Molecular Characteristics of Cancer Stem-like Cells Derived from Human Breast Cancer Cells

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Abstract. *We characterized the cellular properties of cancer stem-like cells (CSLCs) isolated from immortalized MDA-MB453 human breast cancer cells in culture. We showed that although the expression of Octamer-binding transcription factor-4 (OCT4) correlates to stemness in these CSLCs, OCT4 knockdown does not induce their differentiation. Our results suggest that the differentiation program in MDA-MB453 CSLCs is blocked at a step upstream of the transcription of the OCT4 promoter, allowing CSLCs to maintain their population through asymmetric cell division during many repeated passages. Comparative expression analysis indicates that only a subset of genes and signaling pathways known to be associated with survival and maintenance of CSCs are selectively expressed in CSLCs, as compared with non-CSLCs fractionated from the same parental MDA-MB453 cells. These results suggest that selective expression of a limited number of genes may be sufficient for establishment and maintenance of CSLCs with high tumorigenicity.*

Cancer stem cells (CSCs) are a sub-population of tumor cells that possess high tumorigenic activity and stem cell characteristics of unchecked self-renewal and differentiation into various cell types. The accumulation of drug-resistant CSCs correlates to high rates of therapeutic failure observed in cancer patients (1-4). Owing to these stem cell properties, CSCs are thought to play a critical role in growth and maintenance of cancer (1). CSCs were first discovered in 1994 from acute myelogenous leukemia (5) and later in solid tumors of various organs, such as the brain (6), colon (4, 7, 8), liver (9) and lung (10, 11). CSCs establish a micro-stem-cell niche within tumors, in which transit amplifying cells (TACs), non-stem-type cells that rapidly proliferate but have less or no tumorigenic potential, constitute the majority of the tumor mass. CSCs maintain their population primarily through asymmetric cell division, in which a parental SCS is split into a CSC and a non-CSC (12-15). In the course of cancer progression, the relative population of CSCs can increase through symmetric cell division in which a CSC is split into two progeny CSCs. Studies have shown that the relative abundance of CSCs in tumors is closely related with progression of malignant diseases and the failure of conventional therapies to eradicate tumors (1). However, little is known about the mechanism of how CSCs maintain their population in tumors and regulate symmetric *vs.* asymmetric cell division.

Recent studies using breast, lung, prostate and brain cancer cell line(s) identified rare sub-populations that possess properties unique to stem cells, such as high tumorigenic activity and drug resistance (16-21). Interestingly, when cancer stem-like cells (CSLCs) that had been purified to near-homogeneity were re-plated, the majority of them rapidly differentiated and reached a new equilibrium, similar to the original cellular composition in which CSLCs existed in only a small sub-population. It has therefore been proposed that CSLCs present in immortalized cancer cells have a homeostasis mechanism that regulates the balance between asymmetric and symmetric self-renewal divisions (18, 21). Consistently, a recent study using immortalized human lung cancer cells demonstrated asymmetric cell division of CSLCs at a single-cell level. The steady-state

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Key Words: Cancer stem-like cells, OCT4, MDA-MB453, stemness, differentiation, asymmetric cell division.

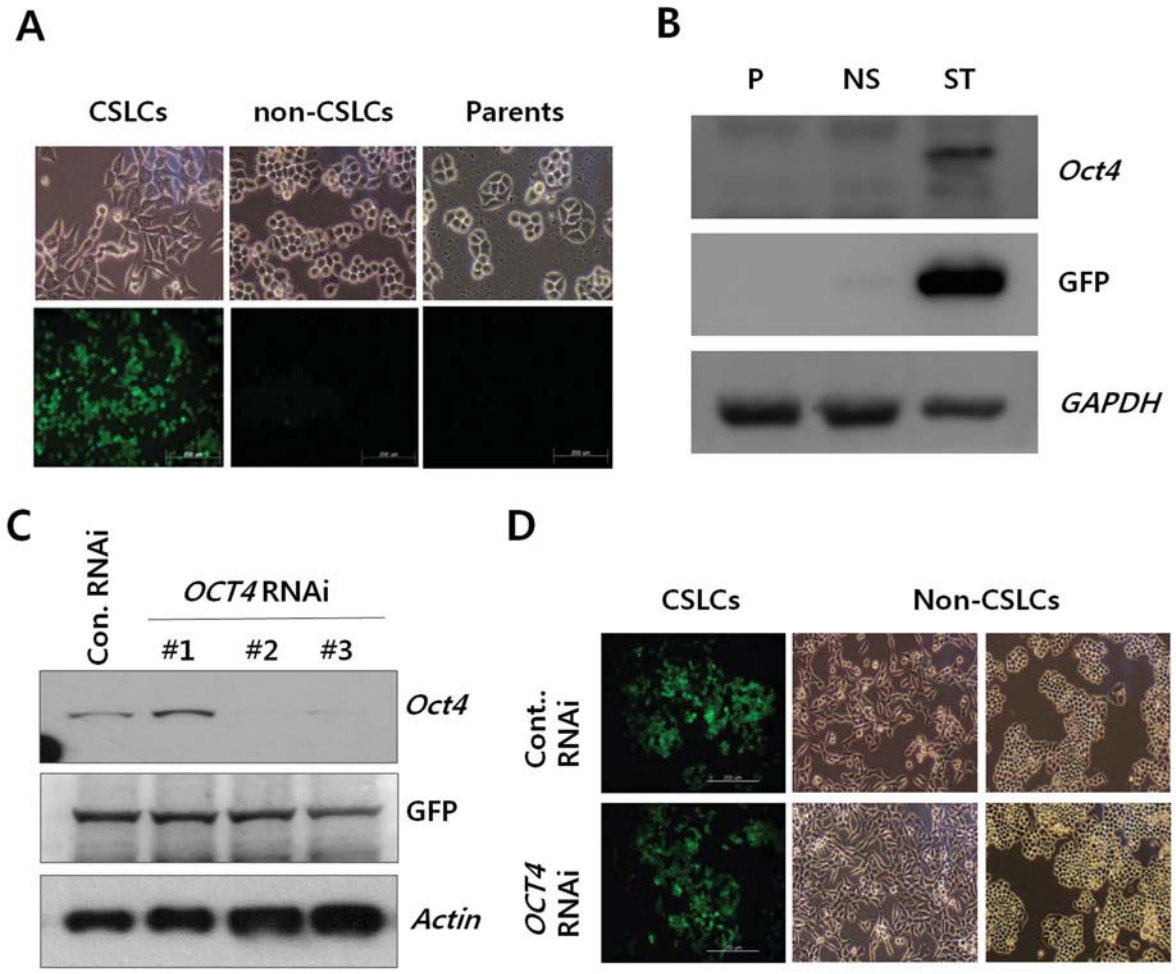

Figure 1. *The knockdown of octamer-binding transcription factor-4 (OCT4) apparently does not induce the differentiation of MDA-MB453 cancer stem-like cells (CSLCs). A: Microscopic analysis of parental MDA-MB453 cells (CSLCs), non-CSLCs, and parental cells. B: Parental MDA-MB453 cells (P), non-CSLCs (NS) and CSCLs (ST) were subjected to immunoblotting with antibodies, as indicated. C: CSCLs transfected with control and OCT4 (#1-3) siRNAs were subjected to immunoblotting with antibodies, as indicated. D: Microscopic analysis of CSLCs transfected with control and OCT4 siRNA #2.*

level of CSLCs in a given cell culture is determined by the balance between asymmetric *vs*. symmetric cell divisions, which is also affected by various factors, such as cell density, cell-to-cell contact, and hypoxic condition (18). A recent study has shown that formation and differentiation of CSCs in cultured cells is in a dynamic equilibrium which is regulated by paracrine signaling between CSCs and non-CSCs (21). The cytokine interleukin-6 (IL-6) was shown to promote the reversion of non-CSCs (that had been differentiated from CSCs) to CSCs (21).

