BioChip J. (2012) 6(1): 99-105
DOI 10.1007/s13206-012-6113-8

Original Research

Analysis of SSEA1$^+$ vs. SSEA1$^-$ fractions of bulk-cultured XENP cell lines

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Received: 2 December 2011 / Accepted: 25 January 2012 / Published online: 20 March 2012

Abstract Previously isolated rat extraembryonic endoderm precursor (XENP) cell lines had been characterized after clonal density plating. The arising colonies had consisted of peripheral XENP cells expressing the surface antigen SSEA1 and the transcription factor Oct4, and inner XENP-derived extraembryonic endoderm cells that were nearly negative for SSEA1 and Oct4. We now sorted bulk-cultured XENP cell lines from two rat strains by FACS into SSEA1$^+$ and SSEA1$^-$ populations and compared their expression profiles by microarray and RT-PCR. In the bulk cultures, the SSEA1$^+$ fraction was only slightly enriched for Oct4, and also slightly enriched for the visceral endoderm markers, Dab2 and Ihh. Both fractions expressed vascular-associated mesodermal markers (VE-cadherin, Flk1). Thus, in regular-density XENP cell cultures, SSEA1 is not suitable as a stem cell marker, and the XENP cells appear to undergo partial somatic differentiation.

Keywords: Stem cells, XENP cells, Extraembryonic endoderm, SSEA1, Oct4, Microarray

Introduction

Recently, extraembryonic endoderm precursor (XENP) cells were isolated from rat blastocysts and proposed to be similar to the committed extraembryonic endoderm precursor of the inner cell mass (ICM) of blastocysts[1]. Because of their propensity to differentiate, the XENP cells could not be cultured in pure form. However, when plated at clonal density, only or almost only XENP cells survived and gave rise to colonies. For the first one to three divisions, the cells remained undifferentiated, but as the colonies expanded, the inner cells flattened out and differentiated into extraembryonic endoderm, the fate that was also observed upon microinjection in vivo. Immunochemistry showed that XENP cells co-expressed the transcription factors Gata6 and Oct4, which is characteristic of the ICM-stage extraembryonic endoderm precursor[2], as well as the carbohydrate surface antigen SSEA1 that is a popular marker of mouse embryonic stem (ES) cells[3]. Like Oct4, SSEA1 was absent or at least greatly reduced in the differentiating flat cells within the colonies arising from the low density-plated XENP cells. This observation suggests that SSEA1 can be used in order to enrich undifferentiated XENP cells by FACS in larger numbers from mass (bulk) cultures.

However, as reported here, in the bulk culture setting, SSEA1 does not only mark XENP cells, and Oct4 is not limited to SSEA1 positive (SSEA1$^+$) cells. Moreover, gene expression analysis suggests a broader differentiation capacity than seen with XENP cells tested in vivo, thus indicating plasticity and emphasizing the need to develop a method to culture pure XENP cells in bulk amounts.

Results and Discussion

Sorting and contribution of feeder cells

Two lines of XENP cells, CX1, from the inbred rat strain Wistar Kyoto (WKY), and CX7, from the out-

