene* **23**, 2950-2966 (2004).
56. Todaro, M., Francipane, M.G., Medema, J.P. & Stassi, G. Colon cancer stem cells: promise of targeted therapy. *Gastroenterology* **138**, 2151-2162 (2010).
57. Zhou, S. et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nature medicine* **7**, 1028-1034 (2001).
58. Martin, C.M. et al. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. *Developmental biology* **265**, 262-275 (2004).
59. Shimano, K. et al. Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1. *The American journal of pathology* **163**, 3-9 (2003).
60. Watanabe, K. et al. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. *FEBS letters* **565**, 6-10 (2004).
61. Corti, S. et al. Identification of a primitive brain–derived neural stem cell population based on aldehyde dehydrogenase activity. *Stem cells* **24**, 975-985 (2006).

62. Huang, E.H. et al. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. *Cancer research* **69**, 3382-3389 (2009).
63. Ginestier, C. et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell stem cell* **1**, 555-567 (2007).
64. Thiery, J.P. & Sleeman, J.P. Complex networks orchestrate epithelial–mesenchymal transitions. *Nature reviews Molecular cell biology* **7**, 131-142 (2006).
65. Kalluri, R. & Weinberg, R.A. The basics of epithelial-mesenchymal transition. *J Clin Invest* **119**, 1420-8 (2009).
66. Zavadil, J. et al. Genetic programs of epithelial cell plasticity directed by transforming growth factor-β. *Proceedings of the National Academy of Sciences* **98**, 6686-6691 (2001).
67. Santisteban, M. et al. Immune-induced epithelial to mesenchymal transition in vivo generates breast cancer stem cells. *Cancer Res* **69**, 2887-95 (2009).
68. Patrawala, L. et al. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene* **25**, 1696-708 (2006).
69. Klarmann, G.J. et al. Invasive prostate cancer cells are tumor initiating cells that have a stem cell-like genomic signature. *Clinical & experimental metastasis* **26**, 433-446 (2009).
70. Celià-Terrassa, T. et al. Epithelial-mesenchymal transition can suppress major attributes of human epithelial tumor-initiating cells. *The Journal of clinical investigation* **122**, 1849-1868 (2012).
71. Huang, S.D. et al. Tumor cells positive and negative for the common cancer stem cell markers are capable of initiating tumor growth and generating both progenies. *PLoS One* **8**, e54579 (2013).
72. Shipitsin, M. et al. Molecular definition of breast tumor heterogeneity. *Cancer cell* **11**, 259-273 (2007).
73. Horster, M.F., Braun, G.S. & Huber, S.M. Embryonic renal epithelia: induction, nephrogenesis, and cell differentiation. *Physiological Reviews* **79**, 1157-1191 (1999).
74. Paria, B.C., Zhao, X., Das, S.K., Dey, S.K. & Yoshinaga, K. Zonula occludens-1 and E-cadherin are coordinately expressed in the mouse uterus with the initiation of implantation and decidualization. *Developmental biology* **208**, 488-501 (1999).
75. Shih, I.-M. et al. The role of E-cadherin in the motility and invasion of implantation site intermediate trophoblast. *Placenta* **23**, 706-715 (2002).
76. Graff, J.R., Gabrielson, E., Fujii, H., Baylin, S.B. & Herman, J.G. Methylation patterns of the E-cadherin 5′ CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *Journal of Biological Chemistry* **275**, 2727-2732 (2000).
77. Nass, S.J. et al. Aberrant methylation of the estrogen receptor and E-cadherin 5′ CpG islands increases with malignant progression in human breast cancer. *Cancer research* **60**, 4346-4348 (2000).
78. Gunasinghe, N.D., Wells, A., Thompson, E.W. & Hugo, H.J. Mesenchymal–epithelial transition (MET) as a mechanism for metastatic colonisation in breast cancer. *Cancer and Metastasis Reviews* **31**, 469-478 (2012).
79. Liu, S. et al. Breast Cancer Stem Cells Transition between Epithelial and Mesenchymal States Reflective of their Normal Counterparts. *Stem Cell Reports* **2**, 78-91 (2014).

80. Sarrio, D., Franklin, C.K., Mackay, A., Reis-Filho, J.S. & Isacke, C.M. Epithelial and mesenchymal subpopulations within normal basal breast cell lines exhibit distinct stem cell/progenitor properties. *Stem Cells* **30**, 292-303 (2012).
81. Li, L. & Clevers, H. Coexistence of quiescent and active adult stem cells in mammals. *Science* **327**, 542-5 (2010).
82. Yu, M. et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science* **339**, 580-4 (2013).
83. Biddle, A. et al. Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. *Cancer Res* **71**, 5317-26 (2011).
84. Gupta, P.B. et al. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell* **146**, 633-44 (2011).
85. Tsai, C.-C., Su, P.-F., Huang, Y.-F., Yew, T.-L. & Hung, S.-C. Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. *Molecular cell* **47**, 169-182 (2012).
86. Ogawa, M. Differentiation and proliferation of hematopoietic stem cells. *Blood* **81**, 2844-2853 (1993).
87. Chen, S. et al. Reversine increases the plasticity of lineage-committed mammalian cells. *Proceedings of the National Academy of Sciences* **104**, 10482-10487 (2007).
88. Bijian, K. et al. Targeting focal adhesion turnover in invasive breast cancer cells by the purine derivative reversine. *Br J Cancer* (2013).
89. Engler, A.J., Sen, S., Sweeney, H.L. & Discher, D.E. Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677-689 (2006).
90. Li, J. et al. MEK/ERK signaling contributes to the maintenance of human embryonic stem cell self-renewal. *Differentiation* **75**, 299-307 (2007).
91. Schust, J., Sperl, B., Hollis, A., Mayer, T.U. & Berg, T. Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. *Chemistry & biology* **13**, 1235-1242 (2006).
92. Azmi, A.S. et al. Systems analysis reveals a transcriptional reversal of the mesenchymal phenotype induced by SNAIL-inhibitor GN-25. *BMC systems biology* **7**, 85 (2013).
93. Shi, Y. et al. Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. *Cell stem cell* **3**, 568-574 (2008).
94. Huangfu, D. et al. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nature biotechnology* **26**, 795-797 (2008).
95. Kornblith, A.B. et al. Impact of azacytidine on the quality of life of patients with myelodysplastic syndrome treated in a randomized phase III trial: a Cancer and Leukemia Group B study. *Journal of Clinical Oncology* **20**, 2441-2452 (2002).
96. Kantarjian, H.M. et al. Multicenter, randomized, open-label, phase III trial of decitabine versus patient choice, with physician advice, of either supportive care or low-dose cytarabine for the treatment of older patients with newly diagnosed acute myeloid leukemia. *Journal of Clinical Oncology* **30**, 2670-2677 (2012).
97. Balch, C. et al. Antimitogenic and chemosensitizing effects of the methylation inhibitor zebularine in ovarian cancer. *Molecular cancer therapeutics* **4**, 1505-1514 (2005).
98. Brueckner, B. et al. Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. *Cancer Research* **65**, 6305-6311 (2005).