Recently, Sajithral *et al*. isolated CSLCs from MDA-MB453 human breast cancer cells by stably expressing Green Fluorescent Protein (GFP) under the promoter of octamer-binding transcription factor-4 (*OCT4*), a homeodomain transcription factor required for the self-renewal of various CSCs (19). It has been shown that the levels of *OCT4* tightly correlate to the differentiation status of the cells (22). Consistently, GFP-positive CSLCs, representing an active recombinant *OCT4* promoter, showed high tumorigenicity (with as few as 100 cells) in immunocompromised mice and exhibited higher resistant to anticancer reagents, hypoxia and acidotic environments, as compared with GFP-negative non-CSCs representing an inactive recombinant *OCT4* promoter. While this suggests that the activity of the OCT4 promoter correlates to the stemness of MDA-MB453 cells, the authors made an unexpected observation that the cells isolated from tumors in grafted mice fully retained GFP signals and the ability to induce tumors in mice. Moreover, when repeatedly re-plated, CSLCs fully retained the expression of GFP and cellular properties of CSCs, such as morphological features (small and round) and the tendency to form spheroids, as compared

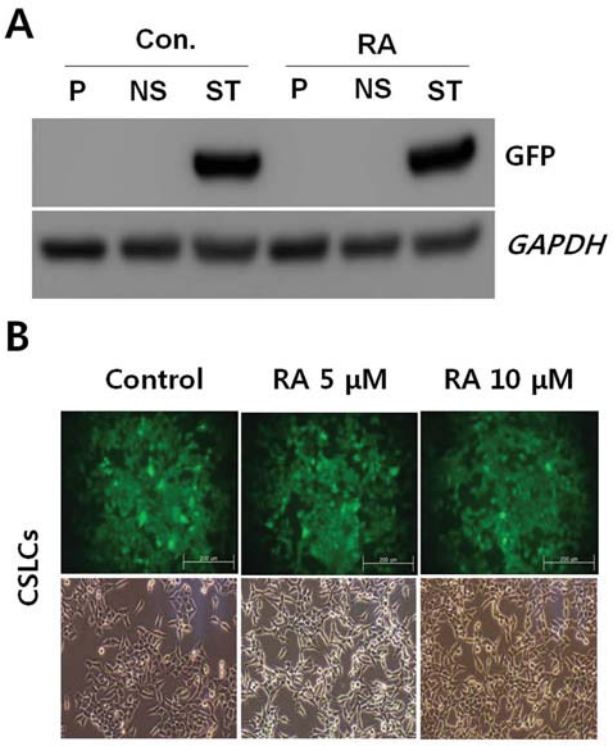

Figure 2. *The treatment of all-trans retinoic acid (ATRA) apparently does not induce differentiation of MDA-MB453 cancer stem-like cells (CSLCs). A: Parental MDA-MB453 cells (P), non-CSLCs (NS) and CSCLs (ST) were treated with 10 μM ATRA for four days (each time per day) and then subjected to immunoblotting with antibodies as indicated. B: MDA-MB453 CSLCs were treated once a day with dimethyl sulfoxide (DMSO) and ATRA for four days and analyzed by microscopy.*

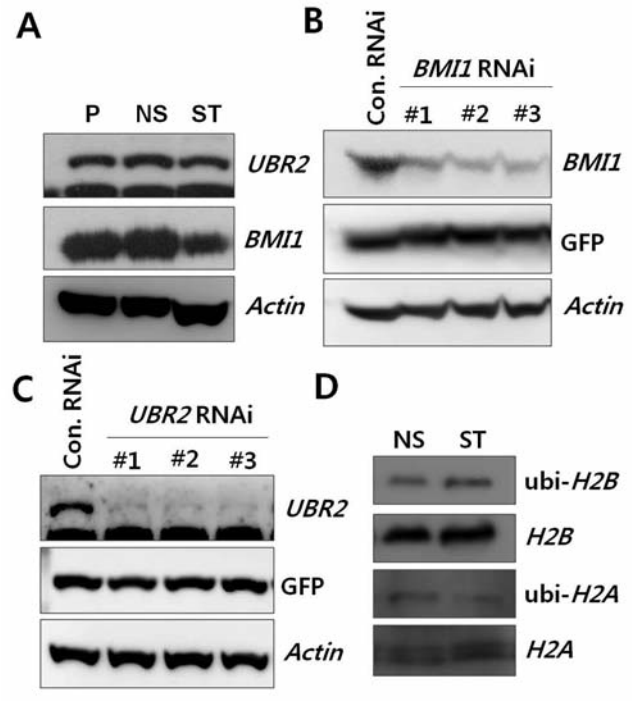

Figure 3. *Characterization of histone ubiquitylation in MDA-MB453 cancer stem-like cells (CSLCs). A: Parental MDA-MB453 cells (P), non-CSLCs (NS) and CSCLs (ST) were subjected to immunoblotting with antibodies, as indicated. B: MDA-MB453 CSCLs transfected with siRNAs (#1-3) against B-lymphoma Mo-MLV insertion region-1 (BMI1) were subjected to immunoblotting with antibodies as indicated. C: MDA-MB453 CSCLs transfected with control or Ubiquitin protein ligase E3 component n-recognin-2 (UBR2) siRNAs (#1-3) were subjected to immunoblotting with antibodies, as indicated. D: Non-CSLCs (NS) and CSCLs (ST) were subjected to immunoblotting with antibodies as indicated.*

with non-CSLCs negative for the expression of GFP. However, the cellular properties of MDA-MB453 CSLCs remain poorly-understood.

In the current study, we characterized the cellular properties of and CSC-specific markers in GFP-positive CSLCs isolated from MDA-MB453 human breast cancer cells, in comparison with GFP-negative non-CSLCs. We also characterized histone ubiquitylation, autophagy, and cellular responses to endoplasmic reticulum stress in MDA-MB453 CSLCs in an attempt to identify their selective role in the maintenance of the stemness of these cells.

Materials and Methods

Cell lines. MDA-MB453 CSLCs (GFP-positive), non-CSLCs (GFP-negative), and parental MDA-MB453 cells were generous gifts from Dr. Edward Prochownik (University of Pittsburgh) and cultured in Dulbecco's modified MEM (DMEM) supplemented with 10% fetal bovine serum (FBS). Starvation was performed by changing the media with Hanks balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA).

Antibodies and other reagents. We used antibodies against the following proteins: GFP, Polo-like kinase 1 (*PLK1*), Centrosomal protein 55KDa (*CEP55*), Microtubule-associated protein 1 light chain 3 (*LC3*) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), *OCT4*, Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), histon *H2A* (Abcam, Cambridge, MA, USA), ubiquitylated *H2A*, ubiquitylated *H2B*, *H2B* (Millipore, Billerica, MA, USA), and β-actin (Sigma, St. Louis, MO, USA). SiRNAs against *OCT4*, B lymphoma Mo-MLV insertion region 1 (*BMI1*) and Ubiquitin protein ligase E3 component n-recognin 2 (*UBR2*) were purchased from the Invitrogen Stealth RNAi™ SiRNA library (Invitrogen, Carlsbad, CA, USA). We purchased all-*trans* retinoic acid (ATRA) and tharpsigargin from Sigma, and bafilomycin A1 from MerkMillipore (Billerica, MA, USA).

Transfection. Cells were reverse-transfected with siRNAs against *OCT4*, *BMI1* and *UBR2* using Lipofectamine™ RNAiMAX (Invitrogen) following the manufacturer's instructions. Briefly, 30 pmol RNAi duplex in 500 μL serum-free Opti-MEM I media was mixed with 5 μl Lipofectamine RNAiMAX in each well of 6-well plates. After 15 minutes at room temperature, 2×10^5 cells in 2.5 ml

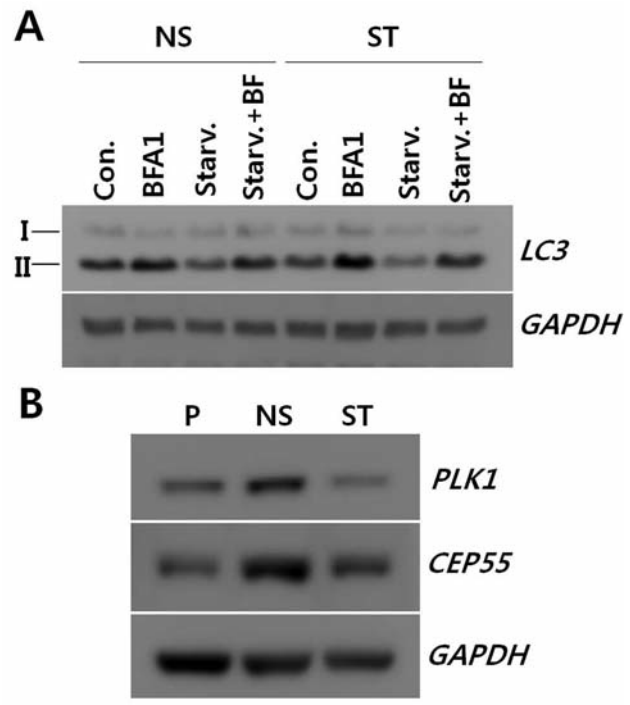

Figure 4. *Cancer stem-like cells (CSLCs) display an increased autophagic flux relative to non-CSLCs. A: Autophagy was induced by 0.2 μM bafilomycin-A1 (2 h), amino acid starvation (2 h), or both treatments in non-CSLCs (NS) and CSCLs (ST), followed by immunoblotting analysis. B: Parental MDA-MB453 cells (P), non-CSLCs (NS) and CSCLs (ST) were subjected to immunoblotting with antibodies, as indicated.*

