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The Intracellular Distribution of the ES Cell Totipotent Markers OCT4 and Sox2 in Adult Stem Cells Differs Dramatically According to Commercial Antibody Used

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ABSTRACT

To characterize ES cells, researchers have at their disposal a list of pluripotent markers, such as OCT4. In their quest to determine if adult stem cell populations, such as MSCs and ASCs, are pluripotent, several groups have begun to report the expression of these markers in these cells. Consistent with this, human ASCs (hASCs) are shown in this study to express a plethora of ES pluripotent markers at the gene and protein level, including OCT4, Sox2, and Nanog. When intracellular distribution is examined in hASCs, both OCT4 and Sox2 are expressed within the nuclei of hASCs, consistent with their expression patterns in ES cells. However, a significant amount of expression can be noted within the hASC cytoplasm and a complete absence of nuclear expression is observed for Nanog. Recent descriptions of OCT4 transcript variants may explain the cytoplasmic expression of OCT4 in hASCs and consistent with this, hASCs do express both the OCT4A and 4B transcript variants at the gene level. However, discrepancies arise when these three pluripotent markers are studied at the protein level. Specifically, distinct differences in intracellular expression patterns were noted for OCT4, Sox2, and Nanog from commercial antibody to commercial antibody. These antibody discrepancies persisted when hMSCs and rat ASCs and MSCs were examined. Therefore, confirming the expression of OCT4, Sox2, and Nanog in adult stem cells with today's commercial antibodies must be carefully considered before the designation of pluripotent can be granted. J. Cell. Biochem. 106: 867–877, 2009.

KEY WORDS: ADIPOSE-DERIVED STEM CELLS; ADULT STEM CELLS; OCT4; Sox2; INTRACELLULAR DISTRIBUTION

Classification and characterization of stem cells in vitro often takes advantage of the expression of distinct intracellular markers. In the case of the embryonic stem cell (ES cell), numerous studies have identified the expression of several unique markers in the course of isolating and identifying these stem cells. Some of these markers, such as OCT4, Sox2, and Nanog have become well known in today's literature and are often cited as one of the first requirements for confirming the presence of totipotent ES cells [Evans and Kaufman, 1981; Nichols et al., 1998; Rossant, 2001; Chambers et al., 2003; Chambers, 2004; Kurosaka et al., 2004; Yates and Chambers, 2005; Loh et al., 2006; Morrison and Brickman, 2006]. OCT4, a POU motif-binding transcription factor was the first such marker to be identified. Studies on it's molecular mechanism has suggested that OCT4 controls the activity of many downstream pluripotent genes within the ES cell, such as Nanog [Catena et al., 2004; Chew et al., 2005; Kuroda et al., 2005; Rodda et al., 2005; Loh et al., 2006]. Recent studies now indicate that OCT4 expression within the ES cell declines as the stem cell population differentiates and loses its pluripotent capacity [Rathjen et al., 1999]. Based on these works, it has almost become the norm to identify the expression of OCT4 when attempting to classify a stem cell population as pluripotent. In fact, in the quest to classify adult stem cell populations, such as MSCs and ASCs, as multipotent or pluripotent, several groups have begun to describe the expression of OCT4 and other ES pluripotent markers [Moriscot et al., 2005; Tai et al., 2005; Tondreau et al., 2005; Izadpanah et al., 2006]. The expression of these totipotent markers in adult stem cells makes it tempting to suggest commonalities between ES and adult stem cell populations and that adult stem cells may possibly possess increased potency—that is, beyond that of two or three cell lineages. Such a possibility would be an exciting one and would allow researchers more freedom from ethical constraints when deciding which stem

Abbreviations used: ES cells, embryonic stem cells; hASCs, human adipose-derived stem cells; rASCs, rat adipose-derived stem cells; hMSCs, human mesenchymal stem cells; rMSCs, rat mesenchymal stem cells; rPAb, rabbit polyclonal antibody; gPAb, goat polyclonal antibody; MAb, monoclonal antibody; IF, immunofluorescence.

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cell population to use in future therapeutic applications. However, the expression of OCT4, Sox2, and Nanog expression as a parameter for reclassifying an adult stem cell as pluripotent must be carefully considered.

In 2001, an adult stem cell population from human lipoaspirates—now termed Adipose-derived Stem Cells/ASCs (first published as PLA cells)—was described in the literature and suggested to be a multipotent stem cell population within the mesodermal germ lineage [Zuk et al., 2001]. However, subsequent studies on ASCs by several groups has suggested possible ectodermal or endodermal germ lineage potential [Safford et al., 2002, 2004; Gimble and Guilak, 2003; Kang et al., 2003, 2004; Yang et al., 2004; Seo et al., 2005; Timper et al., 2006]. In 2006, a study on ASCs isolated from human and primate adipose tissue identified the expression of both OCT4 and Sox2 at the gene and protein level and Rex-1 at the gene level in these stem cells [Izadpanah et al., 2006]. Using indirect immunofluorescence (IF), the authors reported a nuclear expression pattern for both OCT4 and Sox2 in human ASCs (hASCs)—a finding that is consistent with the role of these proteins as transcription factors in ES cells [Nichols et al., 1998; Avilion et al., 2003]. In contrast to this earlier work, this current study, while confirming the nuclear expression of these totipotent markers in hASCs and human MSCs (hMSCs) also notes significant expression of these markers outside the nucleus. With recent articles proposing transcript variants for OCT4, each exhibiting distinct intracellular expression patterns, the nuclear and cytoplasmic expression patterns seen in these adult stem cell populations may also be reflective of multiple OCT4 isoforms. However, the distinct intracellular expression patterns for OCT4, Sox2, and Nanog may be due to the source of the commercial antibody chosen. Specifically, it appears that several commercial antibodies to OCT4, Sox2, and Nanog give conflicting intracellular patterns. Moreover, these conflicting patterns were found to be independent of stem cell source (i.e., ASC vs. MSC, human vs. rodent) and adhesive surface (i.e., plastic vs. glass). Therefore, care should be taken when utilizing IF and today's crop of available antibodies in describing the intracellular distribution of these accepted ES pluripotent markers in adult stem cell populations.

MATERIALS AND METHODS

hASCs and rat ASCs (rASCs) were isolated based on previously published protocols [Zuk et al., 2002]. hASCs, rASCs, and rat MSCs (rMSCs) were maintained in non-inductive Control Medium (DMEM-high glucose, 10% FBS, penicillin, streptomycin—[Zuk et al., 2001]). hMSCs were either purchased from Clonetics and maintained in the manufacturer's recommended medium (MSCGM, 10% FBS, penicillin/streptomycin) or isolated from human bone marrow based on previously published studies [Prockop, 1997; Pittenger et al., 2000]. Two tri-lineage clonal populations expanded from single ASCs were also analyzed: an AOC clonal population exhibiting adipogenic, osteogenic, and chondrogenic potentials and an OCM clonal population with osteogenic, chondrogenic, and skeletal myogenic potentials. The isolation of AOC populations has been previously described [Zuk et al., 2002]. OCM cells were a generous gift of Dr. Min Zhu (Cytori Therapeutics, La Jolla, CA). The isolation and maintenance of these clonal populations was based on a previous study [Zuk et al., 2002]. Stem cell cultures were maintained as above until 80% confluent then passaged using 2.5% trypsin/2.2 mM EDTA (Mediatech Cellgro, Herndon, VA).