99. Fiskus, W. et al. Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells. *Blood* **114**, 2733-2743 (2009).
100. Helin, K. & Dhanak, D. Chromatin proteins and modifications as drug targets. *Nature* **502**, 480-488 (2013).
101. Schenk, T. et al. Inhibition of the LSD1 (KDM1A) demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia. *Nature medicine* **18**, 605-611 (2012).
102. Zou, H.Y. et al. An orally available small-molecule inhibitor of c-Met, PF-2341066, exhibits cytoreductive antitumor efficacy through antiproliferative and antiangiogenic mechanisms. *Cancer research* **67**, 4408-4417 (2007).
103. Ma, P.C., Schaefer, E., Christensen, J.G. & Salgia, R. A selective small molecule c-MET Inhibitor, PHA665752, cooperates with rapamycin. *Clinical cancer research* **11**, 2312-2319 (2005).
104. Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. & Brivanlou, A.H. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nature medicine* **10**, 55-63 (2003).
105. Ying, Q.-L. et al. The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519-523 (2008).
106. Huang, S.-M.A. et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* **461**, 614-620 (2009).
107. Thorne, C.A. et al. Small-molecule inhibition of Wnt signaling through activation of casein kinase 1α. *Nature chemical biology* **6**, 829-836 (2010).
108. Sequist, L.V. et al. Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. *Journal of Clinical Oncology* **31**, 3327-3334 (2013).
109. Chambers, S.M. et al. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nature biotechnology* **27**, 275-280 (2009).
110. Ichida, J.K. et al. A Small-Molecule Inhibitor of Tgf-β Signaling Replaces< i> Sox2</i> in Reprogramming by Inducing< i> Nanog</i>. *Cell stem cell* **5**, 491-503 (2009).
111. Chen, S. et al. Self-renewal of embryonic stem cells by a small molecule. *Proceedings of the National Academy of Sciences* **103**, 17266-17271 (2006).
112. Melo, S. et al. Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing. *Proceedings of the National Academy of Sciences* **108**, 4394-4399 (2011).
113. Chiu, Y.-L. et al. Dissecting RNA-interference pathway with small molecules. *Chemistry & biology* **12**, 643-648 (2005).
114. Watashi, K., Yeung, M.L., Starost, M.F., Hosmane, R.S. & Jeang, K.-T. Identification of small molecules that suppress microRNA function and reverse tumorigenesis. *Journal of Biological Chemistry* **285**, 24707-24716 (2010).
115. Harrison, M.R. et al. A phase II study of 2-methoxyestradiol (2ME2) NanoCrystal® dispersion (NCD) in patients with taxane-refractory, metastatic castrate-resistant prostate cancer (CRPC). *Investigational new drugs* **29**, 1465-1474 (2011).

116. Wahli, W., Braissant, O. & Desvergne, B. Peroxisome proliferator activated receptors: transcriptional regulators of adipogenesis, lipid metabolism and more…. *Chemistry & biology* **2**, 261-266 (1995).
117. Lee, G. et al. T0070907, a selective ligand for peroxisome proliferator-activated receptor γ, functions as an antagonist of biochemical and cellular activities. *Journal of Biological Chemistry* **277**, 19649-19657 (2002).
118. Seargent, J.M., Yates, E.A. & Gill, J.H. GW9662, a potent antagonist of PPARγ, inhibits growth of breast tumour cells and promotes the anticancer effects of the PPARγ agonist rosiglitazone, independently of PPARγ activation. *British journal of pharmacology* **143**, 933-937 (2004).
119. Gidekel, S., Pizov, G., Bergman, Y. & Pikarsky, E. Oct-3/4 is a dose-dependent oncogenic fate determinant. *Cancer cell* **4**, 361-370 (2003).
120. Bass, A.J. et al. SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. *Nature genetics* **41**, 1238-1242 (2009).
121. Belotte, J. et al. Sox2 Gene Amplification Significantly Impacts Overall Survival in Serous Epithelial Ovarian Cancer. *Reproductive Sciences*, 1933719114542021 (2014).
122. Riggi, N. et al. EWS-FLI-1 modulates miRNA145 and SOX2 expression to initiate mesenchymal stem cell reprogramming toward Ewing sarcoma cancer stem cells. *Genes & development* **24**, 916-932 (2010).
123. Foster, K.W. et al. Increase of GKLF messenger RNA and protein expression during progression of breast cancer. *Cancer Research* **60**, 6488-6495 (2000).
124. Kim, J. et al. A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. *Cell* **143**, 313-324 (2010).
125. Suvà, M.L. et al. Reconstructing and Reprogramming the Tumor-Propagating Potential of Glioblastoma Stem-like Cells. *Cell* **157**, 580-594 (2014).
126. Miyoshi, N. et al. Defined factors induce reprogramming of gastrointestinal cancer cells. *Proceedings of the National Academy of Sciences* **107**, 40-45 (2010).
127. Ohm, J.E. et al. Cancer-related epigenome changes associated with reprogramming to induced pluripotent stem cells. *Cancer Res* **70**, 7662-73 (2010).
128. Mapp, A.K., Ansari, A.Z., Ptashne, M. & Dervan, P.B. Activation of gene expression by small molecule transcription factors. *Proceedings of the National Academy of Sciences* **97**, 3930-3935 (2000).
129. Silva, J. et al. Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS biology* **6**, e253 (2008).
130. Hou, P. et al. Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. *Science* **341**, 651-654 (2013).
131. Wang, X. et al. Identification of a novel function of TWIST, a bHLH protein, in the development of acquired taxol resistance in human cancer cells. *Oncogene* **23**, 474-482 (2004).
132. Hebrok, M., Wertz, K. & Füchtbauer, E.-M. < i> M-twist</i> Is an Inhibitor of Muscle Differentiation. *Developmental biology* **165**, 537-544 (1994).
133. Yin, G. et al. Constitutive proteasomal degradation of TWIST-1 in epithelial-ovarian cancer stem cells impacts differentiation and metastatic potential. *Oncogene* **32**, 39-49 (2013).
134. Batlle, E. et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nature cell biology* **2**, 84-89 (2000).