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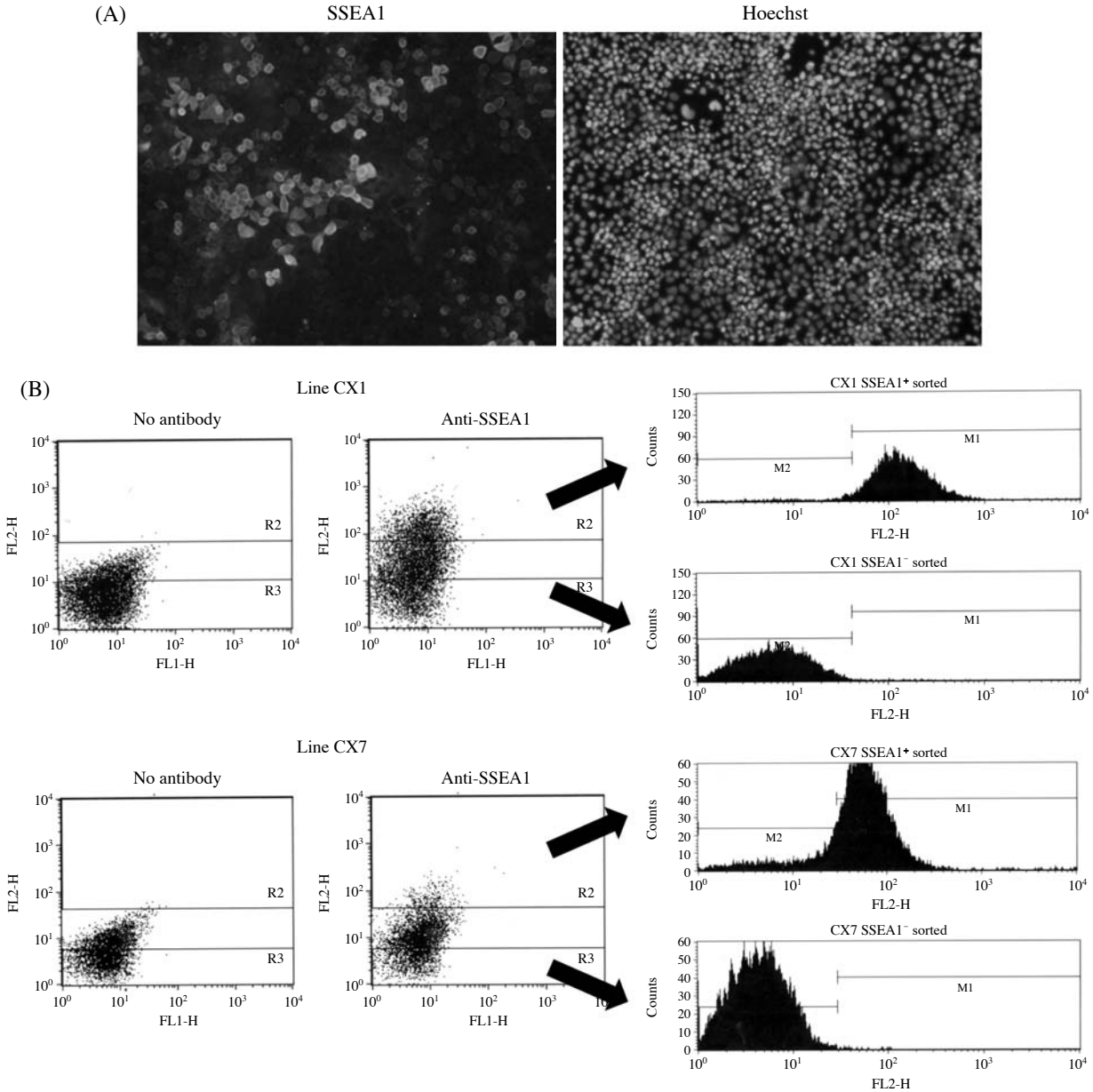

Figure 1. Sorting of XENP cell lines CX1 (WKY) and CX7 (SD) into SSEA1$^-$ and SSEA1$^+$ fractions. A, Representative image of non-sorted, bulk-cultured XENP cells (CX1) stained for SSEA1 (left) or DNA (right). Isotype control staining was negative (not shown). Origin. magn., 10× B, Representative images of the FACS separations.