For RT-PCR analysis, hASC populations at passage 2 or 3, along with AOC and OCM populations were harvested in a commercial lysis buffer (Qiagen RLT Buffer) and total RNA prepared using a commercial kit (RNeasy, Qiagen, Valencia, CA). One microgram of total RNA was converted into cDNA using conventional protocols and commercial enzymes (Promega, Madison, WI). RT-PCR for OCT4, Sox2, and Nanog, in addition to several other established ES markers, was performed using the primer sequences given in Table I. PCR products were resolved by conventional agarose gel-electrophoresis.

For analysis using IF, stem cell populations were plated onto either 8-well glass or plastic chamber slides (Nalge Nunc, Thermo Fisher Scientific, St. Louis, MO). IF analysis was performed on cell cultures at either passage 2 or 3. The cells were plated at a density of 50,000 cells per well and allowed to adhere overnight in Control Medium. The cells were rinsed with 1×PBS and fixed for 15 min at room temperature in 4% paraformaldehyde (Ted Pella, Redding, CA) in 0.1 mM sodium phosphate buffer (pH 7.0). The cells were washed

TABLE I. Primer Sequences

Gene name	Primer sequence
OCT4A	5′: GAAGCTGGAGAAGGAGAAGC
	3′: GAACATGTGTAAGCTGCGGCCCTTG
OCT4B	5′: TGCAGAAAGAACTCGAGCAA
	3′: GAAGGATGTGGTCCGAGTGT
Sox2	5′: CAAGATGCACAACTCGGAGA
	3′: GTTCATGTGCGCGTAACTGT
Nanog	5′: CAAAGGCAAACAACCCACTT
	3′: CTGGATGTTCTGGGTCTGGT
Rex-1	5′: GAAGCAAGCTCCCTTGAA
	3′: GGATGTGGGCTTTCAGGT
Nodal	5′: AGCATGGTTTTGGAGGTGAC
	3′: TTGGTCGGATGAAACTCCTC
Cripto/TDGF-1	5′: GGAATTTGCTCGTCCATCTC
	3′: CATCACAGCCGGGTAGAA
hTERT	5′: GACCATCTTTCTGGGTTCCA
	3′: ACACTCATCAGCCAGTGCAG
	Alternate 5′: AACGTTCCGCAGAGAAA
	Alternate 3′: AAGCGTAGGAAGACGTCG
hTERF1	5′: TCCTCTGCCTCTCTCTTTGC
	3′: CCGCTGCCTTCATTAGAAAG
Connexin 43	5′: GGACATGCACTTGAAGCAGA
	3′: CAGCTTGTACCCAGGAGGAG
Bcrp-1/ABCG2	5′: GCTGCAAGGAAAGATCCAAG
	3′: TTCCTGAGGCCAATAAGGTG
UTF-1	5′: GTCCCCACCGAAGTCTGC
	3′: GGACACTGTCTGGTCGAAGG
	Alternate 3′: TGCTGGTTCAAGGTCAGCA
LEFTYB	5′: AACTTCTGGCAGCAGCTGAG
	3′: CTTGATGCTGACGATCATGG
Msx1	5′: CGGTGTCAAAGTGGAGGACT
	3′: GGCTTACGGTTCGTCTTGTG
cmyc	5′: AGAGAAGCTGGCCTCCTACC
	3′: TCGGTTGTTGCTGATCTGTC
FoxD3	5′: CTCAACGACTGCTTCGTCAA
	3′: TGTTCTCGATGCTGAACGAC
FGF4	5′: GACTACCTGCTGGGCATCAA
	3′: GTTCCCCTTCTTGGTCTTCC
OCT4 pseudogene 3/OCT4PS3	5′: GAGGAGTCCCAGGACATCAA
	3′: ACACTCGGACCACATCCTTC
OCT4 pseudogene 4/OCT4PS4	5′: CCCCGCTGTATGAGTTCTGT
	3′: GCAAGAGGGTTTCTGCTTTG

TABLE II. Known ES Markers

Gene name	Alternate gene name	GenBank
Octamer binding protein 4/OCT4	POU class 5 transcription factor 1/POU5F1	OCT4A: NM_002701, OCT4B: NM_203289
Breast cancer resistant protein/Bcrp-1	ATP binding cassette subfamily G2/ABCG2	NM_004827
Connexin 43	Gap junction protein alpha 1, 43 kDa or GJA1	NM_000165
Connexin 45	Gap junction protein gamma 1, 45 kDa or GJA7	NM_005497
Fibroblast growth factor 4	FGF4	J02986
Cripto-1	Teratoma-derived growth factor-1/TDGF-1	NM_003212
Forkhead box protein D3	FOXD3	NM_012183
Human telomerase reverse transcriptase	hTERT	NM_198255
Left-right determination factor B	LEFTYB	AF081512
Left-right determination factor A/LeftyA	EBAF	AF081513
msh homeobox 1	Msx1	NM_002448
Nanog		NM_024865
Nodal	Nodal homolog	NM_018055
Podocalyxin	PODXL	NM_001018111
Rex1	Zinc finger protein 42/ZFP42	NM_17900
Sry-box containing gene 2	Sox2	NM_003106
Stage-specific antigen isoforms 3 and 4	SSEA-3, SSEA-4	
Telomeric repeat binding factor-1	TERF1 or TRF1	NM_017489
Telomeric repeat binding factor-2	TERF2 or TRF2	AF002999
Tumor rejection antigen isoforms 1–60 and 1–81	TRA1-60, TRA1-81	
Undifferentiated embryonic transcription factor-1	UTF-1	AB011076

with three changes of 1×PBS (5 min each wash) and incubated for 2–3 h at room temperature with primary antibodies diluted in an IF Buffer (1×PBS, 1% BSA, 10% FBS, 0.1% TritonX-100). Alternatively, cells were incubated with primary antibodies overnight at 4°C. A list of ES pluripotent markers examined for expression in hASCs is given in Table II. The primary antibodies used to assess the expression of OCT4A, OCT4B, Sox2, and Nanog, together with the amino acid regions used for antibody production are summarized in Table III. Primary antibody dilutions were empirically determined and, when stipulated, based on the manufacturer's recommended dilutions. Two distinct lots of Millipore anti-OCT4A/B primary antibody were utilized in this study with no differences in intracellular pattern being noted from lot to lot for this antibody (data not shown). Following primary antibody incubation, the cells were washed as above with 1×PBS and incubated for 1 h at room temperature in IF Buffer supplemented with the appropriate FITC-conjugated secondary antibody (Molecular Probes, Invitrogen, Carlsbad, CA—1:100 dilution). The cells were washed again and then prepared for microscopy using an anti-quenching mounting medium containing the nuclear dye DAPI (Vectamount Hardset, Vector Labs, Burlingame, CA). Digital images were acquired using a Spot2 camera at either 200× or 400× magnification.