135. Holliday, R. & Pugh, J.E. DNA modification mechanisms and gene activity during development. *Science* **187**, 226-32 (1975).
136. Christophersen, N.S. & Helin, K. Epigenetic control of embryonic stem cell fate. *The Journal of experimental medicine* **207**, 2287-2295 (2010).
137. Fouse, S.D. et al. Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. *Cell stem cell* **2**, 160-169 (2008).
138. Sharma, S., Kelly, T.K. & Jones, P.A. Epigenetics in cancer. *Carcinogenesis* **31**, 27-36 (2010).
139. Lister, R. et al. Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* **471**, 68-73 (2011).
140. Mikkelsen, T.S. et al. Dissecting direct reprogramming through integrative genomic analysis. *Nature* **454**, 49-55 (2008).
141. Howell, C.Y. et al. Genomic Imprinting Disrupted by a Maternal Effect Mutation in the< i> Dnmt1</i> Gene. *Cell* **104**, 829-838 (2001).
142. Sen, G.L., Reuter, J.A., Webster, D.E., Zhu, L. & Khavari, P.A. DNMT1 maintains progenitor function in self-renewing somatic tissue. *Nature* **463**, 563-567 (2010).
143. Okano, M., Bell, D.W., Haber, D.A. & Li, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99**, 247-257 (1999).
144. Li, J.-Y. et al. Synergistic function of DNA methyltransferases Dnmt3a and Dnmt3b in the methylation of Oct4 and Nanog. *Molecular and cellular biology* **27**, 8748-8759 (2007).
145. Bernstein, B.E. et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**, 315-326 (2006).
146. Schlesinger, Y. et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nature genetics* **39**, 232-236 (2006).
147. Ohm, J.E. et al. A stem cell–like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nature genetics* **39**, 237-242 (2007).
148. Morey, L. & Helin, K. Polycomb group protein-mediated repression of transcription. *Trends in biochemical sciences* **35**, 323-332 (2010).
149. O'Carroll, D. et al. The Polycomb-Group GeneEzh2 Is Required for Early Mouse Development. *Molecular and cellular biology* **21**, 4330-4336 (2001).
150. Varambally, S. et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *nature* **419**, 624-629 (2002).
151. Viré, E. et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* **439**, 871-874 (2005).
152. Hoffmann, M.J. et al. Expression changes in EZH2, but not in BMI-1, SIRT1, DNMT1 or DNMT3B are associated with DNA methylation changes in prostate cancer. *Cancer Biology and Therapy* **6**, 1403 (2007).
153. Hayami, S. et al. Overexpression of LSD1 contributes to human carcinogenesis through chromatin regulation in various cancers. *International Journal of Cancer* **128**, 574-586 (2011).
154. Harris, W.J. et al. The histone demethylase KDM1A sustains the oncogenic potential of MLL-AF9 leukemia stem cells. *Cancer cell* **21**, 473-487 (2012).
155. Siddique, H.R. & Saleem, M. Role of BMI1, a stem cell factor, in cancer recurrence and chemoresistance: preclinical and clinical evidences. *Stem Cells* **30**, 372-8 (2012).

156. Molofsky, A.V., He, S., Bydon, M., Morrison, S.J. & Pardal, R. Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. *Genes & development* **19**, 1432-1437 (2005).
157. Park, I.K. et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423**, 302-5 (2003).
158. Lessard, J. & Sauvageau, G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* **423**, 255-60 (2003).
159. Park, I.-k. et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423**, 302-305 (2003).
160. Lessard, J. & Sauvageau, G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *nature* **423**, 255-260 (2003).
161. Wang, Y. et al. Cancer stem cell marker Bmi-1 expression is associated with basal-like phenotype and poor survival in breast cancer. *World J Surg* **36**, 1189-94 (2012).
162. Lukacs, R.U., Memarzadeh, S., Wu, H. & Witte, O.N. Bmi-1 is a crucial regulator of prostate stem cell self-renewal and malignant transformation. *Cell stem cell* **7**, 682-693 (2010).
163. Kreso, A. et al. Self-renewal as a therapeutic target in human colorectal cancer. *Nature medicine* **20**, 29-36 (2014).
164. Hattori, N. et al. Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. *J Biol Chem* **279**, 17063-9 (2004).
165. Enright, B., Kubota, C., Yang, X. & Tian, X. Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2′-deoxycytidine. *Biology of Reproduction* **69**, 896-901 (2003).
166. Pawlak, M. & Jaenisch, R. De novo DNA methylation by Dnmt3a and Dnmt3b is dispensable for nuclear reprogramming of somatic cells to a pluripotent state. *Genes & development* **25**, 1035-1040 (2011).
167. Roberts, A.B. et al. Type beta transforming growth factor: a bifunctional regulator of cellular growth. *Proceedings of the National Academy of Sciences* **82**, 119-123 (1985).
168. Hirschi, K.K., Rohovsky, S.A. & D'Amore, P.A. PDGF, TGF-β, and heterotypic cell–cell interactions mediate endothelial cell–induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *The Journal of cell biology* **141**, 805-814 (1998).
169. Massagué, J., Cheifetz, S., Endo, T. & Nadal-Ginard, B. Type beta transforming growth factor is an inhibitor of myogenic differentiation. *Proceedings of the National Academy of Sciences* **83**, 8206-8210 (1986).
170. Li, Y. et al. Transforming growth factor-β1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis. *The American journal of pathology* **164**, 1007-1019 (2004).
171. Shangguan, L. et al. Inhibition of TGF-β/Smad Signaling by BAMBI Blocks Differentiation of Human Mesenchymal Stem Cells to Carcinoma-Associated Fibroblasts and Abolishes their Protumor Effects. *Stem cells* **30**, 2810-2819 (2012).
172. Massagué, J. TGFβ in cancer. *Cell* **134**, 215-230 (2008).
173. Arteaga, C. et al. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *Journal of Clinical Investigation* **92**, 2569 (1993).