bred rat strain Sprague Dawley (SD), were grown on Mitomycin C-treated feeder cells in bulk amounts. Immunocytochemistry illustrated that the bulk-cultured cells contained a fraction of SSEA1$^+$ cells (Figure 1A). The cultures were subjected to FACS (Figure 1B), which reproducibly showed that line CX1 contained ~28% of SSEA1$^+$ cells, but line CX7 only ~9%. After sorting, RNA was isolated and used for microarray analysis (Figure 2) and qRT-PCR (Figure 3). Since the feeder cells should contribute to the SSEA1$^-$ fraction, RNA was also prepared from pure feeders. As expected, the microarray comparison of feeder vs. sorted samples revealed many differences (e.g., Figure 2B). For example, we identified smooth muscle myosin as a potentially feeder-specific product, and therefore studied two smooth muscle markers, aSM and SM22, by qRT-PCR. These markers were expressed more than 100x higher in the feeders than in each sorted sample (Figure 3A), implying that feeder cells made only a minor contribution to the RNA yields of the sorted samples.

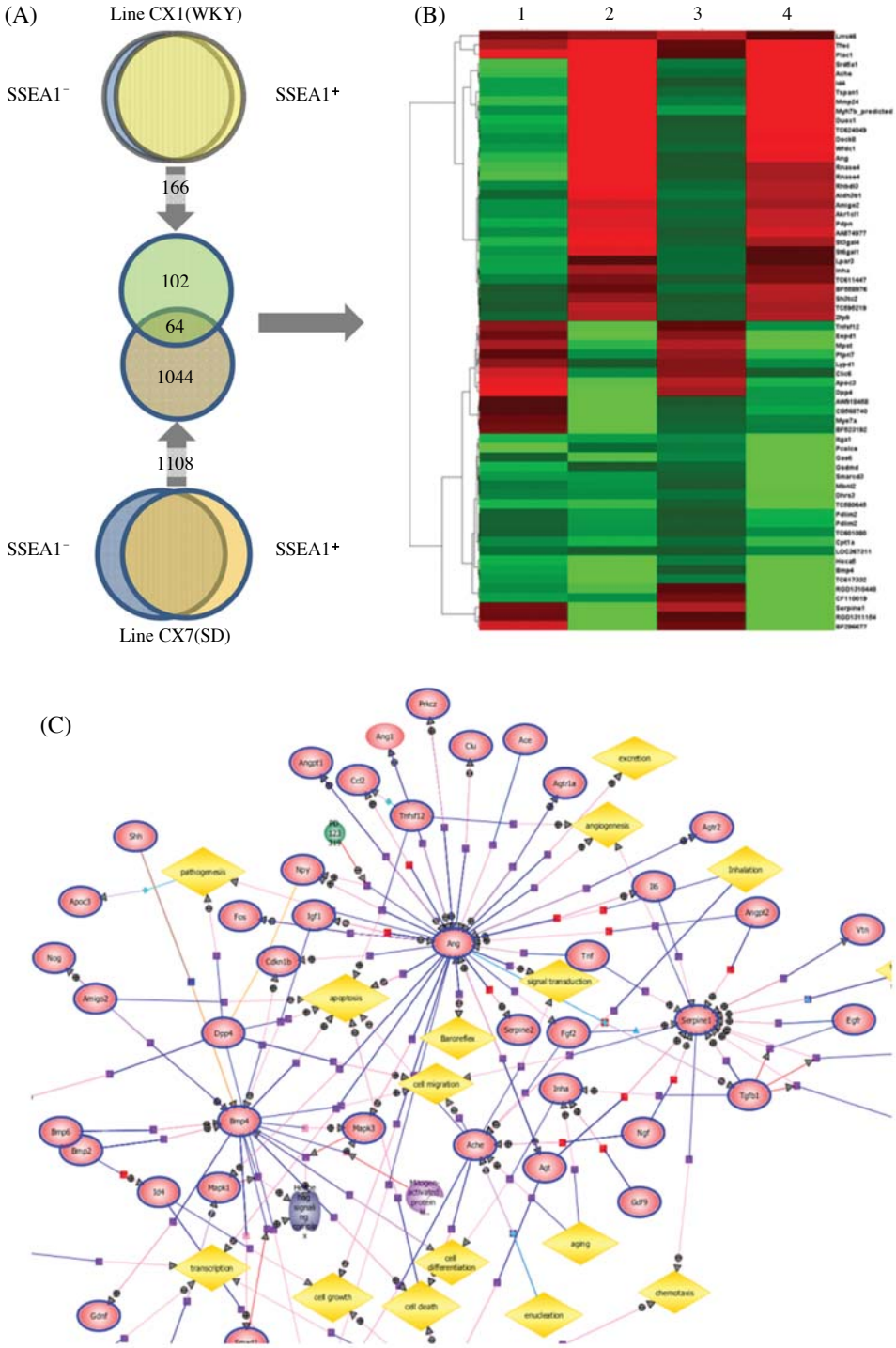