RESULTS AND DISCUSSION

Identification and characterization of human ES cells has resulted in the compilation of a list of "pluripotent markers" (Table II). hASCs were found to express several of these ES markers at both the gene and protein level (Fig. 1). For example, expression could be confirmed for Connexin 43/Cxn43, a gap junction protein thought to regulate communication between stem cells and their supportive cells within the stem cell niche [Wong et al., 2004], in addition to Bcrp-1, a multi-drug resistance transporter expressed in "side population" stem cells from bone marrow, skeletal muscle and NSCs [Lechner et al., 2002; Scharenberg et al., 2002] (Fig. 1A,B). Not only could expression of these two ES markers be confirmed at the protein level using IF, but IF was also able to confirm expression of these markers on the surface of hASCs, consistent with their proposed functions in ES cells. Expression at both the gene and/or

TABLE III. Summary of Commercial Antibodies Used

Antibody name and catalog #	Source	MAb/PAb	Reactivity	Immunogen—amino acid region	Reported IC distribution (OCT4 transcript)
OCT4A MAB4401	Millipore/Chemicon	MAb	Human, mouse	Clone 10H11.2 1–143	Nuclear
OCT4A/4B MAB4305	Millipore/Chemicon	MAb	Human, mouse	Clone 9E.2 143–359	Nuclear
OCT4A Ab18976	Abcam	rPAb	Human, rat	1–140	Nuclear
OCT4A/4B ab19857	Abcam	rPAb	Human, rat	Within 300 to COOH terminus	Nuclear
OCT4A/4B ab27985	Abcam	gPAb	Human, mouse	Within 300 to COOH terminus	Nuclear
OCT4A sc-5279	Santa Cruz	MAb	Human, rat	1–140	Nuclear
OCT4A/4B Sc009	R&D	gPAb	Human, mouse	COOH terminus *E. coli* derived OCT4A/B	Nuclear
Sox2 ab15830	Abcam	rPAb	Human, mouse	1–100	Nuclear
Sox2 MAB2018	R&D	MAb	Human, mouse	135–317 *E. coli* derived Sox2	NA
Nanog ab21603	Abcam	rPAb	Human. mouse	Full-length mouse protein	Nuclear
Nanog AF1997	R&D	gPAb	Human	*E. coli* derived 153–305	NA

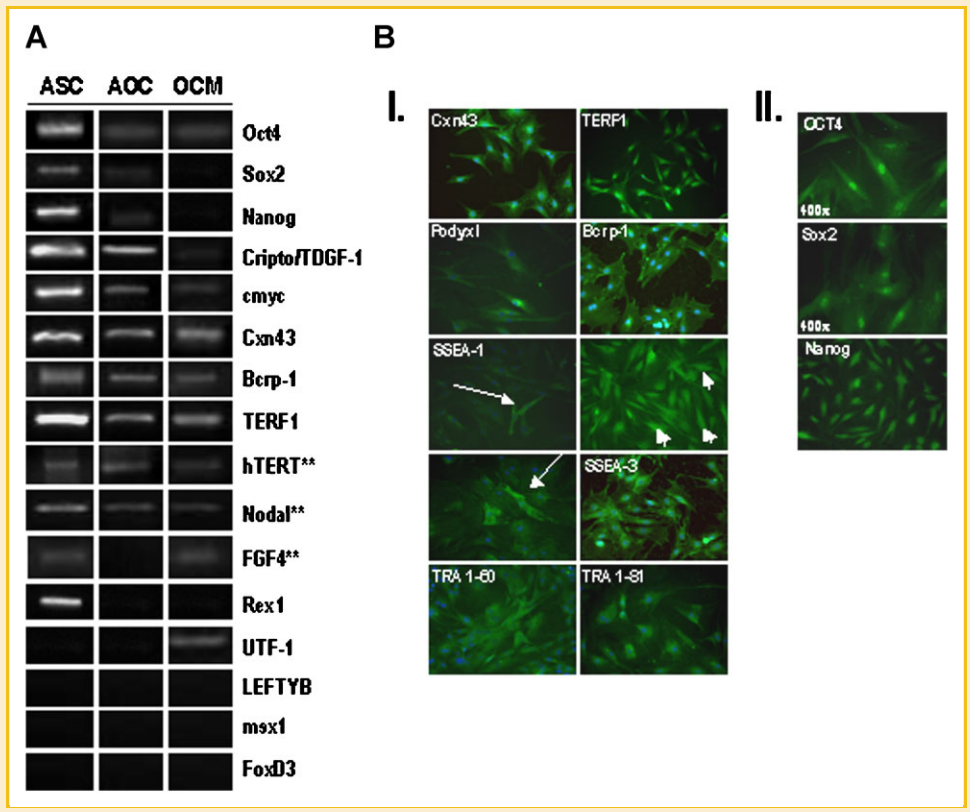

Fig. 1. Human ASCs (hASCs) express numerous ES pluripotent markers. Panel A: hASCs and two clonal populations (*AOC cells/AOC*—adipogenic, osteogenic, and chondrogenic potentials; *OCM cells/OCM*—osteogenic, chondrogenic and skeletal myogenic potential) were assessed using conventional RT-PCR for several established ES pluripotent markers listed in Table II. Expression of hTERT, Nodal and FGF4 was not observed in all hASC populations examined and is denoted as "**". Panel B: IF analysis of hASC populations for several of these markers. Nuclear staining as assessed using DAPI is shown in images where nuclear staining was not observed (blue staining). Elevated expression of SSEA-1 and SSEA-4 in individual hASCs is shown (arrows). Nucleolar expression of Cripto is shown (arrowheads). Magnification is 200× unless otherwise noted.

protein level was also confirmed for the ES marker Podocalyxin/Podyxl a CD34-like protein used to identify early stem cell progenitors [Lee et al., 2008]. hASCs were also found to express the telomere repeat binding factor/TERF-1 at both gene and protein level. Consistent with its role in mediating telomere length and DNA damage [Smogorzewska et al., 2000], TERF-1 expression was found within the hASC nucleus. hASCs also expressed both Nodal and Cripto/TDGF-1, components involved in mesodermal/ectodermal fate determination [Parisi et al., 2003]. Expression of Cripto was observed at both the gene and protein level with hASCs expressing this protein within throughout the cytoplasm and within the nucleus restricted to what appeared to be the nucleolus (Fig. 1A, white arrowhead). Expression of the Nodal gene was variable in hASCs, with a fraction of the total populations assayed (~50%) failing to express this marker. Variability in the expression of hTERT and FGF4 was also observed across the assayed hASC populations. In addition to these markers, hASCs also expressed at the gene level, Rex-1, a transcription factor expressed in inner cell mass cells [Ben-Shushan et al., 1998] and the oncogene c-myc. While hASCs did express several well-characterized ES markers, they did show differential expression with respect to others including undifferentiated embryonic transcription factor-1/UTF-1, Left-right determination factor B/LEFTYB, FoxD3, and msh homeobox-1/Msx1, failing to express these ES markers at the gene level. As such, it appears that while hASCs express many genes associated with pluripotency, they do exhibit some genotypic differences with their ES progenitors.