174. Citri, A. & Yarden, Y. EGF–ERBB signalling: towards the systems level. *Nature reviews Molecular cell biology* **7**, 505-516 (2006).
175. Izumi, Y., Xu, L., di Tomaso, E., Fukumura, D. & Jain, R.K. Tumour biology: herceptin acts as an anti-angiogenic cocktail. *Nature* **416**, 279-280 (2002).
176. Kris, M.G. et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non–small cell lung cancer: a randomized trial. *Jama* **290**, 2149-2158 (2003).
177. Bouche, O. et al. Phase II trial of weekly alternating sequential BIBF 1120 and afatinib for advanced colorectal cancer. *Anticancer Res* **31**, 2271-81 (2011).
178. Ferrarotto, R. & Gold, K.A. Afatinib in the treatment of head and neck squamous cell carcinoma. *Expert opinion on investigational drugs* **23**, 135-143 (2014).
179. Lin, C.-I., Whang, E.E., Lorch, J.H. & Ruan, D.T. Autophagic activation potentiates the antiproliferative effects of tyrosine kinase inhibitors in medullary thyroid cancer. *Surgery* **152**, 1142-1149 (2012).
180. Wang, X.-k. et al. Afatinib Enhances the Efficacy of Conventional Chemotherapeutic Agents by Eradicating Cancer Stem–like Cells. *Cancer research* (2014).
181. Marshall, J. et al. A Phase I, open-label, dose escalation study of afatinib, in a 3-week-on/1-week-off schedule in patients with advanced solid tumors. *Investigational new drugs* **31**, 399-408 (2013).
182. Burrows, R.C., Wancio, D., Levitt, P. & Lillien, L. Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. *Neuron* **19**, 251-267 (1997).
183. Paez, J.G. et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**, 1497-1500 (2004).
184. Moore, M.J. et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *Journal of clinical oncology* **25**, 1960-1966 (2007).
185. Bean, J. et al. MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. *Proceedings of the National Academy of Sciences* **104**, 20932-20937 (2007).
186. Yano, S. et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor–activating mutations. *Cancer research* **68**, 9479-9487 (2008).
187. Gastaldi, S. et al. Met signaling regulates growth, repopulating potential and basal cell-fate commitment of mammary luminal progenitors: implications for basal-like breast cancer. *Oncogene* **32**, 1428-40 (2013).
188. Neuss, S., Becher, E., Wöltje, M., Tietze, L. & Jahnen-Dechent, W. Functional expression of HGF and HGF receptor/c-met in adult human mesenchymal stem cells suggests a role in cell mobilization, tissue repair, and wound healing. *Stem cells* **22**, 405-414 (2004).
189. Vermeulen, L. et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nature cell biology* **12**, 468-476 (2010).
190. Wend, P., Holland, J.D., Ziebold, U. & Birchmeier, W. in Seminars in cell & developmental biology 855-863 (Elsevier, 2010).
191. Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A. & Birchmeier, C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376**, 768-771 (1995).

192. Hu, Z., Evarts, R., Fujio, K., Marsden, E. & Thorgeirsson, S. Expression of hepatocyte growth factor and c-met genes during hepatic differentiation and liver development in the rat. *The American journal of pathology* **142**, 1823 (1993).
193. Puri, N. et al. c-Met is a potentially new therapeutic target for treatment of human melanoma. *Clinical cancer research* **13**, 2246-2253 (2007).
194. Debili, N. et al. Effects of the recombinant hematopoietic growth factors interleukin-3, interleukin-6, stem cell factor, and leukemia inhibitory factor on the megakaryocytic differentiation of CD34+ cells. *Blood* **82**, 84-95 (1993).
195. Lopez, M.J. & Porter, K.A. Inflammatory breast cancer. *Surgical Clinics of North America* **76**, 411-429 (1996).
196. Korkaya, H. et al. Activation of an IL6 inflammatory loop mediates trastuzumab resistance in HER2+ breast cancer by expanding the cancer stem cell population. *Mol Cell* **47**, 570-84 (2012).
197. Singh, J.K. et al. Targeting CXCR1/2 significantly reduces breast cancer stem cell activity and increases the efficacy of inhibiting HER2 via HER2-dependent and-independent mechanisms. *Clinical Cancer Research* **19**, 643-656 (2013).
198. Hartman, Z.C. et al. Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the proinflammatory cytokines IL-6 and IL-8. *Cancer research* **73**, 3470-3480 (2013).
199. Artavanis-Tsakonas, S., Rand, M.D. & Lake, R.J. Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-6 (1999).
200. Marson, A. et al. Wnt signaling promotes reprogramming of somatic cells to pluripotency. *Cell stem cell* **3**, 132 (2008).
201. Chen, B. et al. Small molecule–mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nature chemical biology* **5**, 100-107 (2009).
202. Bodine, P.V. et al. A small molecule inhibitor of the Wnt antagonist secreted frizzled-related protein-1 stimulates bone formation. *Bone* **44**, 1063-1068 (2009).
203. Wang, H., Hao, J. & Hong, C.C. Cardiac induction of embryonic stem cells by a small molecule inhibitor of Wnt/β-catenin signaling. *ACS chemical biology* **6**, 192-197 (2010).
204. You, L. et al. Inhibition of Wnt-2-mediated signaling induces programmed cell death in non-small-cell lung cancer cells. *Oncogene* **23**, 6170-6174 (2004).
205. Laiosa, C.V., Stadtfeld, M., Xie, H., de Andres-Aguayo, L. & Graf, T. Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBPα and PU. 1 transcription factors. *Immunity* **25**, 731-744 (2006).
206. Apelqvist, Å. et al. Notch signalling controls pancreatic cell differentiation. *Nature* **400**, 877-881 (1999).
207. Wang, S. et al. Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* **21**, 63-75 (1998).
208. Maillard, I., Fang, T. & Pear, W.S. Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annu. Rev. Immunol.* **23**, 945-974 (2005).
209. Ichida, J.K. et al. Notch inhibition allows oncogene-independent generation of iPS cells. *Nature chemical biology* (2014).
210. De La O, J.-P. et al. Notch and Kras reprogram pancreatic acinar cells to ductal intraepithelial neoplasia. *Proceedings of the National Academy of Sciences* **105**, 18907-18912 (2008).