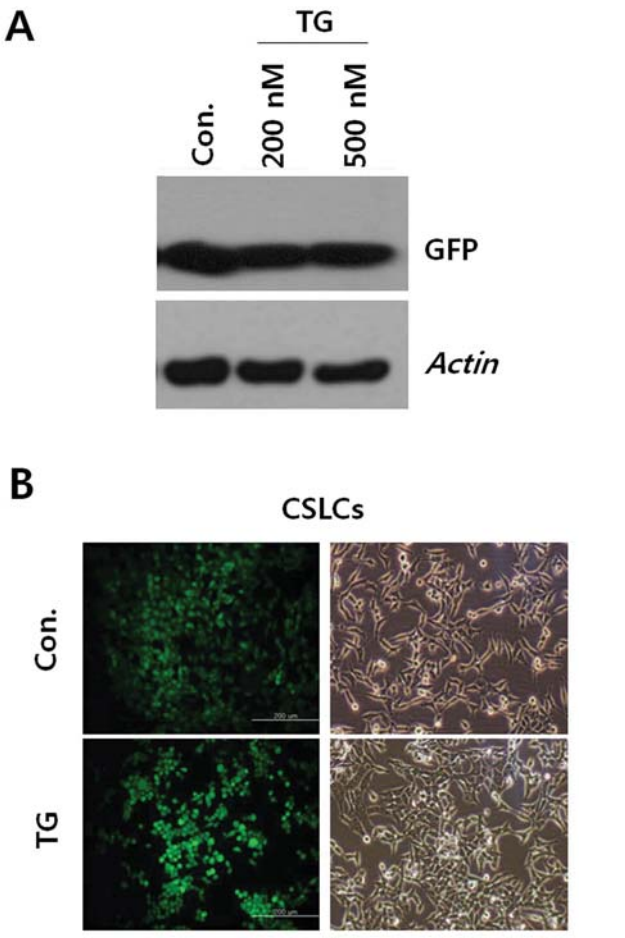

Figure 5. *Characterization of cellular responses to endoplasmic reticulum (ER) stress in MDA-MB453 cancer stem-like cells (CSLCs). A: CSLCs were treated with dimethyl sulfoxide (DMSO) or thapsigargin (TG) for 6 h, as indicated and subjected to immunoblotting with antibodies, as indicated. B: CSLCs were treated with DMSO and thapsigargin (20 nM) for one day and analyzed by fluorescent microscopy.*

growth media without antibiotics were added to each well. Experiments were conducted 72 h after transfection. For fluorescent microscopy, cells were plated on glass coverslips and processed using the same protocols. Cells were fixed with 3.7% paraformaldehyde before microscopic analysis.

Immunoblotting. Subconfluent cells were washed with ice-cold phosphate-buffered saline twice and lysed with 1% Nonidet P-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinine, and 20 mg/ml leupeptin). Lysates were cleared by centrifugation for 20 min at 4˚C, and protein concentration was determined by Bio-Rad protein assays (Bio-Rad, Hercules, CA, USA). The lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electronically transferred to polyvinylidene difluoride (PVDF) membrane. Western blotting was carried out using horseradish peroxidase-conjugated IgG as a secondary antibody and enhanced chemiluminescence (ECL) system for detection.

Microarray analysis. Total RNAs were isolated from two pairs of GFP-positive CSLCs and GFP-negative non-CSLCs on different days. Two batches of RNAs isolated on different days were combined for microarray analysis. Microarray analysis was performed using the Illumina HumanHT-12 v4 Expression BeadChip (Illumina, Inc., San Diego, CA, USA http://www.illumina.com/). RNAs were isolated using TRIzol (Invitrogen) according to the manufacturer's instructions and labeled using biotin. Biotinylated cRNA were prepared from 0.55 μg total RNA using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin,TX, USA). Following fragmentation, 0.75 μg of cRNAs were hybridized to the Illumina HumanHT-12 Expression Beadchip according to the protocols provided by the manufacturer. Export processing and analysis of array data were performed using Illumina GenomeStudio v2009.2 (Gene Expression Module v1.5.4) in comparison between GFP-positive CSCLs and GFP-negative non-CSLCs, and signaling pathway analysis was performed using DAVID (http://david.abcc.ncifcrf.gov/home.jsp). The GEOArchive files were deposited in the Gene Expression Omnibus (GEO) of NCBI (http://www.ncbi.nlm.nih.gov/geo/) under the accession number of GSE43336. We analyzed only the probes that fulfill the criteria in

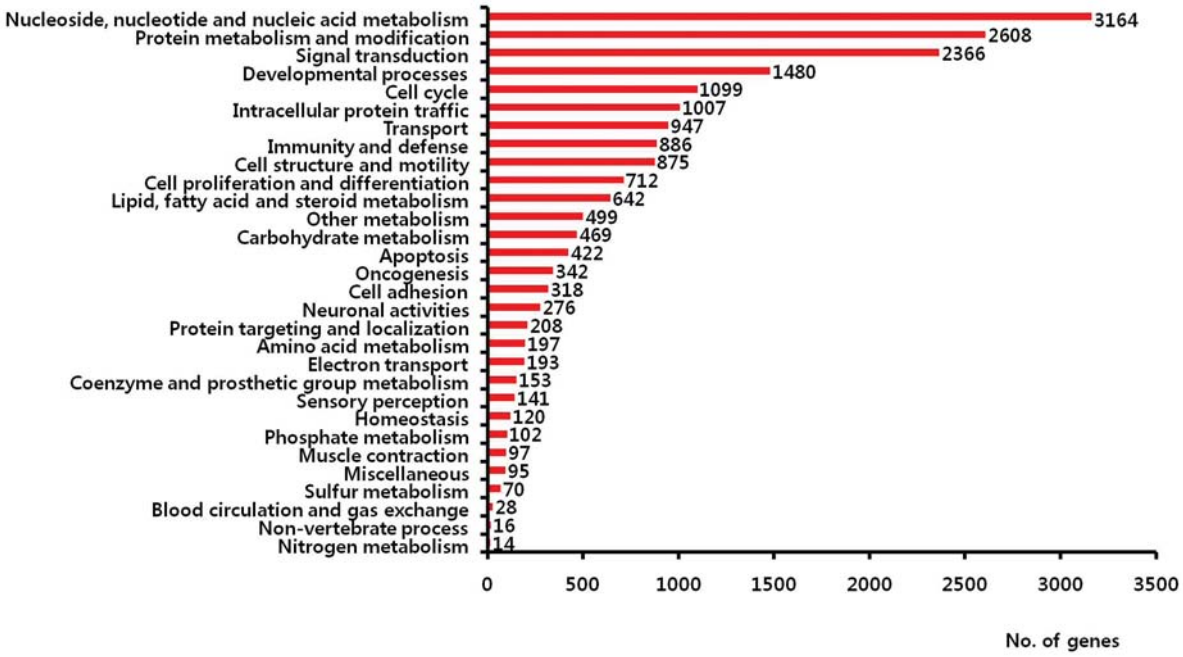

Figure 6. *Gene-oncology-based classifiaction of genes differentially expressed by more than 2-fold between cancer stem-like cells (CSLCs) and non-CSLCs.*

which the detection probability-value (pval) is lower than 0.05 in more than 50% of all samples. Quantile normalization was applied for normalization of gene expression value. The correlation coefficient between the log fold-change of raw data and that after quantile normalization was 0.981, suggesting the value was adjusted without significant loss from raw data. Through the quantile normalization, all probe intensities conformed to the same distribution for all sample arrays. Fold-Change (FC) was calculated by comparing the expression in the CSC-like groups with that in the non-CSC group.