Because the hASC population is potentially a heterogenous population, the expression of these ES markers may be due to the presence of contaminating populations with increased potency profiles. To assess this, the expression of these ES markers was also examined in two tri-lineage clonal populations derived from single ASCs: an AOC clonal population exhibiting adipogenic, osteogenic, and chondrogenic potentials (AOCs) and an OCM clonal population with osteogenic, chondrogenic, and skeletal myogenic potentials (OCMs) [Zuk et al., 2002]. Due to their derivation from hASCs, the two clonal populations exhibited an ES marker expression profile similar to their "parental" populations. Both AOC and OCM populations expressed Cxn43, Bcrp-1, TERF-1, cmyc, and Cripto, although the expression of Cripto was significantly weaker in the OCM population (Fig. 1A). Like their parental counterparts, AOC and OCM clones also failed to express LETFYB, FoxD3, and msx-1. Finally, while the expression of Nodal and hTERT was variable across hASC populations, the parental populations used to derive the AOC and OCM clones did express these markers and, as such, expression of these two ES markers was confirmed in their clonal

derivatives. However, differential expression was observed in the two clonal populations. Consistent with its decrease in later stage pluripotent stem cells [Rogers et al., 1991], both the AOC and OCM clonal populations, with their more restricted potentials failed to express Rex-1. Surprisingly, expression of UTF-1 was observed in OCM clones while this marker was not expressed in either the AOC clone or parental ASC populations.

Characterization of murine and human ES cells often describes the expression of stage-specific antigens/SSEAs and the tumor rejection antigen/TRA proteins [Draper et al., 2002; Carpenter et al., 2003]. All hASC populations examined in this study were found to express significant levels of SSEA-3, SSEA-4, TRA1-60, and TRA1-81 (Fig. 1B, Panel I). A cell surface expression pattern for SSEA-3 and SSEA-4 was observed in all assayed hASC populations, consistent with their proposed expression on the ES cell surface (see Fig. 1B, Panel I: SSEA-3), whereas the expression patterns for both TRA1-60 and TRA1-81 were consistent with a cytoplasmic localization. Staining levels for SSEA-3, TRA1-60, and TRA1-81 were found to be homogenous throughout each hASC population examined, with each cell expressing similar levels of these proteins. However, increased expression of SSEA-4 could be observed in a few individual hASCs within the sample (<10% of the total ASCs imaged; Fig. 1B, Panel I—see arrows). Curiously, a small number of hASC populations also appeared to express low levels of SSEA-1—a cell surface antigen typically associated with murine ES cells [Ginis et al., 2004]. However, increased expression of SSEA-1 has been reported on differentiating human ES cells [Noaksson et al., 2005]. Like SSEA-4, elevated expression of SSEA-1 could be observed in a few hASCs found within the sample (Fig. 1B, Panel I—see arrows) and may indicate the presence of distinct subpopulations within the hASC sample.

In addition to these established ES markers, hASC populations and their two clonal derivatives also expressed the "trinity" of ES markers—OCT4, Sox2, and Nanog—at both the gene and protein level. Initially identified during mouse embryogenesis [Scholer et al., 1990], OCT4 is thought to be crucial for ES self-renewal and pluripotency as OCT4-knockout blastocysts fail to adopt a pluripotent phenotype and alterations in OCT4 levels in ES cells can direct their differentiation fate [Niwa et al., 2000]. Through its complexing with the transcription factor Sox-2, OCT4 is thought to control many downstream embryonic genes, including Nanog, a homeodomain protein involved in murine and human ES cell pluripotency [Rodda et al., 2005; Loh et al., 2006]. Recent studies have confirmed expression of OCT4 in adult stem cells such as primate and hASCs [Izadpanah et al., 2006], umbilical cord blood stem cells [Kang et al., 2004], and MSCs [Moriscot et al., 2005; Tai et al., 2005] and expression of Sox2 has also recently been described in ASCs [Izadpanah et al., 2006]. Consistent with these previous works, expression of OCT4, Sox2, and Nanog could be confirmed at the gene level in hASCs (Fig. 1A). Expression of these three markers was also confirmed in the AOC clonal derivative. However, decreased expression of OCT4, Sox2, and Nanog was observed in this clonal population. Such a decrease is consistent with previous works that indicate restriction of lineage is associated with drops in pluripotent marker expression levels [Rathjen et al., 1999]. Real-time PCR was able to confirm lower expression levels of both OCT4 and Nanog in the AOC clonal population (OCT4 -42.95 ± 31.83 decrease vs. hASCs, Nanog -46.08 ± 4.81 decrease vs. hASCs). The expression of OCT4, Sox2, and Nanog differed significantly in the OCM population. Like hASCs and AOC cells, OCM cells did express OCT4. However, expression of both Sox2 and Nanog was not observed in these clonal cells and real-time PCR also confirmed this absence. Variations in OCT4 expression levels are thought to play a role in the restriction of ES potency and their differentiation of ectodermal and endodermal lineages [Niwa et al., 2000]. Since the AOC and OCM clonal populations represent more restricted ASC populations, the decreased expression levels of OCT4 and Nanog in AOC cells, together with the absence of Sox2 and Nanog in the OCM clone may be a result of this loss of potency or, alternatively, may play a more direct role in their restriction. Subsequent studies will be required to elucidate the role of these transcription factors in ASC potency.