211. Shih, I.-M. & Wang, T.-L. Notch signaling, γ-secretase inhibitors, and cancer therapy. *Cancer research* **67**, 1879-1882 (2007).
212. Fan, X. et al. NOTCH Pathway Blockade Depletes CD133-Positive Glioblastoma Cells and Inhibits Growth of Tumor Neurospheres and Xenografts. *Stem cells* **28**, 5-16 (2010).
213. Wislet-Gendebien, S. et al. Plasticity of cultured mesenchymal stem cells: Switch from nestin-positive to excitable neuron-like phenotype. *Stem cells* **23**, 392-402 (2005).
214. Judson, R.L., Babiarz, J.E., Venere, M. & Blelloch, R. Embryonic stem cell–specific microRNAs promote induced pluripotency. *Nature biotechnology* **27**, 459-461 (2009).
215. Subramanyam, D. et al. Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. *Nature biotechnology* **29**, 443-448 (2011).
216. Kong, W. et al. MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. *Mol Cell Biol* **28**, 6773-84 (2008).
217. Guttilla, I. et al. Prolonged mammosphere culture of MCF-7 cells induces an EMT and repression of the estrogen receptor by microRNAs. *Breast cancer research and treatment* **132**, 75-85 (2012).
218. Bao, B. et al. Notch-1 induces epithelial–mesenchymal transition consistent with cancer stem cell phenotype in pancreatic cancer cells. *Cancer letters* **307**, 26-36 (2011).
219. Iliopoulos, D. et al. Loss of miR-200 inhibition of Suz12 leads to polycomb-mediated repression required for the formation and maintenance of cancer stem cells. *Molecular cell* **39**, 761-772 (2010).
220. Liu, C. et al. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nature medicine* **17**, 211-215 (2011).
221. Iliopoulos, D., Jaeger, S.A., Hirsch, H.A., Bulyk, M.L. & Struhl, K. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *Molecular cell* **39**, 493-506 (2010).
222. Deiters, A. Small molecule modifiers of the microRNA and RNA interference pathway. *The AAPS journal* **12**, 51-60 (2010).
223. Gumireddy, K. et al. Small-Molecule Inhibitors of MicroRNA miR-21 Function. *Angewandte Chemie International Edition* **47**, 7482-7484 (2008).
224. Young, D.D., Connelly, C.M., Grohmann, C. & Deiters, A. Small molecule modifiers of microRNA miR-122 function for the treatment of hepatitis C virus infection and hepatocellular carcinoma. *Journal of the American Chemical Society* **132**, 7976-7981 (2010).
225. Ward, P.S. & Thompson, C.B. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer cell* **21**, 297-308 (2012).
226. Gordan, J.D., Thompson, C.B. & Simon, M.C. HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer cell* **12**, 108-113 (2007).
227. Yang, M.-H. & Wu, K.-J. TWIST activation by hypoxia inducible factor-1 (HIF-1). *Cell Cycle* **7**, 2090-2096 (2008).
228. Sahlgren, C., Gustafsson, M.V., Jin, S., Poellinger, L. & Lendahl, U. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proceedings of the National Academy of Sciences* **105**, 6392-6397 (2008).
229. Dong, C. et al. Loss of FBP1 by Snail-mediated repression provides metabolic advantages in basal-like breast cancer. *Cancer cell* **23**, 316-331 (2013).