Results

MDA-MB453 CSLCs overexpress OCT4 and are apparently resistant to differentiation induced by OCT4 knockdown. We repeatedly cultured MDA-MB453 CSLCs and characterized their cellular properties in comparison with non-CSLCs that had been fractionated from the same parental MDA-MB453 cells (19). Consistent with the previous study, immunoblotting and fluorescent microscopy showed that the expression of GFP in MDA-MB453 CSLCs correlated to stem cell-like morphological features, such as larger diameters, fibrillary shape, and growth without extensive cell-to-cell adhesion (Figure 1A and B). By contrast, the lack of GFP expression in non-CSLCs correlated to distinct morphological features, such as round shape with a bright edge and growth in aggregates (perhaps as a consequence of strong cell-to-cell adhesion) (Figure 1A). The parental MDA-MB453 cells displayed a morphology similar to that of non-CSLCs (Figure 1A).

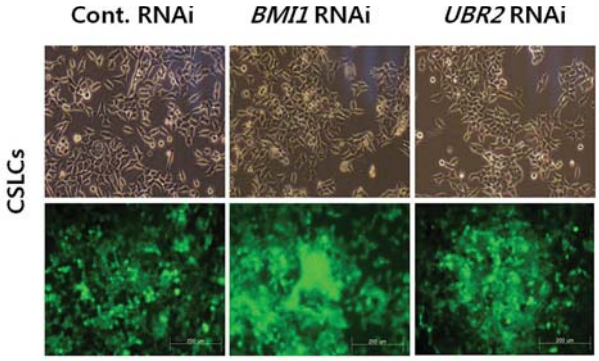

Figure 7. *Microscopic analysis of cancer stem-like cells transfected with control, BMI1(#2) and UBR2(#2) siRNAs.*

A previous study has shown that the activity of the *OCT4* promoter, as determined by the expression of GFP, correlated to the tumorigenesis of MDA-MB453 CSLCs (19). As MDA-MB453 CSLCs contain two types of the *OCT4* promoter, the exogenous promoter expressing GFP and the endogenous promoter expressing *OCT4*, we tested whether GFP-positive CSLCs contain a higher transcription activity for the endogenous *OCT4* promoter. Immunoblotting analysis showed that MDA-MB453 CSLCs contained a higher level of endogenous *OCT4* as compared with GFP-negative non-stem control cells (Figure 1B). This suggested

Table I. *Most highly-expressed genes in cancer stem-like cell (CSLC) populations compared with non-CSLC populations.*

RefSeq_ID	Gene symbol	ST./NS. FC.	Definition
NM_001001391.1	*CD44*	157.94	CD44 molecule (Indian blood group)
NM_000693.1	*ALDH1A3*	141.85	Aldehyde dehydrogenase-1 family, member A3
XM_001134012.2	*FABP5L2*	113.45	Fatty acid binding protein 5-like 2
NM_175617.3	*MT1E*	107.03	Metallothionein-1E
NM_005245.3	*FAT1*	92.66	FAT tumor suppressor homolog 1 (Drosophila)
NM_004207.2	*SLC16A3*	80.35	Solute carrier family-16, member-3 (monocarboxylic acid transporter-4)
NM_006829.2	*C10orf116*	73.67	Chromosome 10 open reading frame-116
NM_000693.2	*ALDH1A3*	70.96	Aldehyde dehydrogenase-1 family, member A3
NM_004864.1	*GDF15*	70.11	Growth differentiation factor-15
NM_005245.3	*FAT1*	65.11	FAT tumor suppressor homolog-1 (Drosophila)
NM_000389.2	*CDKN1A*	58.69	Cyclin-dependent kinase inhibitor-1A (p21, Cip1)
NM_001444.1	*FABP5*	55.33	Fatty acid binding protein-5 (psoriasis-associated)
NM_012242.2	*DKK1*	54.72	Dickkopf homolog-1 (Xenopus laevis)
NM_002531.2	*NTSR1*	53.81	Neurotensin receptor-1 (high affinity)
NM_005329.2	*HAS3*	51.37	Hyaluronan synthase-3
NM_002627.3	*PFKP*	51.13	Phosphofructokinase, platelet
NM_000067.1	*CA2*	43.21	Carbonic anhydrase-II
NM_201397.1	*GPX1*	37.34	Glutathione peroxidase-1
NM_004862.2	*LITAF*	36.57	Lipopolysaccharide-induced TNF factor
NM_005330.3	*HBE1*	34.40	Hemoglobin, epsilon-1

that the stemness of MDA-MB453 CSLCs involves the induction of unknown transcription factors that induce the expression of *OCT4*. Previous studies showed that the level of *OCT4* is highly sensitive to both stemness and differentiation of embryonic stem cells (22, 23). We therefore asked whether the treatment of siRNA against *OCT4* would induce the differentiation of MDA-MB453 CSLCs. The *OCT4*-knockdown CSLCs fully retained the expression of GFP, as well as the aforementioned stem cell-like morphological features (Figure 1C and D; data not shown). These results suggest that MDA-MB453 CSLCs overexpress OCT4 and are resistant to differentiation induced by *OCT4* knockdown. However, we do not exclude the possibility that these stem cell-like phenotypes involve partial activation and progression of a differentiation program that was not obviously detected in our current analysis.

MDA-MB453 CSCLs are apparently resistant to differentiation induced by ATRA. Retinoic acid (RA), a metabolite of vitamin A (retinol), can induce the differentiation of various types of CSCs (24, 25) and has been used to treat acute promyelocytic leukemia, a stem cell malignancy (26, 27). The activity of RA in the differentiation of CSCs is attributed to its binding to the nuclear transcription factor retinoic acid receptor (RAR) and the activation of genetic programs that modulate cell proliferation, differentiation and death (28, 29). Immunoblotting and fluorescence analysis of GFP and morphological measurements suggested that the treatment of ATRA for four days did not significantly affect the expression of GFP (Figure 2A and B) and the stem cell-like morphology of MDA-MB453 CSLCs (Figure 2B).

Characterization of histone ubiquitylation in MDA-MB453 CSLCs. The changes in gene expression through global epigenetic modifications contribute to the establishment and maintenance of CSCs (30, 31). The polycomb transcriptional repressor BMI1, a component of a Polycomb E3 ubiquitin ligase complex, mediates the ubiquitylation of histones H2A and H2B and plays an important role in maintaining the global gene expression pattern in various CSCs (32, 33). The ubiquitin ligase UBR2 is a substrate recognition component of the N-end rule pathway and can mediate ubiquitylation of *H2A* and H2B in germ and somatic cells (34). To test a possible role of these epigenetic modifiers in the stemness of MDA-MB453 cells, we compared their expression in GFP-positive and GFP-negative MDA-MB453 cells. Immunoblotting analysis indicated that the selective expression of BMI1 and UBR2 is not required to maintain the stemness of MDA-MB453 CSLCs (Figure 3A). Moreover, siRNA-based knockdown of *BMI1* or *UBR2* did not affect the levels of GFP and stem cell-like morphology in MDA-MB453 CSLCs (Figure 3B, C and Figure 6). In addition, immunoblotting analysis of total and ubiquitin-conjugated H2A and H2B indicated no significant differences between CSLCs and non-stem cell controls (Figure 3D). These results suggest that selective ubiquitylation of histones by BMI1 and UBR2 is not critical for maintaining the stem cell population of MDA-MB453 cell line.