Figure 2. Identification of common differences in SSEA1⁺ vs. SSEA1⁻ cell fractions of the two XENP cell lines. A, Venn diagram. B, Hierarchical clustering analysis: 1, CX7 SSEA1⁺/SSEA1⁻; 2, CX7 SSEA1⁻/Feeder; 3, CX1 SSEA1⁺/SSEA1⁻; 4, CX1 SSEA1/Feeder. C, Gene network analysis (selected region).

We then compared the SSEA1⁺ and SSEA1⁻ fractions by global expression analysis (microarray) and qRT-PCR of selected lineage markers.

Hierarchical clustering and network analysis

In lines CX1 and CX7, 166 and 1108 genes, respective-

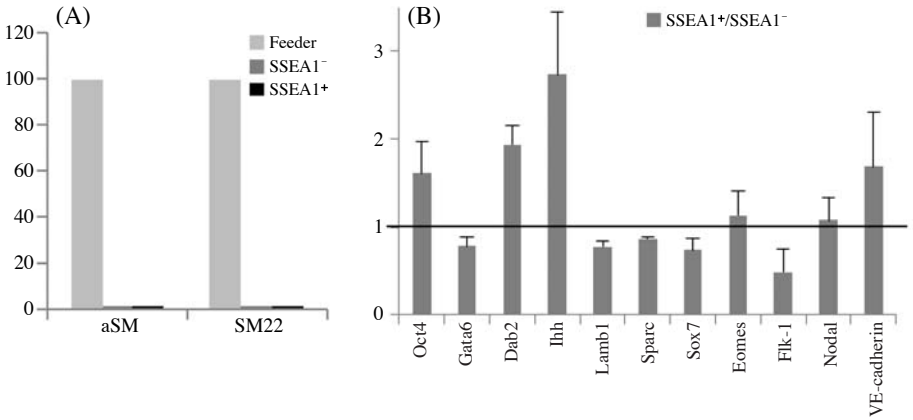

Figure 3. Expression of selected lineage markers (qRT-PCR). A, Two markers (aSM=Smooth muscle α-actin; SM22=transgelin) that are specifically expressed in the feeder cells. B, Relative expression (SSEA1+/SSEA1⁻) of selected lineage markers that were not expressed in feeders.

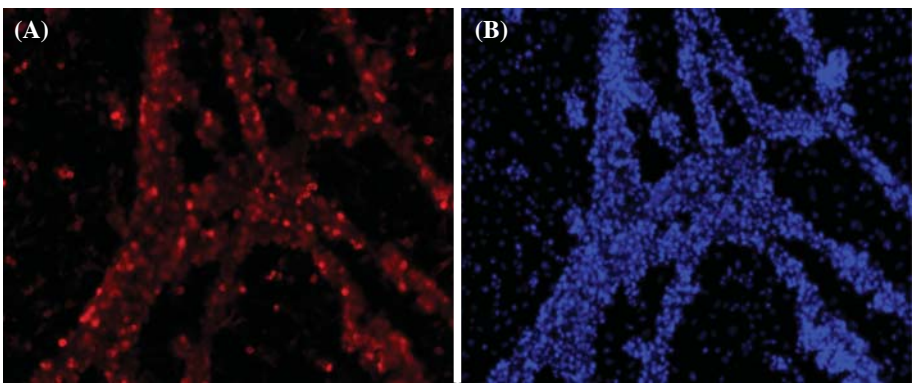

Figure 4. Immunostaining of older (>10 days) XENP cell cultures forming ducts. A, Anti-SSEA1; B, Hoechst dye. Original magnification, 10×. Isotype control was negative (not shown).