230. Cipolleschi, M.G., Sbarba, P.D. & Olivotto, M. The role of hypoxia in the maintenance of hematopoietic stem cells. *Blood* **82**, 2031-2037 (1993).
231. Holzwarth, C. et al. Low physiologic oxygen tensions reduce proliferation and differentiation of human multipotent mesenchymal stromal cells. *BMC cell biology* **11**, 11 (2010).
232. Morrison, S.J. et al. Culture in reduced levels of oxygen promotes clonogenic sympathoadrenal differentiation by isolated neural crest stem cells. *The Journal of Neuroscience* **20**, 7370-7376 (2000).
233. Ceradini, D.J. & Gurtner, G.C. Homing to hypoxia: HIF-1 as a mediator of progenitor cell recruitment to injured tissue. *Trends in cardiovascular medicine* **15**, 57-63 (2005).
234. Cannito, S. et al. Redox mechanisms switch on hypoxia-dependent epithelial–mesenchymal transition in cancer cells. *Carcinogenesis* **29**, 2267-2278 (2008).
235. Covello, K.L. et al. HIF-2α regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes & development* **20**, 557-570 (2006).
236. Soeda, A. et al. Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1α. *Oncogene* **28**, 3949-3959 (2009).
237. Poon, E., Harris, A.L. & Ashcroft, M. Targeting the hypoxia-inducible factor (HIF) pathway in cancer. *Expert reviews in molecular medicine* **11**, e26 (2009).
238. Kirkpatrick, J. et al. in J Clin Oncol (Meeting Abstracts) 2065 (2007).
239. Matei, D. et al. Activity of 2 methoxyestradiol (Panzem< sup>®</sup> NCD) in advanced, platinum-resistant ovarian cancer and primary peritoneal carcinomatosis: A Hoosier Oncology Group trial. *Gynecologic oncology* **115**, 90-96 (2009).
240. Hu, C.-J., Wang, L.-Y., Chodosh, L.A., Keith, B. & Simon, M.C. Differential roles of hypoxia-inducible factor 1α (HIF-1α) and HIF-2α in hypoxic gene regulation. *Molecular and cellular biology* **23**, 9361-9374 (2003).
241. Heddleston, J.M., Li, Z., McLendon, R.E., Hjelmeland, A.B. & Rich, J.N. The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype. *Cell Cycle* **8**, 3274-84 (2009).
242. Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F. & Maity, A. Regulation of glut1 mRNA by hypoxia-inducible factor-1 Interaction between H-ras and hypoxia. *Journal of Biological Chemistry* **276**, 9519-9525 (2001).
243. Fox, C.J., Hammerman, P.S. & Thompson, C.B. Fuel feeds function: energy metabolism and the T-cell response. *Nature Reviews Immunology* **5**, 844-852 (2005).
244. Wang, F. & Tong, Q. SIRT2 suppresses adipocyte differentiation by deacetylating FOXO1 and enhancing FOXO1's repressive interaction with PPARγ. *Molecular biology of the cell* **20**, 801-808 (2009).
245. Reka, A.K. et al. Peroxisome proliferator-activated receptor-γ activation inhibits tumor metastasis by antagonizing Smad3-mediated epithelial-mesenchymal transition. *Molecular cancer therapeutics* **9**, 3221-3232 (2010).
246. Yin, F. et al. Troglitazone inhibits growth of MCF-7 breast carcinoma cells by targeting G1 cell cycle regulators. *Biochemical and biophysical research communications* **286**, 916-922 (2001).
247. Kubota, T. et al. Ligand for peroxisome proliferator-activated receptor γ (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. *Cancer Research* **58**, 3344-3352 (1998).

248. Zhou, B. et al. Troglitazone attenuates TGF-β1-induced EMT in alveolar epithelial cells via a PPARγ-independent mechanism. *PloS one* **7**, e38827 (2012).
249. Pegg, A.E. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer research* **48**, 759-774 (1988).
250. Huang, Y., Pledgie, A., Casero, R.A., Jr. & Davidson, N.E. Molecular mechanisms of polyamine analogs in cancer cells. *Anticancer Drugs* **16**, 229-41 (2005).
251. Zhao, F., Song, C.-P., He, J. & Zhu, H. Polyamines improve K+/Na+ homeostasis in barley seedlings by regulating root ion channel activities. *Plant physiology* **145**, 1061-1072 (2007).
252. Cirenajwis, H. et al. Reduction of the putative CD44+CD24- breast cancer stem cell population by targeting the polyamine metabolic pathway with PG11047. *Anticancer Drugs* **21**, 897-906 (2010).
253. Gupta, P.B. et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* **138**, 645-59 (2009).
254. Mehrpour, M. Inhibition of the autophagic flux by salinomycin in breast cancer stem-like/progenitor cells interferes with their maintenance. *Autophagy* **9**, 714-729 (2013).
255. Fuchs, D., Daniel, V., Sadeghi, M., Opelz, G. & Naujokat, C. Salinomycin overcomes ABC transporter-mediated multidrug and apoptosis resistance in human leukemia stem cell-like KG-1a cells. *Biochemical and biophysical research communications* **394**, 1098-1104 (2010).
256. Riccioni, R. et al. The cancer stem cell selective inhibitor salinomycin is a p-glycoprotein inhibitor. *Blood Cells, Molecules, and Diseases* **45**, 86-92 (2010).
257. Lu, D. et al. Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemia cells. *Proceedings of the National Academy of Sciences* **108**, 13253-13257 (2011).
258. Mitani, M., Yamanishi, T. & Miyazaki, Y. Salinomycin: a new monovalent cation ionophore. *Biochemical and biophysical research communications* **66**, 1231-1236 (1975).
259. Sachlos, E. et al. Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. *Cell* **149**, 1284-97 (2012).
260. Wang, P.S. et al. Dopamine antagonists and the development of breast cancer. *Archives of general psychiatry* **59**, 1147-1154 (2002).

Chapter 2 References

1. Curtis, C. et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* **486**, 346-352 (2012).
2. Network, C.G.A. Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61-70 (2012).
3. Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J. & Clarke, M.F. Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences* **100**, 3983-3988 (2003).
4. Visvader, J.E. & Lindeman, G.J. Cancer stem cells: current status and evolving complexities. *Cell Stem Cell* **10**, 717-28 (2012).
5. Mani, S.A. et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704-15 (2008).
6. Chaffer, C.L. et al. Poised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. *Cell* **154**, 61-74 (2013).