Table II. *Cancer stem cell markers, membrane transporters and Wnt/β-catenin signaling genes.*

RefSeq_ID	Gene symbol	ST./NS. FC.	Definition
CSC markers			
NM_002354.2	*EPCAM*	1.5	Epithelial cell adhesion molecule, mRNA.
NM_001001391.1	*CD44*	157.9	CD44 molecule (Indian blood group), transcript variant-4
NM_001001392.1	*CD44*	16.3	CD44 molecule (Indian blood group), transcript variant-5
NM_000610.3	*CD44*	1.3	CD44 molecule (Indian blood group), transcript variant-1
NM_013230.2	*CD24*	6.0	CD24 molecule
NM_000689.3	*ALDH1A1*	–14.9	Aldehyde dehydrogenase-1 family, member A1
NM_000689.3	*ALDH1A1*	–25.9	Aldehyde dehydrogenase-1 family, member A1
NM_000693.1	*ALDH1A3*	141.9	Aldehyde dehydrogenase-1 family, member A3
NM_000693.2	*ALDH1A3*	71.0	Aldehyde dehydrogenase-1 family, member A3
NM_000210.2	*ITGA6*	1.1	Integrin, alpha 6, transcript variant 2
NM_006017.1	*CD133*	20.3	Prominin 1
Membrane transporter			
NM_019112.3	*ABCA7*	2.3	ATP-binding cassette, sub-family A, member-7
NM_000927.3	*ABCB1*	1.4	ATP-binding cassette, sub-family B(MDR/TAP), member-1
NM_000392.1	*ABCC2*	2.7	ATP-binding cassette, sub-family C (CFTR/MRP), member-2
NM_003786.2	*ABCC3*	7.9	ATP-binding cassette, sub-family C (CFTR/MRP), member-3
Wnt/ b-catenine signaling			
NM_181050.1	*AXIN1*	–2.2	Axin-1, transcript variant-2
NM_033637.2	*BTRC*	1.7	Beta-transducin repeat containing, transcript variant-1
NM_003505.1	*FZD1*	–1.2	Frizzled homolog 1 (*Drosophila*)
NM_003505.1	*FZD1*	–1.3	Frizzled homolog 1 (*Drosophila*)
NM_177435.1	*PPARD*	1.0	Peroxisome proliferative activated receptor, delta, transcript variant-2,
NM_006238.2	*PPARD*	1.0	Peroxisome proliferative activated receptor, delta, transcript variant-1,
NM_025216.2	*WNT10A*	–1.7	Wingless-type MMTV integration site family, member 10A
NM_003394.2	*WNT10B*	1.1	Wingless-type MMTV integration site family, member 10B
NM_057168.1	*WNT16*	5.4	Wingless-type MMTV integration site family, member 16
NM_024494.1	*WNT2B*	1.2	Wingless-type MMTV integration site family, member 2B, transcript variant WNT-2B2
NM_024494.1	*WNT2B*	1.1	Wingless-type MMTV integration site family, member 2B, transcript variant WNT-2B2
NM_006522.3	*WNT6*	–1.9	Wingless-type MMTV integration site family, member 6
NM_058238.1	*WNT7B*	–1.1	Wingless-type MMTV integration site family, member 7B
NM_002467.3	*MYC*	1.6	v-myc myelocytomatosis viral oncogene homolog (avian)
NM_002467.3	*MYC*	1.5	v-myc myelocytomatosis viral oncogene homolog (avian)
NM_053056.2	*CCND1*	–1.3	Cyclin D1
NM_012242.2	*DKK1*	54.7	Dickkopf homolog 1 (*Xenopus laevis*)
NM_012242.2	*DKK1*	2.2	Dickkopf homolog 1 (*Xenopus laevis*)
NM_014421.2	*DKK2*	–1.0	Dickkopf homolog 2 (*Xenopus laevis*)
NM_001098209.1	*β-catenine*	1.6	Catenin (cadherin-associated protein), beta 1, 88kDa, transcript variant-2
NM_001098209.1	*β-catenine*	1.4	Catenin (cadherin-associated protein), beta 1, 88kDa, transcript variant-2

Characterization of autophagic activity in MDA-MB453 CSLCs. Macroautophagy (hereafter autophagy) is a highly conserved bulk protein degradation pathway in eukaryotes that is induced in response to various cellular stresses, such as starvation, and mediates the degradation of cytosolic long-lived proteins and organelles (35). During autophagy, cytoplasmic macromolecules and organelles are engulfed by autophagosomes, which involves the conjugation of the ubiquitin-like protein LC3-I with phospholipid phosphotidylethanolamine (PE) to generate the active form, LC3-II, whose PE moiety is anchored to autophagosomal membranes. Cargo-loaded, LC3-II-positive autophagosomes are fused with lysosomes wherein the content is degraded by lysosomal hydrolases, producing fuels and amino acids. Recent studies have demonstrated that increased autophagic activity plays an important role in tumorigenesis and regulation of self-renewal of CSCs including breast CSCs (36-38). To test the potential function of the autophagy in long-term maintenance of stem cell subpopulation in the MDA-MB453 cell line, we determined autophagic activity in CSLCs. Immunoblotting analysis revealed a moderate but significant increase in the level of LC3-II in GFP-positive cells relative to GFP-negative cells (Figure 4A, lanes 5 *vs*. 1). The reduced level of *LC3*-II may be caused by either a reduced synthesis of LC3-I or an increased turnover of *LC3*-II. The treatment with bafilomycin A1, an inhibitor of the

Table III. *Hedgehog and Notch signaling genes.*

RefSeq_ID	Gene symbol	ST./NS. FC.	Definition
Hedgehog signaling			
NM_005269.1	*GLI1*	–1.3	Glioma-associated oncogene homolog 1 (zinc finger protein)
NM_000168.2	*GLI3*	–1.8	GLI-Kruppel family member GLI3 (Greig cephalopolysyndactyly syndrome)
NM_138465.3	*GLI4*	1.0	GLI family zinc finger 4
NM_005631.3	*SMO*	–1.4	Smoothened homolog (*Drosophila*)
NM_000193.2	*SHH*	NQ	Sonic hedgehog homolog (*Drosophila*)
NM_000264.2	*PTCH1*	NQ	Patched homolog 1 (*Drosophila*), transcript variant 1c
NM_003738.3	*PTCH2*	NQ	Patched homolog 2 (*Drosophila*)
Notch signaling			
NM_005618.3	*DLL1*	–1.5	Delta-like 1 (*Drosophila*)
NM_016941.2	*DLL3*	–9.8	Delta-like 3 (*Drosophila*), transcript variant 1
NM_003744.5	*NUMB*	–1.2	Numb homolog (*Drosophila*), transcript variant 3
NM_001005744.1	*NUMB*	–1.2	Numb homolog (*Drosophila*), transcript variant 2
NM_017617.3	*NOTCH1*	–1.1	Notch homolog 1, translocation-associated (*Drosophila*)
NM_024408.2	*NOTCH2*	–1.4	Notch homolog 2 (*Drosophila*)
NM_203458.3	*NOTCH2NL*	–1.2	Notch homolog 2 (*Drosophila*) N-terminal like
NM_203458.3	*NOTCH2NL*	–1.3	Notch homolog 2 (*Drosophila*) N-terminal like
NM_000435.1	*NOTCH3*	1.6	Notch homolog 3 (*Drosophila*)
NM_000214.1	*JAG1*	–1.1	Jagged 1 (Alagille syndrome)
NM_145159.1	*JAG2*	–1.6	Jagged 2, transcript variant 2
NM_002226.3	*JAG2*	–1.7	Jagged 2, transcript variant 1
NM_032492.3	*JAGN1*	1.1	Jagunal homolog 1 (*Drosophila*)
NM_020892.1	*DTX2*	2.1	Deltex homolog 2 (*Drosophila*)
NM_020892.1	*DTX2*	1.2	Deltex homolog 2 (*Drosophila*)
NM_001429.2	*EP300*	–2.5	E1A binding protein p300
NM_001527.2	*HDAC2*	1.2	Histone deacetylase 2
NM_001527.2	*HDAC2*	1.0	Histone deacetylase 2
NM_001040708.1	*HEY1*	–3.6	Hairy/enhancer-of-split related with YRPW motif 1, transcript variant 2
NM_001040708.1	*HEY1*	–32.6	Hairy/enhancer-of-split related with YRPW motif 1, transcript variant 2
NM_012259.1	*HEY2*	–6.7	Hairy/enhancer-of-split related with YRPW motif 2

fusion between autophagosomes and lysosomes, resulted in a moderate but significant accumulation of LC3-II in CSLCs relative to non-CSLCs (Figure 4A, lanes 6 *vs.* 2), suggesting that the stemness of MDA-MB453 cells may involve an enhanced autophagic flux. Consistently, the treatment with starvation media for 2 h, a strong inducer of autophagy, resulted in a reduced level of LC3-II in CSLCs (Figure 4A, lanes 7 *vs.* 3). These results suggest that the stem cell population of MDA-MB453 cells contain an increased autophagic flux relative to non-stem cell subpopulation.