ly, were significantly differently expressed between the SSEA1+ and SSEA1⁻ fractions, and amongst those differences, 64 were seen in both cell lines (=39% in line CX1 and 6% in line CX7) (Figure 2A). These numbers suggest that the two cell lines differed somewhat in their degrees of differentiation. The most significant common differences are given in Table 1. Unexpectedly, the key XENP cell marker, Oct4, was absent from that list, and it was well present in both fractions. Inspection of the microarray data also suggested that Ihh and Dab2, two visceral endoderm markers, were moderately enriched in the SSEA1+ fraction. Apart from that, none of the differentially expressed genes raised particular interest, therefore hierarchical clustering (Figure 2B) and subsequent network analysis were performed (Figure 2C). The latter revealed a potential network of interactions around the pro-angiogenesis factor Angiogenin (itself ~3 fold enriched in the SSEA1⁻ fraction). This was reminiscent of the formation of vessel-like structures of unknown identity previously observed in older XENP cell cultures[1], which prompted us to repeat the SSEA1 immunocytochemistry with aged XENP cell cultures. Interestingly, this experiment revealed intense and widespread staining of the vessels for SSEA1 (Figure 4). The vessel formation prompted us to include vascular and other somatic markers into subsequent qRT-PCR analysis.

XENP stem cell markers

The molecular signature of XENP cell was previously defined as Oct4+/Gata6+/Gata4+/SSEA1+/Nanog⁻/Sox2⁻/CDX2⁻; also, Sox7, Eom, and Rex1 were identified as likely XENP cell markers[1]. None of these markers is unique for XENP cells. With this limitation in mind, we noted that in the present study, none of the tested XENP cell markers (Gata6, Oct4, Eom, Sox7) was restricted to the SSEA1+ fraction. Gata6, and Sox7 were, if anything, marginally more abundant in the SSEA1⁻ fraction, while Oct4 showed a moderate enrichment in the SSEA1+ fraction (Figure 3B). These RT-PCR data, which are all in agreement with the microarray results, may indicate that a fraction of the XENP cells was negative for SSEA1 or that the "XENP markers" were separately expressed in various XENP-derived cell types.

Extraembryonic endoderm markers

Newly arising XENP cell colonies are initially negative for markers of differentiated extraembryonic en-

Table 1. Selection of mRNAs most highly enriched in either SSEA1$^+$ or SSEA1$^-$ fraction.