When examined at the protein level, hASCs were found to express all three pluripotent ES markers (Fig. 1B, Panel II). The intracellular distribution of OCT4 and Sox2 were similar in each hASC population examined, with both proteins localizing to the nucleus, consistent with their roles as transcription factors. However, a significant fraction of OCT4 and Sox2 could be localized to the cytoplasm in these cells. In contrast to OCT4 and Sox2, restriction solely to the cytoplasm was observed for Nanog in all hASC populations examined. The expression of these three transcription factors in the cytoplasm of hASCs and the absence of Nanog in the hASC nucleus is intriguing and could suggest that these three genes have differing roles in hASCs versus ES cell. However, recent articles have identified transcript variants for OCT4 [Takeda et al., 1992; Atlasi et al., 2008] and have proposed distinct intracellular distribution patterns for them. At least 11 transcript variants have been proposed for OCT4 [Liedtke et al., 2008] and work by Cauffman has identified two specific variants that differ in their temporal and spatial patterns within the embryo [Cauffman et al., 2006]. These two variants, OCT4A and OCT4B, show identity between exons 2 through 5 but differ in their absence of an ~90 amino acid N-terminus in the truncated OCT4B variant [Takeda et al., 1992]. Functionally, it has been proposed that ES self-renewal and stem cell properties (i.e., potency) are provided by the OCT4A variant with its nuclear localization consistent with being a transcription factor, while the function of OCT4B, localized to the stem cell's cytoplasm remains unknown [Cauffman et al., 2006; Lee et al., 2006]. Therefore, the expression of OCT4 in both the nuclear and cytoplasmic compartments of hASCs is consistent with the presence of these two variants. It remains unknown if Sox2 and Nanog possess similar variants that are expressed differentially within the stem cell.

With this knowledge of OCT4 variants, expression of this marker at the gene level was re-examined using variant specific primers. PCR products were obtained in both the parental hASC and clonal populations using primer sets specific to the OCT4A N-terminus and to a region of the gene common to both OCT4A and OCT4B (Fig. 2A). Expression of these two variants was confirmed by sequencing the resulting PCR product in order to eliminate the possibility of false positives attributed to the transcription of OCT4 pseudogenes. To date, there are at least six known OCT4 pseudogenes [Takeda et al., 1992], many of which are transcribed in cancers and stem cell lines

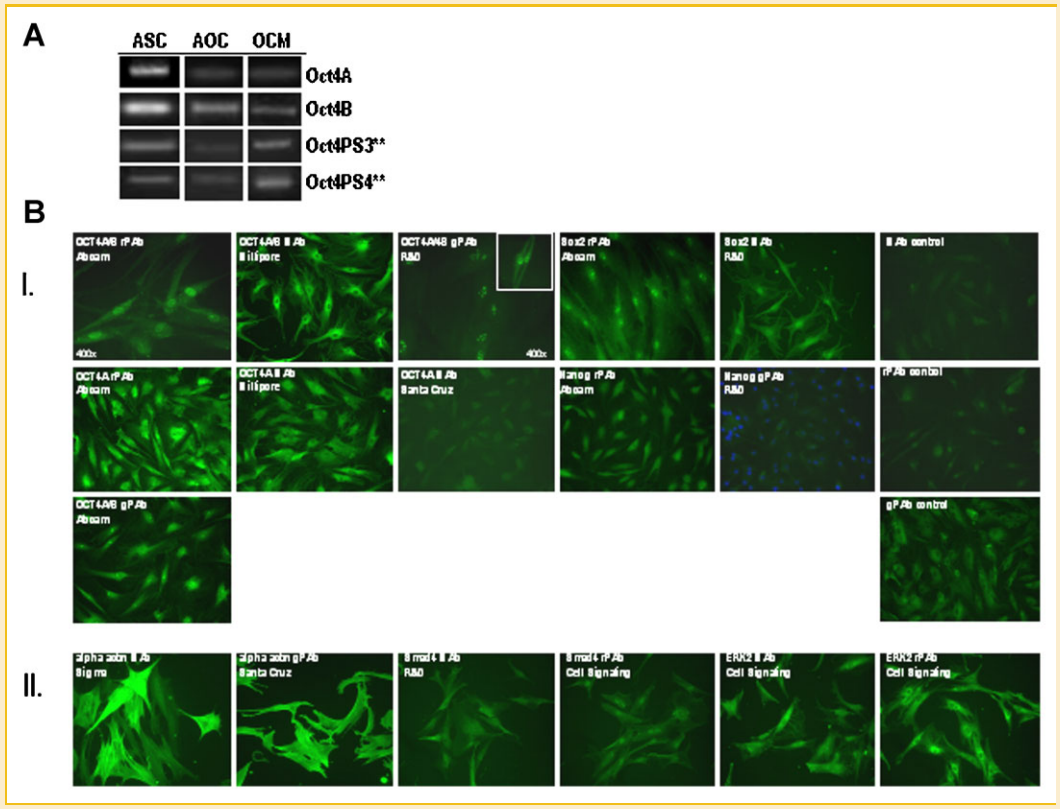

Fig. 2. Intracellular expression patterns of Oct4A, Oct4B, Sox2, and Nanog in hASCs differ dramatically with commercial antibody source. Panel A: Gene expression of the longer OCT4A transcript variant and the truncated OCT4B variant, together with two OCT4 pseudogenes (OCT4PS3 and OCT4PS4) at the gene level in hASCs. Expression of OCT4PS3 and OCT4PS4 was not observed in all hASC populations examined and is denoted as "**". Panel B—Group I: Intracellular expression patterns of OCT4, Sox2, and Nanog in hASCs using several commercial antibodies (Abcam, Millipore, R&D Systems, and Santa Cruz). Commercial antibodies specific to the N-terminus of OCT4A and to a region common to both OCT4A and OCT4B (OCT4A/B) were used. Non-specific fluorescence due to secondary antibody interaction is also shown (far right column). Expression of the nucleolar marker Nucleoplasmin is shown (OCT4A/B gPAb R&D image—inset panel). Panel B—Group II: Expression of alpha actin, Smad4, and ERK2 using several commercial antibody sources is shown as a control. Monoclonal antibody—MAb, rabbit polyclonal antibody—rPAb, goat polyclonal antibody—gPAb. Magnification is 200× unless otherwise noted.

[Suo et al., 2005; Lin et al., 2007]. As such, the expression of two OCT4 pseudogenes (pseudogenes 3 and 4—NG_oo5793, NG_005794, respectively) was also assessed in hASC and its clonal derivatives (Fig. 2A).