7. Lim, E. et al. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nature medicine* **15**, 907-913 (2009).
8. Thiery, J.P., Acloque, H., Huang, R.Y. & Nieto, M.A. Epithelial-mesenchymal transitions in development and disease. *Cell* **139**, 871-90 (2009).
9. Morel, A.P. et al. Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One* **3**, e2888 (2008).
10. Park, S.Y., Gönen, M., Kim, H.J., Michor, F. & Polyak, K. Cellular and genetic diversity in the progression of in situ human breast carcinomas to an invasive phenotype. *The Journal of clinical investigation* **120**, 636-644 (2010).
11. Hurt, E.M., Kawasaki, B.T., Klarmann, G.J., Thomas, S.B. & Farrar, W.L. CD44+ CD24− prostate cells are early cancer progenitor/stem cells that provide a model for patients with poor prognosis. *British journal of cancer* **98**, 756-765 (2008).
12. Lee, T.K.W. et al. CD24< sup>+</sup> Liver Tumor-Initiating Cells Drive Self-Renewal and Tumor Initiation through STAT3-Mediated NANOG Regulation. *Cell stem cell* **9**, 50-63 (2011).
13. Zhang, C., Li, C., He, F., Cai, Y. & Yang, H. Identification of CD44+ CD24+ gastric cancer stem cells. *Journal of cancer research and clinical oncology* **137**, 1679-1686 (2011).
14. Li, C. et al. Identification of pancreatic cancer stem cells. *Cancer research* **67**, 1030-1037 (2007).
15. Gao, M., Choi, Y., Kang, S., Youn, J. & Cho, N. CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. *Oncogene* **29**, 2672-2680 (2010).
16. Cho, R.W. et al. Isolation and molecular characterization of cancer stem cells in MMTV-Wnt-1 murine breast tumors. *Stem cells* **26**, 364-371 (2008).
17. Bar, E.E. et al. Cyclopamine-Mediated Hedgehog Pathway Inhibition Depletes Stem-Like Cancer Cells in Glioblastoma. *Stem cells* **25**, 2524-2533 (2007).
18. Lim, J., Lee, K.-m., Shim, J. & Shin, I. CD24 regulates stemness and the epithelial to mesenchymal transition through modulation of Notch1 mRNA stability by p38MAPK. *Archives of biochemistry and biophysics* (2014).
19. Niessen, K. et al. Slug is a direct Notch target required for initiation of cardiac cushion cellularization. *The Journal of cell biology* **182**, 315-325 (2008).
20. Zavadil, J., Cermak, L., Soto-Nieves, N. & Bottinger, E.P. Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. *Embo j* **23**, 1155-65 (2004).
21. Ginestier, C. et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell stem cell* **1**, 555-567 (2007).
22. Croker, A.K. & Allan, A.L. Inhibition of aldehyde dehydrogenase (ALDH) activity reduces chemotherapy and radiation resistance of stem-like ALDHhiCD44+ human breast cancer cells. *Breast cancer research and treatment* **133**, 75-87 (2012).
23. Ricardo, S. et al. Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. *Journal of clinical pathology* **64**, 937-946 (2011).
24. Liu, S. et al. Breast Cancer Stem Cells Transition between Epithelial and Mesenchymal States Reflective of their Normal Counterparts. *Stem Cell Reports* **2**, 78-91 (2014).
25. Mikkelsen, T.S. et al. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* **448**, 553-560 (2007).

26. Mikkelsen, T.S. et al. Dissecting direct reprogramming through integrative genomic analysis. *Nature* **454**, 49-55 (2008).
27. Fillmore, C.M. & Kuperwasser, C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res* **10**, R25 (2008).
28. Yoneda, T., Sasaki, A. & Mundy, G.R. Osteolytic bone metastasis in breast cancer. *Breast cancer research and treatment* **32**, 73-84 (1994).
29. Miyoshi, N. et al. Defined factors induce reprogramming of gastrointestinal cancer cells. *Proceedings of the National Academy of Sciences* **107**, 40-45 (2010).
30. Grimshaw, M.J. et al. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. *Breast Cancer Res* **10**, R52 (2008).
31. Git, A. et al. PMC42, a breast progenitor cancer cell line, has normal-like mRNA and microRNA transcriptomes. *Breast Cancer Res* **10**, R54 (2008).
32. Ackland, M.L., Michalczyk, A. & Whitehead, R.H. PMC42, a novel model for the differentiated human breast. *Exp Cell Res* **263**, 14-22 (2001).
33. Gupta, P.B. et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* **138**, 645-59 (2009).
34. Bijian, K. et al. Targeting focal adhesion turnover in invasive breast cancer cells by the purine derivative reversine. *Br J Cancer* (2013).
35. Hayward, P., Kalmar, T. & Arias, A.M. Wnt/Notch signalling and information processing during development. *Development* **135**, 411-424 (2008).
36. Chen, S. et al. Reversine increases the plasticity of lineage-committed mammalian cells. *Proceedings of the National Academy of Sciences* **104**, 10482-10487 (2007).
37. Krause, D.S. & Crispino, J.D. Molecular pathways: induction of polyploidy as a novel differentiation therapy for leukemia. *Clin Cancer Res* **19**, 6084-8 (2013).
38. Tanaka, T.U. et al. Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. *Cell* **108**, 317-329 (2002).
39. Favata, M.F. et al. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *Journal of Biological Chemistry* **273**, 18623-18632 (1998).
40. Slack-Davis, J.K. et al. Cellular characterization of a novel focal adhesion kinase inhibitor. *Journal of biological chemistry* **282**, 14845-14852 (2007).
41. Zhao, B. et al. Modulation of kinase-inhibitor interactions by auxiliary protein binding: crystallography studies on Aurora A interactions with VX-680 and with TPX2. *Protein Sci* **17**, 1791-7 (2008).
42. Morabito, A. et al. Vandetanib (ZD6474), a dual inhibitor of vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) tyrosine kinases: current status and future directions. *Oncologist* **14**, 378-90 (2009).
43. Luistro, L. et al. Preclinical profile of a potent gamma-secretase inhibitor targeting notch signaling with in vivo efficacy and pharmacodynamic properties. *Cancer Res* **69**, 7672-80 (2009).
44. Chen, B. et al. Small molecule–mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nature chemical biology* **5**, 100-107 (2009).
45. Yakes, F.M. et al. Cabozantinib (XL184), a novel MET and VEGFR2 inhibitor, simultaneously suppresses metastasis, angiogenesis, and tumor growth. *Molecular cancer therapeutics* **10**, 2298-2308 (2011).