Gene symbol	Description	Accession #	SSEA1$^+$ /SSEA1$^-$ (Line CX1)	SSEA1$^+$ /SSEA1$^-$ (Line CX7)	Average
Prg2	proteoglycan 2, bone marrow	NM_031619	2.82	6.81	4.81
Wfdc1	WAP four-disulfide core domain 1	NM_133581	0.43	0.29	0.36
Pitx2	paired-like homeodomain 2	NM_019334	0.47	0.28	0.37
Nfix	nuclear factor I/X (CCAAT-binding transcription factor)	NM_030866	0.42	0.11	0.26
Lgals2	lectin, galactoside-binding, soluble, 2	NM_133599	3.45	11.82	7.63
Pcolce	procollagen C-endopeptidase enhancer	NM_019237	0.36	0.12	0.24
Tspan1	tetraspanin 1	NM_001004236	0.43	0.27	0.35
Inha	inhibin alpha	NM_012590	0.41	0.26	0.33
Svs4	seminal vesicle secretory protein 4	NM_012662	0.43	0.14	0.28
Rnase4	ribonuclease, RNase A family 4	NM_020082	0.49	0.18	0.34
Mmp24	matrix metallopeptidase 24	NM_031757	0.37	0.19	0.28
Lgi4	leucine-rich repeat LGI family, member 4	NM_199499	0.45	0.29	0.37
Ang	angiogenin, ribonuclease, RNase A family, 5	BC166436	0.49	0.21	0.35
Chl1	PREDICTED: Rattus norvegicus cell adhesion molecule with homology to L1CAM (Chl1), mRNA [XM_001077843]	XM_001077843	0.42	0.17	0.30
Lzts1	leucine zipper, putative tumor suppressor 1	NM_153470	0.21	0.33	0.27
Ache	acetylcholinesterase	NM_172009	0.42	0.19	0.31
Igsf1	immunoglobulin superfamily, member 1	NM_175763	0.32	0.43	0.38
Srd5a1	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	NM_017070	0.37	0.17	0.27
F2	coagulation factor II (thrombin)	NM_022924	2.85	4.77	3.81
Myh7b_ predicted	Rattus norvegicus similar to KIAA1512 protein (LOC311570), mRNA [XM_230774]	XM_230774	0.28	0.31	0.30
Nid1	nidogen 1	XM_213954	0.28	0.10	0.19
Myh3	myosin, heavy chain 3, skeletal muscle, embryonic	NM_012604	0.42	0.30	0.36
Tfec	transcription factor EC	NM_022379	2.28	3.82	3.05
Cdkn2a	cyclin-dependent kinase inhibitor 2A	NM_031550	0.40	0.20	0.30
Ldhb	lactate dehydrogenase B	NM_012595	0.26	0.22	0.24
Plac1	placenta-specific 1	NM_001024894	2.14	6.82	4.48
Pdpn	podoplanin	NM_019358	0.44	0.25	0.34
Igsf1	immunoglobulin superfamily, member 1	BC060309	0.31	0.30	0.31
Nog	noggin	NM_012990	0.25	0.20	0.22
Srd5a1	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	NM_017070	0.34	0.14	0.24
Etv5	ets variant 5	NM_001107082	2.02	5.93	3.97
Dram	damage-regulated autophagy modulator	NM_001173427	0.35	0.34	0.35

doderm cells, such as laminin b2, collagen 4, and SSEA3, but express those markers upon in vitro differentiation[1]. Further, XENP cell cultures express the extraembryonic endoderm marker Sox7 and the differentiation markers Sparc, Lamb1, Ihh, Dab2, and Hnf4a. As already mentioned, microarray analysis had indicated that visceral endoderm markers Dab2 and Ihh were enriched in the SSEA1$^+$ fraction. This was confirmed by the RT-PCR, whereas the parietal endoderm markers Lamb1 and Sparc were not appreciably different between the fractions (Figure 3B).

The moderate enrichment of visceral endoderm markers in the SSEA1$^+$ fraction contrasts with the previous analysis of early XENP cell colonies, in which the XENP-derived extraembryonic endoderm cells were negative for SSEA1[1]. It is known that immature visceral endoderm does not, but mature visceral endoderm does, express SSEA1[4]; thus it appears that in the regular-density bulk cultures, XENP cells during their visceral endodermal differentiation first lost SSEA1 and then regained it, like in vivo.

Somatic cell markers

As mentioned above, we wondered whether bulk XENP cultures contain vascular or even other somatic cell markers, and therefore analyzed a selected set by qRT-PCR.

The bulk XENP cell line cultures indeed contained the vascular markers, Flk1 and VE-cadherin (Figure 3B). These markers were present in both fractions, although there was a (non-significant) trend towards enrichment in the SSEA1$^+$ fraction (VE-cadherin) or SSEA1$^-$ fraction (Flk1). As already mentioned, smooth muscle markers (SM22, aSM) were not present at noticeable levels. We also analyzed neuroectodermal markers, including Nestin, Tubb3, and Sox2, which, however, were present at lower levels than in the feeder cells, without a clear preference for one cell fraction (not shown).