Confirmation of OCT4, Sox2, and Nanog expression is possible today using a variety of commercial monoclonal and polyclonal antibodies (for a summary see Table III). Each commercial antibody details localization of these markers to the nucleus in ES cells. However, in assessing the expression patterns of these three ES markers in hASCs using several sources of antibody, an interesting finding was observed. As shown in Figure 2B, Panel I and summarized in Table IV, the intracellular expression patterns of these markers differed dramatically with respect to the commercial antibody chosen. For example, processing of hASCs using a polyclonal antibody from Abcam specific to both the OCT4A and OCT4B variants (i.e., OCT4A/4B) localized these proteins to both the

TABLE IV. Summary of Intracellular Distributions of Totipotent Markers in Adult Stem Cells

Marker name	hASCs	hMSCs
OCT4A MAb (Chemicon)	Cytoplasmic – fine punctate (no nuclear)	Cytoplasmic – punctate (no nuclear)
OCT4A/4B MAb (Chemicon)	Cytoplasmic + cytoskeletal and surface (no nuclear)	Cytoplasmic, nuclear and surface
OCT4A PAb (Abcam)	Cytoplasmic + cytoskeletal (nuclear reaction – background)	Weak cytoplasmic
OCT4A/4B PAb (Abcam)	Cytoplasmic – punctuate and nuclear	Cytoplasmic – punctate (no nuclear)
OCT4A/4B gPAb (R&D)	Nucleolar	Nucleolar
OCT4A/4B gPAb (Abcam)	Cytoplasmic – punctate and nucleolar	Cytoplasmic – punctuate and surface
OCT4A MAb (Santa Cruz)	Minimal reaction cytoplasmic and nuclear	Minimal reaction cytoplasmic and nuclear
Sox2 PAb (Abcam)	Cytoplasmic – punctuate, nucleoplasmic + nucleolar	Cytoplasmic and perinuclear
Sox2 MAb (R&D)	Cytoplasmic + cytoskeletal perinuclear and surface	Weak cytoplasmic weak nuclear – background ?
Nanog PAb (Abcam)	Cytoplasmic – punctate (no nuclear)	Weak cytoplasmic (no nuclear)
Nanog gPAb (RD)	Minimal reaction	Very weak, background reaction

nucleus and cytoplasm (also shown in Fig. 1B). The nuclear distribution pattern was diffuse and characteristic of expression through the nucleoplasm and the cytoplasmic staining pattern was punctate and homogenous. Such a staining pattern in the hASCs is consistent with the proposed distributions of the OCT4A and 4B variants in ES cells [Lee et al., 2006]. However, when hASCs were processed using a polyclonal Abcam antibody specific to the OCT4A transcript, a dramatically different intracellular pattern was noted, with a strong cytoplasmic signal being observed, together with the absence of nuclear staining. Any nuclear staining that was observed was very weak and determined to be due to secondary antibody cross-reactivity. Since the OCT4A variant is thought to be a transcription factor responsible for the pluripotency and self-renewal of ES cells, its absence from the nuclei of hASCs using this antibody is interesting. An absence of nuclear staining was also noted upon processing of hASCs using two monoclonal antibodies from Millipore (formerly Chemicon). Like the Abcam antibodies, these two antibodies were specific to the OCT4A variant and the OCT4A/4B variants. The distribution of OCT4A/4B variant in hASCs using this Millipore antibody has been previously described in the literature [Izadpanah et al., 2006]. Rather than detecting nuclear expression, both antibodies resulted in a similar intracellular pattern—a punctate, cytoplasmic localization with a filamentous/cytoskeletal pattern. The discrepancies continued using antibodies from Santa Cruz and R&D Systems. The OCT4A-specific monoclonal antibody from Santa Cruz was recently recommended by Liedtke and colleagues [Liedtke et al., 2008] since it is specific to the OCT4A N-terminus and is a monoclonal antibody. However, this antibody failed to detect significant amounts of OCT4A expression in the hASC samples and, in particular, no nuclear staining could be seen. The most unusual intracellular pattern was observed when using a goat polyclonal from R&D specific to the OCT4A/4B variants. With this antibody, a weak cytoplasmic localization was noted together with a nuclear pattern reminiscent of the nucleolus. To confirm this nucleolar pattern, hASCs were also processed using an antibody specific to the nucleolar protein nucleostemin (Fig. 2B—inset). A similar intracellular pattern was also seen using a goat polyclonal to OCT4A/4B purchased from Abcam. The apparent nucleolar expression of OCT4A/4B was not due interference from the secondary antibody, as hASCs processed with anti-goat secondaries alone failed to show this nucleolar localization.

Differences in IC pattern were also noted using commercial antibodies for both Sox2 and Nanog. As seen with OCT4, both cytoplasmic and nuclear staining was observed in hASCs for Sox2 using a polyclonal antibody from Abcam. The cytoplasmic expression was diffuse and homogenous and the nuclear pattern appeared to be comprised of both a diffuse nucleoplasmic distribution together with nucleolar compartmentalization. Interestingly, the nucleolar expression of Sox2 appeared to be dependent upon hASC passage number. Both cytoplasmic and nuclear expression was observed in freshly isolated hASCs not subject to passaging in vitro (i.e., passage 0—data not shown) and in early passage populations (i.e., passage 1, passage 2—passage 2 samples shown in Fig. 2B). However, later passage hASCs (i.e., passage 4,