Although the mechanisms underlying asymmetrical cell division of CSCs remain poorly-understood, recent studies showed that a singular organelle, called a midbody derivative, that forms between two daughter cells during cell division, marks CSCs after asymmetric division and, thus, plays a role in the maintenance of the pluripotency of CSCs (39). It has been proposed that the selective accumulation of midbody derivatives in CSCs is caused by reduced autophagic activity in stem cell progenies (39). In contrast to the results from the study by Kuo *et al.*, our immunobloting analysis revealed that CSLCs contained reduced levels of PLK1 and CEP55, major components of midbody derivatives, as compared with non-CSLCs (Figure 4B). These results suggest that selective segregation by differential degradation of midbody derivatives is not required to maintain the stemness in the MDA-MB453 cell line. The reduced levels of PLK1 and *CEP55* may be caused by an increased level of autophagic flux.

Characterization of cellular responses to endoplasmic reticulum (ER) stress in MDA-MB453 CSLCs. The ER is an organelle into which approximately one-third of cellular proteins destined to the secretory pathway, are translocated. As these ER-targeted proteins should be properly folded and thus undergo post-translational modifications within the lumen, the function of the ER is highly sensitive to various cellular stresses. Recent studies suggested that appropriate response to ER stress, caused by culture conditions, are important for embryonic stem cells to survive and maintain their pluripotency (40-42). To test whether GFP-positive and -negative MDA-MB453 cells exhibit differential responses to ER stress, we treated CSLCs and non-CSLCs with the ER

Table IV. *Bone morphogenetic proteins (BMP) and Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) signaling-associated genes.*

RefSeq_ID	Gene symbol	ST./NS. FC.	Definition
BMP signaling			
NM_006131.1	*BMP1*	–1.1	Bone morphogenetic protein 1, transcript variant BMP1-5
NM_006129.2	*BMP1*	–1.4	Bone morphogenetic protein 1, transcript variant BMP1-3
NM_017593.3	*BMP2K*	–2.3	BMP2 inducible kinase, transcript variant 2
NM_017593.3	*BMP2K*	–5.6	BMP2 inducible kinase, transcript variant 2
NM_198892.1	*BMP2K*	–6.4	BMP2 inducible kinase, transcript variant 1
NM_001202.2	*BMP4*	2.2	Bone morphogenetic protein 4, transcript variant 1
NM_130851.1	*BMP4*	2.1	Bone morphogenetic protein 4, transcript variant 3
NM_001718.4	*BMP6*	–1.7	Bone morphogenetic protein 6
NM_001719.1	*BMP7*	–2.5	Bone morphogenetic protein 7 (osteogenic protein 1)
NM_001720.3	*BMP8B*	–1.2	Bone morphogenetic protein 8b
NM_004329.2	*BMPR1A*	–1.8	Bone morphogenetic protein receptor, type IA
NM_001204.5	*BMPR2*	–2.0	Bone morphogenetic protein receptor, type II (serine/threonine kinase)
NM_001204.5	*BMPR2*	–2.3	Bone morphogenetic protein receptor, type II (serine/threonine kinase)
NM_005903.5	*SMAD5*	1.5	SMAD family member 5, transcript variant 1
NM_005585.3	*SMAD6*	–7.0	SMAD family member 6, transcript variant 1
NM_005904.2	*SMAD7*	–1.8	SMAD family member 7
NM_005904.2	*SMAD7*	–5.5	SMAD family member 7
JAK/STAT signaling			
NM_002227.2	*JAK1*	4.2	Janus kinase 1
NM_004972.2	*JAK2*	–2.0	Janus kinase 2 (a protein tyrosine kinase)
NM_139266.1	*STAT1*	1.4	Signal transducer and activator of transcription 1, 91kDa, transcript variant beta
NM_007315.2	*STAT1*	1.2	Signal transducer and activator of transcription 1, 91kDa (STAT1), transcript variant alpha, mRNA.
NM_007315.2	*STAT1*	–1.2	Signal transducer and activator of transcription 1, 91kDa, transcript variant alpha
NM_005419.2	*STAT2*	1.2	Signal transducer and activator of transcription 2, 113kDa
NM_213662.1	*STAT3*	3.6	Signal transducer and activator of transcription 3 (acute-phase response factor), transcript variant 3
NM_139276.2	*STAT3*	2.5	Signal transducer and activator of transcription 3 (acute-phase response factor), transcript variant 1
NM_213662.1	*STAT3*	2.4	Signal transducer and activator of transcription 3 (acute-phase response factor), transcript variant 3
NM_003151.2	*STAT4*	1.3	Signal transducer and activator of transcription 4
NM_003153.3	*STAT6*	1.6	Signal transducer and activator of transcription 6, interleukin-4 induced

stressor thapsigargin, an inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase. Immunoblotting and fluorescence analyses showed that the levels of GFP and morphological features of both cell types were resistant to thapsigargin-induced ER stress (Figure 5A and B). This result, together with those described above, collectively suggest that the differentiation program in MDA-MB453 CSLCs is blocked, through an unknown mechanism, at a step upstream of the transcription of the *OCT4* promoter, allowing for CSLCs to maintain their rare population through asymmetric cell division during many repeated passages.

Global gene expression profiling of MDA-MB453 CSLCs. As the results described above indicate that many characteristics known to specify CSCs are not conserved in CSCs isolated from MDA-MB453 cells, we used microarray analysis to identify signature pathways that define the stemness of this specific cell type. Approximately 1,959 out of 31,424 genes in total were differentially expressed in GFP-positive cells relative to GFP-negative cells, amongst which 1,069 were up-regulated, whereas 890 were down-regulated by more than 2-fold. Gene ontology-based classification revealed that CSLCs differentially expressed the genes functionally involved in malignancy of cancer cells, such cell cycle, immunity and defense, motility, and proliferation and differentiation (Figure 7). As the differential expression of these genes may contribute to high tumorigenic activity of GFP-positive MDA-MB453 cells (19), we further categorized them into several functional groups (Tables I-IX). When known breast CSC markers were analyzed, GFP-positive cells exhibited higher levels for Clusters of differentiation 44 (*CD44*), Aldehyde dehydrogenase-1A3 (*ALDH1A3*) and *CD133*, but not Epithelial cell adhesion molecule (*EPCAM*) and Integrin-α6 (43-46) (Table II). Interestingly, the stemness of MDA-MB453 cells did not present a significant correlation to the Hedgehog, Wnt, and Notch pathways that have been extensively characterized for their essential role in self-renewal of CSCs (47-50) (Tables II and III). Instead, we observed a strong up-regulation of the Janus kinase-1 (*JAK1*)/Signal transducer and activator of transcription-3

Table V. *Jun oncogene (JUN)/ dachshund homolog1 (DACH1)/p21, and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB)/ inhibitor of NFκB (IκB).*

RefSeq_ID	Gene symbol	ST./NS. FC.	Definition
JUN/DACH/p21			
NM_002228.3	*JUN*	–1.2	Jun oncogene
NM_000389.2	*p21*	58.7	Cyclin-dependent kinase inhibitor 1A (p21, Cip1), transcript variant 1,
ILMN_2386053	*DACH1*	NQ	Dachshund homolog 1 (*Drosophila*), transcript variant 2
NFκB			
NM_003998.2	*NFKB1*	–1.1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NM_002502.3	*NFKB2*	–1.1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100), transcript variant 2
NM_001077493.1	*NFKB2*	–1.2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100), transcript variant 3
NM_020529.1	*NFKBIA*	–3.0	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NM_002503.3	*NFKBIB*	1.2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta, transcript variant 1
NM_001001716.1	*NFKBIB*	1.1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta, transcript variant 2
NM_139239.1	*NFKBID*	1.1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta
NM_004556.2	*NFKBIE*	–1.2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
NM_013432.3	*NFKBIL2*	–1.2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 2
NM_201612.1	*IKBIP*	1.4	IKBKB interacting protein, transcript variant 2
NM_153687.2	*IKBIP*	1.4	IKBKB interacting protein, transcript variant 1
NM_201612.1	*IKBIP*	1.2	IKBKB interacting protein, transcript variant 2
NM_003640.2	*IKBKAP*	1.0	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein
NM_001556.1	*IKBKB*	–1.4	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
NM_014002.2	*IKBKE*	1.2	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
NM_001099856.1	*IKBKG*	1.3	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma, transcript variant 2