Conclusions

Bulk-cultured XENP cell lines displayed a more complex pattern than previously described for colonies arising after low-density seeding[1]. In "regular" (=bulk-amount, moderate-density, steady-state) XENP cell cultures, cells with mesodermal lineage markers (vascular endothelial) were detected and hence XENP cells appear to exhibit lineage plasticity. This would contrast with the observation that XENP cells do not contribute to the somatic lineages in vivo[1], but be in line with the fact that the presumed in vivo equivalent of XENP cells, the ICM-stage committed extraembryonic endoderm precursor, can be experimentally converted towards the epiblast[6,7].

We also conclude that SSEA1 cannot be used to enrich XENP cells in bulk culture. Although we observed the enrichment of a number of genes, none of them could be specifically linked with the undifferentiated XENP cells. The SSEA1$^+$ fraction contained both mature visceral endoderm and mesoderm, and SSEA1 expression was seen in morphologically differentiated vessel-like structures. Further, Oct4 was not only found in the SSEA1$^+$ but also the SSEA1$^-$ fraction (which also contained endoderm and mesoderm). Whether the Oct4 in the SSEA1$^-$ fraction is associated with SSEA1$^-$ XENP cells or perhaps with early mesoderm cells[8] needs further study. Taken together, it is not so much the differences between the SSEA1$^+$ and SSEA1$^-$ fractions that appear noticeable but the fact that all markers of interest were significantly present in both fractions. It remains an important goal to find a method to culture bulk amounts of XENP cells in pure undifferentiated form.

Materials and Methods

Culture of XENP cells and sorting

Cells were cultured in 100 mm dishes as previously described[1], i.e. on a layer of Mitomycin C-treated feeder cells (line Li1) in the presence of 15% FCS and 1000 μ/mL mouse LIF. The cells were split at about each 3 days. For sorting, the cells were trypsinized, washed with PBS, and incubated at room temperature (RT) for 30 minutes with an SSEA1 antibody (clone 480, Santa Cruz Biotechnology, CA, USA) diluted 1 : 20 in PBS. After two washes with PBS, the cells were incubated at RT for 30 minutes with TRITC-conjugated anti-mouse IgG (T5393, Sigma, St. Louis, MO, USA) diluted 1 : 100 in PBS. After two more washes with PBS, the cells were resuspended in PBS/1% FBS, sorted with a FACS Vantage SE flow cytometer (Becton Dickinson), and the data were analyzed with CellQuest software (Becton Dickinson).

Microarray and analysis of microarray data

RNA was isolated with TRIZOL (Invitrogen, Carlsbad, CA), and the quality was tested by electrophoresis. The samples were labeled and processed following the Agilent Technologies One-Color Microarray-Based Gene Expression Analysis Protocol version 5.5 with a total RNA input of 200 ng. Samples were hybridized to the Agilent Whole Rat Genome Oligo microarray (AMADID 014879, Agilent Technologies), which contains 41,000 probes measuring around 21,000 unique genes. The microarrays were scanned and data were extracted with Feature Extraction (version 10.7.3.1, Agilent Technologies, CA). Data quality was confirmed using the Agilent quality control metrics. The mRNA microarray output text files were loaded into, and processed by, GeneSpring GX 11.5.1 (Agilent) using a quantile normalization method. The normalized and log transformed intensity values were analyzed using GeneSpring GX. Fold change filters included the requirement that the genes be present in at least 200% of controls for up-regulated genes and lower than 50% of controls for down-regulated genes. Expression profile data were clustered into groups of genes that exhibited similar behavior across the chemical treatment experiments using GeneSpring GX. We utilized an algorithm based on Euclidean distance and average linkage in order to separate genes with similar patterns. Functional analysis was performed using Array2Go[9], GOEAST[10], and DAVID tools[11].