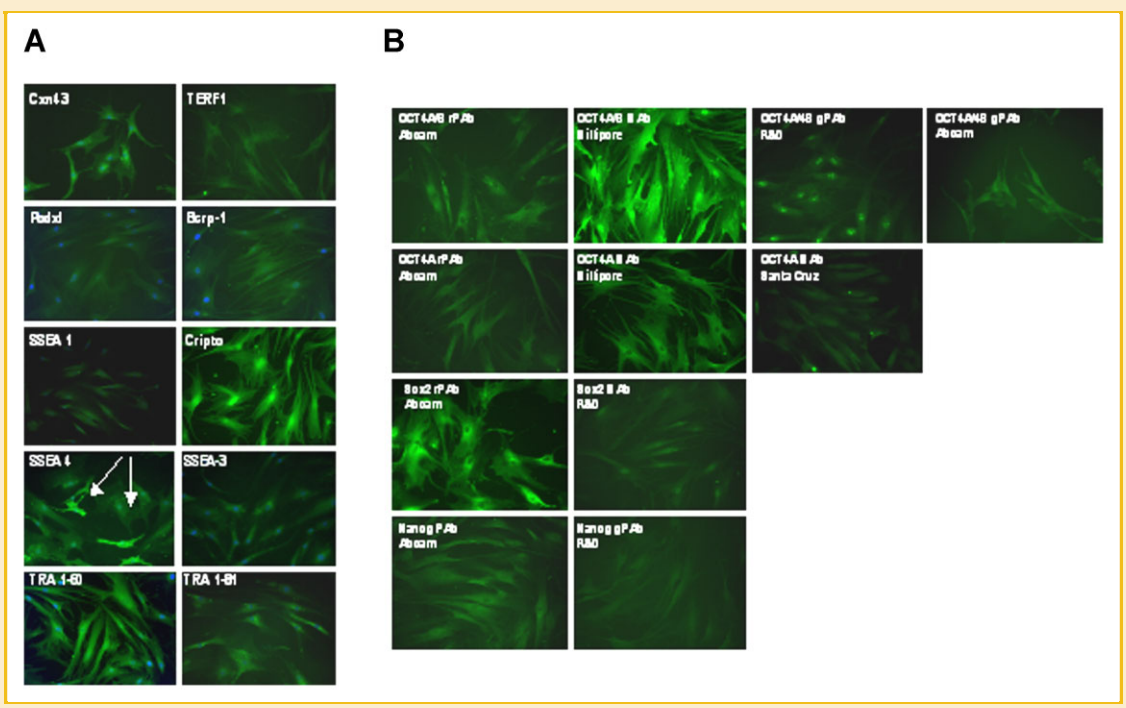

Fig. 3. Intracellular expression patterns of pluripotent ES markers in human MSCs—Antibody source discrepancies still persist. Panel A: Intracellular expression of several ES markers in hMSC populations. Elevated expression of SSEA-4 in individual hMSCs is shown (arrows). Nuclear staining as assessed using DAPI is shown in images where nuclear staining was not observed (blue staining). Panel B: Intracellular expression patterns of OCT4A, OCT4B, Nanog, and Sox2 using Abcam, Millipore, R&D Systems, and Santa Cruz commercial antibodies. Monoclonal antibody—MAb, rabbit polyclonal antibody—rPAb, goat polyclonal antibody—gPAb. Magnification is 200× unless otherwise noted.

passage 5) appeared to lose this nuclear expression pattern while retaining their cytoplasmic distribution (data not shown). A cytoplasmic and nucleoplasmic expression pattern was also obtained in hASCs using a Sox2 monoclonal from R&D. However, the nucleolar localization was lost with this antibody. Moreover, this antibody also appeared to detect a possible surface distribution for Sox2 in addition to its localization within the cytoplasm. Finally, as shown previously in Figure 1, use of a polyclonal to Nanog from Abcam localized this protein exclusively to the cytoplasmic compartment and failed to find any Nanog within the nuclei. In contrast, use of a goat polyclonal from R&D failed to detect any significant expression of this marker in hASCs. These discrepancies appeared to be confined to these three ES markers and different commercial sources for three other eukaryotic proteins (alpha actin, Smad4, and ERK2) produced very similar intracellular patterns (Fig. 2B—Panel II). Taken together, the IF results indicate that the intracellular expression of OCT4, Sox2, and Nanog may differ dramatically depending on the commercial antibody chosen.

These antibody discrepancies were not restricted to hASC populations as differences in the expression patterns of OCT4, Sox2, and Nanog were also noted when hMSCs purchased from Clonetics were assessed as an alternate human adult stem cell population. MSCs and ASCs, both mesenchymal in origin, are considered by some to be similar stem cell populations localized to different locations. Like hASCs, hMSCs expressed many established ES markers, like Cxn43, Cripto, SSEA-4, TRA1-80, and TRA1-60 (Fig. 3A). The expression patterns of Cxn43 and Cripto in hMSCs were similar to hASCs, as was the augmented expression of SSEA-4 in a select few hMSCs, again suggesting the presence of subpopulations within adult stem cell cultures. However, differences in expression levels and patterns were noted between hMSCs and hASCs for a few markers. Specifically, the hMSC populations examined in this study did not express significant levels of Podyxl and showed weak expression of Bcrp-1. Furthermore, while hMSCs did express TERF-1, albeit at a low level, expression of this marker was not found in the nucleus. Finally, the expression of SSEA-3 in hMSCs differed dramatically from the surface expression patterns of hASCs, with a more cytoplasmic expression pattern being observed in hMSCs.

The differences in SSEA-3, TERF-1, Podyxl, and Bcrp-1 in hMSCs appear to support the theory that hASCs and hMSCs, while similar in many respects, are two distinct stem cell populations. Consistent with this, differing intracellular patterns of OCT4, Sox2, and Nanog were also noted in hMSCs (Fig. 3B and Table IV). For example, a few hMSCs did express OCT4 within their nuclei when processed with

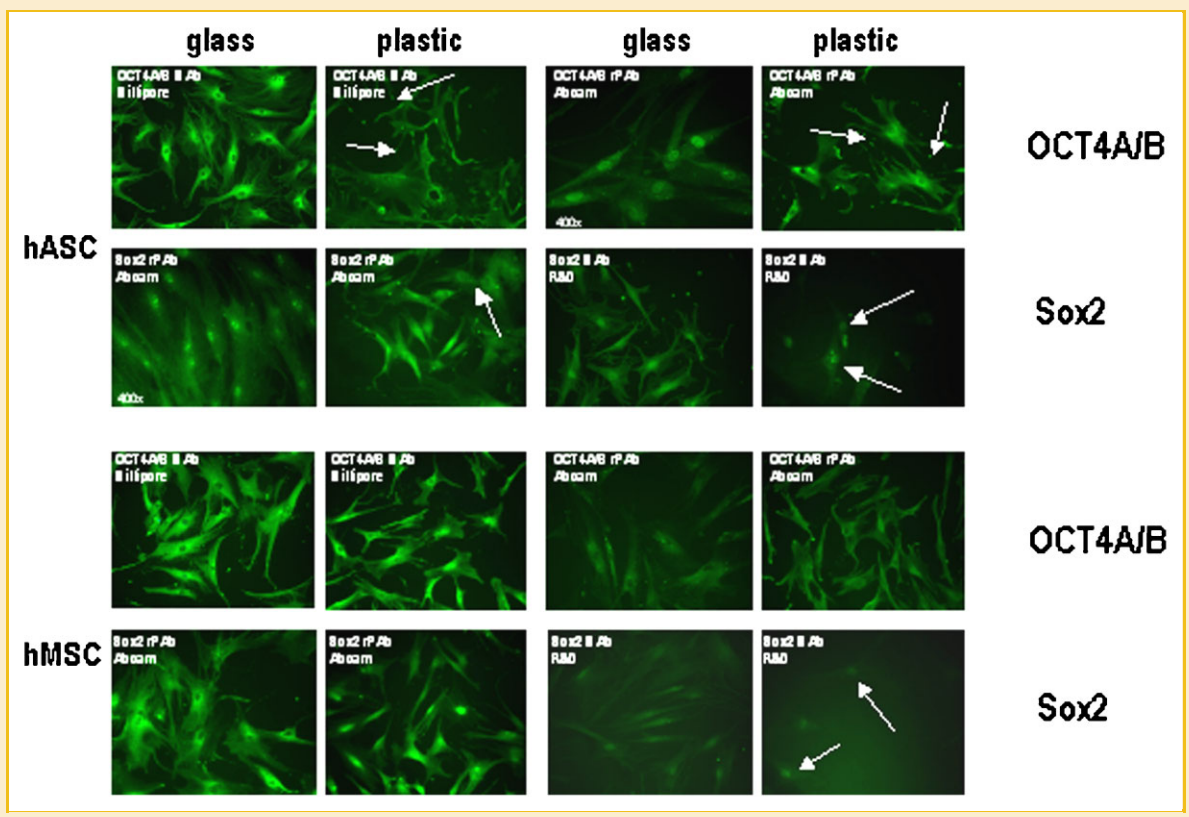

Fig. 4. Antibody discrepancies are independent of adhesive surface—glass versus plastic. Intracellular expression of OCT4A/B and Sox2 in hASCs (top panels) and hMSCs (bottom panels) upon adhesion to glass or plastic surfaces. OCT4A/B expression was assessed using both a monoclonal antibody from Millipore (OCT4A/B MAb, Millipore) and a rabbit polyclonal from Abcam (OCT4A/B rPAb, Abcam). Sox2 expression was assessed using both a monoclonal antibody from R&D Systems (Sox2 MAb, R&D) and a rabbit polyclonal from Abcam (Sox2 rPAb, Abcam). Shifts in intracellular expression are shown (arrows) and described in the text. Magnification is 200× unless otherwise noted.