46. Verstovsek, S. et al. in ASH Annual Meeting Abstracts 553 (2007).
47. Santoro, M. et al. Ret oncogene activation in human thyroid neoplasms is restricted to the papillary cancer subtype. *Journal of Clinical Investigation* **89**, 1517 (1992).
48. Marotta, L.L. et al. The JAK2/STAT3 signaling pathway is required for growth of CD44+ CD24–stem cell–like breast cancer cells in human tumors. *The Journal of clinical investigation* **121**, 2723 (2011).
49. Keen, N. & Taylor, S. Aurora-kinase inhibitors as anticancer agents. *Nat Rev Cancer* **4**, 927-36 (2004).
50. Lee, D.-F. et al. Regulation of embryonic and induced pluripotency by aurora kinase-p53 signaling. *Cell stem cell* **11**, 179-194 (2012).
51. Carmena, M. & Earnshaw, W.C. The cellular geography of aurora kinases. *Nat Rev Mol Cell Biol* **4**, 842-54 (2003).
52. Weier, H. & Mao, J. Meta-analysis of Aurora Kinase A (AURKA) Expression Data Reveals a Significant Correlation between Increased AURKA Expression and Distant Metastases in Human ER-positive Breast Cancers. *J Data Mining Genomics Proteomics* **4**, 2153-0602.1000127 (2013).
53. Hamidi, T. et al. Nupr1-aurora kinase a pathway provides protection against metabolic stress-mediated autophagic-associated cell death. *Clinical Cancer Research* **18**, 5234-5246 (2012).
54. Zou, Z. et al. Aurora kinase A inhibition-induced autophagy triggers drug resistance in breast cancer cells. *Autophagy* **8**, 1798-1810 (2012).
55. Chefetz, I., Holmberg, J.C., Alvero, A.B., Visintin, I. & Mor, G. Inhibition of Aurora-A kinase induces cell cycle arrest in epithelial ovarian cancer stem cells by affecting NFκB pathway. *Cell Cycle* **10**, 2206-2214 (2011).
56. Walsby, E., Walsh, V., Pepper, C., Burnett, A. & Mills, K. Effects of the aurora kinase inhibitors AZD1152-HQPA and ZM447439 on growth arrest and polyploidy in acute myeloid leukemia cell lines and primary blasts. *haematologica* **93**, 662-669 (2008).
57. Davoli, T. & de Lange, T. The causes and consequences of polyploidy in normal development and cancer. *Annual review of cell and developmental biology* **27**, 585-610 (2011).
58. Li, J. et al. A dominant-negative form of mouse SOX2 induces trophectoderm differentiation and progressive polyploidy in mouse embryonic stem cells. *Journal of Biological Chemistry* **282**, 19481-19492 (2007).
59. Argetsinger, L.S. et al. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell* **74**, 237-244 (1993).
60. Wagner, K.-U. & Rui, H. Jak2/Stat5 signaling in mammogenesis, breast cancer initiation and progression. *Journal of mammary gland biology and neoplasia* **13**, 93-103 (2008).
61. Bromberg, J.F. et al. < i> Stat3</i> as an Oncogene. *Cell* **98**, 295-303 (1999).
62. Ying, Q.-L., Nichols, J., Chambers, I. & Smith, A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115**, 281-292 (2003).
63. Lin, L. et al. Evaluation of STAT3 Signaling in ALDH+ and ALDH+/CD44+/CD24− Subpopulations of Breast Cancer Cells. *PloS one* **8**, e82821 (2013).
64. Bretz, N.P. et al. CD24 controls Src/STAT3 activity in human tumors. *Cellular and Molecular Life Sciences* **69**, 3863-3879 (2012).

65. Baumann, P. et al. CD24 interacts with and promotes the activity of c-src within lipid rafts in breast cancer cells, thereby increasing integrin-dependent adhesion. *Cellular and Molecular Life Sciences* **69**, 435-448 (2012).
66. Noman, M.Z. et al. Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression. *Cancer Res* **71**, 5976-86 (2011).
67. Mehrpour, M. Inhibition of the autophagic flux by salinomycin in breast cancer stem-like/progenitor cells interferes with their maintenance. *Autophagy* **9**, 714-729 (2013).
68. Maycotte, P. et al. STAT3-mediated autophagy dependence identifies subtypes of breast cancer where autophagy inhibition can be efficacious. *Cancer research* **74**, 2579-2590 (2014).
69. Yoon, S. et al. STAT3 transcriptional factor activated by reactive oxygen species induces IL6 in starvation-induced autophagy of cancer cells. *Autophagy* **6**, 1125-1138 (2010).
70. Korkaya, H. et al. Activation of an IL6 inflammatory loop mediates trastuzumab resistance in HER2+ breast cancer by expanding the cancer stem cell population. *Mol Cell* **47**, 570-84 (2012).
71. Cufí, S. et al. Autophagy positively regulates the CD44+ CD24-/low breast cancer stem-like phenotype. *Cell cycle* **10**, 3871-3885 (2011).
72. Sandilands, E., Serrels, B., Wilkinson, S. & Frame, M.C. Src-dependent autophagic degradation of Ret in FAK-signalling-defective cancer cells. *EMBO reports* **13**, 733-740 (2012).
73. Lin, C.-I., Whang, E.E., Lorch, J.H. & Ruan, D.T. Autophagic activation potentiates the antiproliferative effects of tyrosine kinase inhibitors in medullary thyroid cancer. *Surgery* **152**, 1142-1149 (2012).
74. Choi, D.S. et al. Chloroquine eliminates cancer stem cells through deregulation of Jak2 and DNMT1. *STEM CELLS* (2014).
75. Huang, M. et al. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* **72**, 567-572 (1988).
76. Takebe, N., Harris, P.J., Warren, R.Q. & Ivy, S.P. Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. *Nature reviews Clinical oncology* **8**, 97-106 (2010).
77. Richard, J. et al. Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. *The Lancet* **329**, 1398-1402 (1987).
78. Sachlos, E. et al. Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. *Cell* **149**, 1284-97 (2012).
79. Dontu, G., Al-Hajj, M., Abdallah, W.M., Clarke, M.F. & Wicha, M.S. Stem cells in normal breast development and breast cancer. *Cell proliferation* **36**, 59-72 (2003).
80. Alaoui-Jamali, M.A. et al. A novel experimental heme oxygenase-1-targeted therapy for hormone-refractory prostate cancer. *Cancer Res* **69**, 8017-24 (2009).
81. Xu, Y. et al. Filamin A regulates focal adhesion disassembly and suppresses breast cancer cell migration and invasion. *J Exp Med* **207**, 2421-37 (2010).