Real-time quantitative RT-PCR

See Table 2 for the primers.

Table 2. Primers used for RT-PCR.

Gene	GenBank #	Forward	Reverse	Anneal. Temp.	Product (bp)
aSM	NM_019183.1	CGCCATCAGGAACCTCGAGA	CAAAGCCCGCCTTACAGA	57.9	103
BMP4	NM_012827.2	TGATACCTGAGACCGGGAAG	AGAAGTGTCGCCTCGAAGTC	57.6	109
Dab2	NM_024159.1	CTTTGCCTCAGAACCTCCAG	AACAGGTGTCCAAGGTCCTG	58.0	175
Eomes	XM_001061749	TGTTCGTGGAAGTGGTTCTG	TTGCCCTGCATGTTATTGTC	57.6	97
Flk-1	NM_013062.1	ATGAACTGCCCTTGGATGAG	CATCTGCCTCAATCACTTGG	57.2	132
Gata6	NM_019185.1	GTAAGATGAACGGCCTCAGC	GGTTGTGGTGTGACAGTTGG	57.9	101
Ihh	NM_053384.1	AGCTCACCCCCAACTACAATC	AGTTCAGACGGTCCTTGCAG	58.4	99
Lam.b1	NM_001106721.1	CCTAACGTGGTCGGAAGAAC	ATATGCCCTGGAAACAGTGG	57.9	154
Mesdc2	NM_001008345.1	CCTTGAGAACCTTTGGCTTG	CCCCTGAACTGATGAGAACG	57.9	196
Nanog	NM_028016.2	AGCAGAAGATGCGGACTGTG	CATCTGCTGGAGGCTGAGG	59.6	92
Nestin	NM_012987.1	GATCGCTCAGATCCTGGAAG	AAGAGAAGCCTGGGAACCTC	57.9	142
Nodal	NM_001106394.1	CTTCTCCAAACCTGCTGGAC	AGTTCTGCCCAGTCACATCC	57.8	194
Oct4	XM_228354	GGTGGAGGAAGCTGACAACAAC	GGCAATGCTAGTGATCTGCTGC	61.3	172
SM22	NM_031549.2	CCACAAACGACCAAGCCTTTT	CGGCTCATGCCATAGGATG	60.53	66
Sox2	NM_001109181.1	CAACTCGGAGATCAGCAAGC	CATGAGCGTCTTGGTTTTCC	58.6	160
Sox7	NM_001106045.1	CAAGGATGAGAGGAAACGTC	CTCTGCCTCATCCACATAGG	55.3	130
Sparc	NM_012656.1	CAAACATGGCAAGGTGTGTG	AGTGGCAGGAAGAGTCGAAG	57.6	136
Tubb3	NM_139254.2	GGGCCTTTGGACACCTATTC	TGCAGGCAGTCACAATTCTC	58.0	157
VEcadh	NM_001107407.1	GGCCAACGAATTGGATTCTA	GTTTACTGGCACCACGTCCT	57.9	196

Immunocytochemistry

Cells were treated with 10% Neutral Buffered Formalin for 15 minutes at RT, then with PBS/0.2% Triton X-100 (PBST)/5% Normal Donkey Serum for 30 minutes at RT, and then with the antibody (mouse anti-SSEA1, Santa Cruz, cat. no. sc-21702; 1 : 200 in PBS /5% donkey serum) or isotype control overnight at 4 ˚C. The incubation steps were separated by washes with PBS. Finally, the cells were incubated with Alexa-Fluor 555 Goat anti-mouse antibody (Invitrogen, cat. no. A21424, 1 : 500) and Hoechst dye (Sigma 33258) for 1 hour at RT.

Acknowledgements We thank KOSEF (project number 0000179) for support.

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