the OCT4A/B Millipore monoclonal while nuclear localization was never observed in hASC populations when using this monoclonal antibody. Differences in the intracellular expression patterns of Sox2 and Nanog were also observed between hASC and hMSC populations. However, the conclusion that hASCs and hMSCs are unique stem cell populations based on their distinct expression patterns of OCT4, Sox2, and Nanog must be considered carefully owing to similar commercial antibody discrepancies observed in hMSCs as that seen in hASCs. For example, similar to hASCs, differing intracellular staining patterns were observed in hMSCs processed with antibodies specific to the OCT4A variant and the OCT4A/B variant. Differing intracellular expression patterns, including a lack of expected nuclear expression were also observed in hMSCs using the commercial antibodies to Sox2 and Nanog. Based on these IF findings, it is difficult to definitively describe the expression patterns of these three ES markers in human adult stem cells.

Adhesion-based mechanisms are known to control gene expression in both somatic and stem cell populations. For example, integrin-based pathways can alter MAPK signal transduction pathways [Stupack and Cheresh, 2002] and adhesion is thought to play a significant role in stem cell potency [Rohwedel et al., 1998; Cool and Nurcombe, 2005; Hakuno et al., 2005]. While the discrepancies between the many OCT4, Sox2, and Nanog commercial antibodies are disturbing, they may result from something as simple as the adhesive surface (i.e., glass vs. plastic) altering the intracellular distributions of these proteins. As a result, the expression of OCT4A/4B and Sox2 were examined in both hASC and hMSC populations adhered to plastic substrates. As shown in Figure 4, adhesion to plastic was capable of changing the intracellular distributions of OCT4A/4B and Sox2, suggesting that adhesive surface can affect the expression of these ES markers. For example, adherence of hASCs and hMSCs to plastic appeared to result in an expression pattern characteristic of surface expression. Adherence of hASCs to plastic also appeared to shift the expression of OCT4A/4B from the nucleus entirely to the cytoplasm. Furthermore, while adherence to plastic did not appear to affect the overall expression pattern of Sox2 in hMSCs, a shift of Sox2 from the nucleus to the cytoplasm was seen in hASCs. However, as seen with glass surfaces, antibody-to-antibody variation was still observed on these plastic surfaces. Finally, antibody variation was not just restricted to human adult stem cell populations as differing OCT4A/4B intracellular patterns were also observed from antibody-to-antibody source when rat ASC and MSC populations were examined (Fig. 5 and Table V). While it is not unreasonable to expect changes in intracellular patterns between human and rodent stem cells, there remains a complete lack of consistency between each OCT4A/4B commercial antibody.

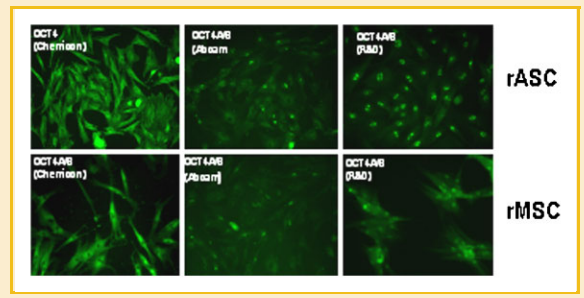

Fig. 5. OCT4A/B antibody discrepancies are independent of stem cell source—human versus rodent. Expression of OCT4A/B using three commercial antibody sources (Millipore, R&D, and Abcam) are shown in rat ASC (rASC) and rat MSC (rMSC) populations. Magnification is 200× unless otherwise noted.

The expression of multiple ES markers, including OCT4, Sox2, and Nanog, within ASCs and MSCs is exciting and may lead many to speculate that these adult stem cells share many characteristics with the totipotent ES cell—including increased potency. On the surface, the expression of these ES markers outside of the nucleus is not concerning. Several studies have described changes in intracellular patterns as a result of differentiation, proliferation, or apoptosis. For example, a redistribution of nuclear proteins to the cytoplasm has been observed upon differentiation of hematopoietic cells and is thought to play a role in the differentiation program of these cells [Tu et al., 2003]. Nucleolar expression of Nanog has been described in goat trophectoderm stem cells [He et al., 2006] and sequestration of transcription factors in the nucleoli of cancer and stem cells is thought to be a mechanism of their inactivation [Tsai and McKay, 2002]. The existence of transcript variants with distinct roles and expression patterns within the stem cell has also been proposed [Cauffman et al., 2006; Lee et al., 2006; Atlasi et al., 2008]. Finally, on a basic level, analysis of these stem cells using adhesion-based IF may give false positives and/or negatives. Reasons such as these could be used to describe the observed individual intracellular patterns of this study. However, the fact that these patterns change so dramatically among antibody sources is concerning and not likely to be due to any adhesive-induced or functionally dependent mechanism. Therefore, without definitive functional assays that measure the activity of these ES factors directly, the temptation for the adult stem cell researcher to call their stem cell population pluripotent based solely on their in vitro expression should be curbed. With the advanced level of research being performed on adult stem cells it is not surprising that we should find the expression of several pluripotent ES markers within the adult stem cell. The question becomes . . . is this expression indicative of increased plasticity or just a clever distraction?

TABLE V. OCT4A/B Distribution—Comparison of Human Versus Rat Stem Cells

Marker name	hASCs	rASCs	hMSCs	rMSCs
OCT4A/4B MAb (Chemicon)	Cytoplasmic (no nuclear)	Cytoplasmic and surface (no nuclear)	Cytoplasmic, nuclear and surface	Cytoplasmic and surface (no nuclear)
OCT4A/4B PAb (Abcam)	Nuclear and cytoplasmic	Nucleolar with some cytoplasmic	Cytoplasmic (no nuclear)	Nucleolar with some cytoplasmic
OCT4A/4B gPAb (R&D)	Nucleolar	Nucleolar	Nucleolar	Nucleolar

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