Table VI. *Autophagy-associated genes.*

RefSeq_ID	Gene symbol	ST./NS. FC.	Definition
Autophagy			
NM_002931.3	*RING1*	1.3	Ring finger protein 1
NM_005180.5	*BMI1*	–1.1	BMI1 polycomb ring finger oncogene
NM_003766.2	*BECN1*	1.5	Beclin 1, autophagy related
NM_003900.3	*SQSTM1*	2.7	Sequestosome 1
NM_031482.3	*ATG10*	–1.6	ATG10 autophagy related 10 homolog (*S. cerevisiae*)
NM_031482.3	*ATG10*	–1.9	ATG10 autophagy related 10 homolog (*S. cerevisiae*)
NM_004707.2	*ATG12*	1.6	ATG12 autophagy related 12 homolog (*S. cerevisiae*)
NM_004707.2	*ATG12*	1.2	ATG12 autophagy related 12 homolog (*S. cerevisiae*)
NM_017974.3	*ATG16L1*	–1.9	ATG16 autophagy related 16-like 1 (*S. cerevisiae*), transcript variant 2
NM_030803.5	*ATG16L1*	–2.1	ATG16 autophagy related 16-like 1 (*S. cerevisiae*), transcript variant 1
NM_033388.1	*ATG16L2*	–1.5	ATG16 autophagy related 16-like 2 (*S. cerevisiae*)
NM_015104.1	*ATG2A*	–1.3	ATG2 autophagy related 2 homolog A (*S. cerevisiae*)
NM_018036.5	*ATG2B*	–1.3	ATG2 autophagy related 2 homolog B (*S. cerevisiae*)
NM_022488.3	*ATG3*	–1.1	ATG3 autophagy related 3 homolog (*S. cerevisiae*)
NM_178270.1	*ATG4A*	1.2	ATG4 autophagy related 4 homolog A (*S. cerevisiae*), transcript variant 2
NM_178270.1	*ATG4A*	1.2	ATG4 autophagy related 4 homolog A (*S. cerevisiae*), transcript variant 2
NM_178326.2	*ATG4B*	–1.1	ATG4 autophagy related 4 homolog B (*S. cerevisiae*), transcript variant 2
NM_032852.2	*ATG4C*	–1.0	ATG4 autophagy related 4 homolog C (*S. cerevisiae*), transcript variant 7
NM_032885.4	*ATG4D*	–1.0	ATG4 autophagy related 4 homolog D (*S. cerevisiae*)
NM_004849.1	*ATG5*	1.3	ATG5 autophagy related 5 homolog (*S. cerevisiae*)
NM_006395.1	*ATG7*	1.1	ATG7 autophagy related 7 homolog (*S. cerevisiae*)
NM_001077198.1	*ATG9A*	–1.0	ATG9 autophagy related 9 homolog A (*S. cerevisiae*), transcript variant 1
NM_001077198.1	*ATG9A*	–1.0	ATG9 autophagy related 9 homolog A (*S. cerevisiae*), transcript variant 1
NM_007278.1	*GABARAP*	–1.1	GABA(A) receptor-associated protein
NM_031412.2	*GABARAPL1*	4.2	GABA(A) receptor-associated protein like 1
NM_007285.6	*GABARAPL2*	1.5	GABA(A) receptor-associated protein-like 2
NM_007285.6	*GABARAPL2*	1.1	GABA(A) receptor-associated protein-like 2

Table VII. *Midbody deritives, hypoxia and epithelial–mesenchymal transition (EMT)-associated genes.*

RefSeq_ID	Gene symbol	ST./NS. FC.	Definition
Midbody derivatives			
NM_018131.3	*CEP55*	–1.2	Centrosomal protein 55 kDa
NM_005030.3	*PLK1*	–1.3	Polo-like kinase 1 (*Drosophila*)
Hypoxia			
NM_001530.2	*HIF1A*	1.8	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor), transcript variant 1
NM_181054.1	*HIF1A*	1.6	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor), transcript variant 2
NM_001530.2	*HIF1A*	1.5	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor), transcript variant 1
NM_017902.2	*HIF1AN*	1.5	Hypoxia inducible factor 1, alpha subunit inhibitor
NM_001430.3	*EPAS1*	7.4	Endothelial PAS domain protein 1
NM_178426.1	*ARNT*	–1.4	Aryl hydrocarbon receptor nuclear translocator, transcript variant 2
NM_014862.3	*ARNT2*	1.8	Aryl-hydrocarbon receptor nuclear translocator 2
NM_001030272.1	*ARNTL*	1.8	Aryl hydrocarbon receptor nuclear translocator-like, transcript variant 2,
NM_022051.1	*EGLN1*	–1.6	Egl nine homolog 1 (*C. elegans*)
NM_053046.2	*EGLN2*	–1.3	Egl nine homolog 2 (*C. elegans*), transcript variant 1
NM_080732.1	*EGLN2*	–1.5	Egl nine homolog 2 (*C. elegans*), transcript variant 3
NM_006389.2	*HYOU1*	1.2	Hypoxia up-regulated 1
NM_006389.2	*HYOU1*	1.1	Hypoxia up-regulated 1
NM_001099668.1	*HIGD1A*	–1.3	HIG1 hypoxia inducible domain family, member 1A, transcript variant 1,
NM_001099668.1	*HIGD1A*	–1.4	HIG1 hypoxia inducible domain family, member 1A, transcript variant 1,
NM_014056.1	*HIGD1A*	–1.5	HIG1 domain family, member 1A
NM_138820.2	*HIGD2A*	–1.5	HIG1 hypoxia inducible domain family, member 2A
EMT			
NM_004360.2	*CDH1*	5.1	Cadherin 1, type 1, E-cadherin (epithelial)
NM_005985.2	*SNAI1*	NQ	Snail homolog 1 (*Drosophila*)
NM_000474.3	*TWIST1*	–5.8	Twist homolog 1 (*Drosophila*)
XM_945603.1	*TWIST2*	1.0	PREDICTED: twist homolog 2 (*Drosophila*), transcript variant 2

(*STAT3*) and Bone morphogenetic protein-4 (*BMP4*) in GFP-positive cells, indicating their potential role in the establishment and asymmetric cell division of MDA-MB453 CSLCs (Table IV). However, no significant difference was observed for cell fate determinants (*e.g.* Numb homolog protein (*NUMB*), Partitioning defective-6A (*PARD6A*), protein kinase C-iota (*PRKCI*), Lethal giant larvae-1 (*LLGL1*) and Serine/threonine kinase-11 (*STK11*)) known to control symmetric/asymmetric cell division (13, 51-55) (Table IX), suggesting that the MDA-MB453 cell line maintains asymmetry-only cell division of its stem cell sub-population through a mechanism independent of the differential transcription of these determinants. Finally, we observed up-regulation of the signaling pathways in focal adhesion, carcinogenesis, extracellular matrix (ECM) interaction, and actin cytoskeleton regulation, which may contribute to the more aggressive behavior of MDA-MB453 CSLCs (56-58).

Taken together, our immunoblotting and microarray data suggested that many factors that have been demonstrated to play important roles in survival and, thus the maintenance of pluripotent CSCs, are not conserved in our CSLCs derived from breast cancer cell lines. These results imply that only limited factors are required for establishment of pluripotency and control of asymmetric/symmetric cell division of CSC, while other genes in signal pathways or processes may play roles in other processes, for example CSC survival and interaction with the environment.

Discussion

In this study, we characterized the nature of the stemness of CSLCs, in comparison with non-CSLCs, that had been isolated from the same parental MDA-MB453 human breast cancer cells. We show that although *OCT4* expression is essential to maintain the CSLC population in MDA-MB453 cells, the function of *OCT4* is not required to maintain stemness. Instead, our results are consistent with the possibility that an essential differentiation program is arrested, through an unknown mechanism, at a step upstream of the transcription of *OCT4*. This model is further supported by the results that MDA-MB453 CSLCs are apparently resistant to differentiation induced by the treatment of ATRA or the knockdown of *BMI1*. It would be, therefore, of interest to identify a component of the *OCT4*-dependent pathway

Table VIII. *Retinoic acid (RA) signaling and miRNA genes.*

RefSeq_ID	Gene symbol	ST./NS. FC.	Definition
RA signaling			
NM_000964.2	*RARA*	1.9	Retinoic acid receptor, alpha, transcript variant 1
NM_002957.3	*RXRA*	1.5	Retinoid X receptor, alpha
NM_021976.3	*RXRB*	1.7	Retinoid X receptor, beta
NM_004585.3	*RARRES3*	–17.1	Retinoic acid receptor responder (tazarotene induced) 3
NM_001878.2	*CRABP2*	14.9	Cellular retinoic acid binding protein 2
NM_001444.1	*FABP5*	55.3	Fatty acid binding protein 5 (psoriasis-associated)
NM_001444.1	*FABP5*	4.1	Fatty acid binding protein 5 (psoriasis-associated)
XM_001134012.2	*FABP5L2*	113.4	PREDICTED: fatty acid binding protein 5-like 2
XM_001721172.1	*FABP5L2*	21.3	PREDICTED: fatty acid binding protein 5-like 2
MiRNAs			
NR_031575.1	*MIR1185-1*	–1.1	MicroRNA 1185-1
NR_031597.1	*MIR1228*	–1.5	MicroRNA 1228
NR_031654.1	*MIR1253*	1.4	MicroRNA 1253.
NR_029694.1	*MIR125B2*	1.1	MicroRNA 125b-2
NR_031695.1	*MIR1282*	1.4	MicroRNA 1282
NR_029697.1	*MIR129-2*	–1.1	MicroRNA 129-2
NR_029706.1	*MIR185*	1.6	MicroRNA 185
NR_031730.1	*MIR1909*	–1.4	MicroRNA 1909
NR_031742.1	*MIR1978*	4.0	MicroRNA 1978
NR_029627.1	*MIR214*	–2.2	MicroRNA 214
NR_029635.1	*MIR221*	1.2	MicroRNA 221
NR_029498.1	*MIR25*	–1.3	MicroRNA 25
NR_030582.1	*MIR300*	–1.1	MicroRNA 300
NR_029886.1	*MIR330*	1.4	MicroRNA 330
NR_029906.1	*MIR345*	–1.1	MicroRNA 345
NR_030313.1	*MIR586*	–1.6	MicroRNA 586
NR_030329.1	*MIR599*	1.0	MicroRNA 599
NR_030338.1	*MIR607*	–1.1	MicroRNA 607
NR_030365.1	*MIR635*	1.1	MicroRNA 635
NR_030368.1	*MIR638*	–1.2	MicroRNA 638
NR_030375.1	*MIR645*	–1.2	MicroRNA 645
NR_029481.1	*MIRLET7D*	–1.5	MicroRNA let-7d

Table IX. *Asymmetric cell division-associated and other genes.*

RefSeq_ID	Gene symbol	ST./NS. FC.	Definition
Asymmetric cell division			
NM_003744.5	*NUMB*	–1.2	Numb homolog (*Drosophila*), transcript variant 3
NM_001005744.1	*NUMB*	–1.2	Numb homolog (*Drosophila*), transcript variant 2
NM_001037281.1 P	*ARD6A*	–1.1	Par-6 partitioning defective 6 homolog alpha (*C. elegans*), transcript variant 2
NM_016948.2	*PARD6A*	–1.2	Par-6 partitioning defective 6 homolog alpha (*C. elegans*), transcript variant 1
NM_002740.5	*PRKCI*	–1.2	Protein kinase C, iota
NM_004140.3	*LLGL1*	2.1	Lethal giant larvae homolog 1 (*Drosophila*)
NM_000455.4	*STK11*	–1.5	Serine/threonine kinase 11
NM_017617.3	*NOTCH1*	–1.1	Notch homolog 1, translocation-associated (*Drosophila*)
NM_024408.2	*NOTCH2*	–1.4	Notch homolog 2 (*Drosophila*)
Other genes			
NM_001621.3	*AHR*	1.9	Aryl hydrocarbon receptor
NM_001621.2	*AHR*	–1.0	Aryl hydrocarbon receptor
NM_001540.2	*HSPB1*	–1.1	Heat shock 27kDa protein 1
NM_000245.2	*MET*	1.2	Met proto-oncogene (hepatocyte growth factor receptor), transcript variant 2
NM_005180.5	*BMI1*	–1.1	BMI1 polycomb ring finger oncogene
NM_015255.1	*UBR2*	–1.1	Ubiquitin protein ligase E3 component n-recognin 2
NM_000600.1	*IL6*	–1.6	Interleukin 6 (interferon, beta 2) (IL6)
NM_000565.2	*IL6R*	1.0	Interleukin 6 receptor, transcript variant 1

critical to maintain the stemness of MDA-MB453 CSLCs. In this study, we correlated the stemness of MDA-MB453 cells to the expression of GFP and stem cell-like morphological features, such as larger diameters, fibrillary shape, and growth without extensive cell-to-cell adhesion (see Figure 1A). However, we do not completely exclude the possibility that these stem cell-like phenotypes involve partial activation and progression of a differentiation program that is not obviously detected in our current analysis.

Studies have shown that the stemness of CSCs requires the selective regulation of major cellular pathways or systems, including epigenetic modification of histones, autophagy, and cellular responses to ER stress (30, 36-38, 41, 42, 59, 60). In contrast to previous studies indicating the importance of histone ubiquitylation (32, 33), our results suggest that MDA-MB453 CSLCs contain a normal level of ubiquitin-conjugated histone H2A and H2B, relative to non-CSLCs, and resistant to differentiation induced by knockdown of *BMI1* whose function has been shown to be essential to maintain the global gene expression pattern in various CSCs (32, 33). In addition, we find that MDA-MB453 CSLCs contain a moderately but significantly increased level of autophagic flux relative to non-stem cell subpopulation, as determined by the synthesis of LC3-II, an active/lipidated form of LC3-I, and the conversion/activation of *LC3*-I to LC3-II, in normal and starvation media. This finding is largely in agreement with previous studies that showed that increased autophagic activity correlated to tumorigenesis and the self-renewal of various CSCs (36-38).

We tested to what degree the expression profile of CSC-specific pathways and proteins is conserved in CSLCs fractionated from immortalized cells in culture. Overall, our results suggest that only a limited number of those CSC markers are selectively expressed in MDA-MB453 CSLCs. We therefore propose that only a limited number of genes may be required to be selectively expressed in order to establish pluripotency and to control asymmetric/symmetric cell division of MDA-MB453 CSLCs, whereas some CSC-specific genes may be secondary to CSC survival and interaction with the microenvironment in tumors. Of note amongst the most selectively expressed genes in MDA-MB453 CSLCs is *ALDH1A3,* encoding the enzyme aldehyde dehydrogenase whose expression correlates to the activity of CSCs in breast tumors (45). Another example of known CSC-specific genes that are selectively expressed in MDA-MB453 CSLCs is *JAK1*, a tyrosine kinase essential for signaling for some type-I and type-II cytokines, and *STAT3*, a transcription factor that mediates signaling from cytokines and growth factors (61, 62). Amongst the genes that are down-regulated in CSCs, we observed the down-regulation of *BMP2* and *BMP7*, belonging to the transforming growth factor-β (TGF-β) superfamily of proteins, that have been shown to negatively-regulate the tumorigenecity of CSCs (63-65). It should be noted that *JAK1*, *STAT3*, *BMP2*, and *BMP7* work together in the cytokine signaling pathway that has been shown to regulate the reversible conversion between CSCs and non-CSCs (66-70), indicating that these genes may play a role in maintaining the stemness of MDA-MB453 CSLCs. Whereas MDA-MB453 CSLCs selectively expressed a limited number of known CSC markers, the majority of CSC markers did not show significant selectivity in CSLCs relative to non-CSLCs. These include the genes in the core stem cell signaling pathways, such as the Wnt/β-catenin pathway that regulates cell-cell communication and telomerase in CSCs (71), the Sonic Hodgehog pathway that has been involved in maintaining the tumorigenicity of CSCs (72), and the Notch pathway that regulates the differentiation of CSCs (73). These results suggest that the stemness of MDA-MB453 CSLCs may not strictly depend on the core stem cell signaling pathways.

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