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SOX2, OCT3/4 and NANOG Expression and Cellular Plasticity in Rare Human Somatic Cells Requires CD73

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Abstract

Endogenous Plastic Somatic (ePS) cells isolated from adult human tissues exhibit extensive lineage plasticity *in vitro* and *in vivo*. Here we visualize these rare ePS cells in a latent state, i.e. lacking SOX2, OCT3/4 and NANOG (SON) expression, in non-diseased breast specimens through immunohistochemical analysis of previously identified ePS-specific biomarkers ($CD73^+$, $EpCAM^+$ and $CD90^-$). We also report a novel mechanism by which these latent ePS cells acquire SON expression and plasticity *in vitro*. Four extracellular factors are necessary for the acquisition of SON expression and lineage plasticity in ePS cells: adenosine (which is produced by the 5' ectonucleotidase CD73 and activates in turn the PKA-dependent IL6/STAT3 pathway through the adenosine receptor ADORA2b), IL-6, FGF2 and ACTIVIN A. Blocking any pathway component renders ePS cells incapable of SON expression and lineage plasticity. Notably, hESCs do not use adenosine or IL-6 nor they express CD73 or ADORA2b and inhibition of adenosine signaling does not ablate their plasticity. Therefore, the data presented here delineate novel circuitry and physiological signals for accessing SON expression in rare, undifferentiated human cells.

Graphical abstract

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Conflict of interest

None

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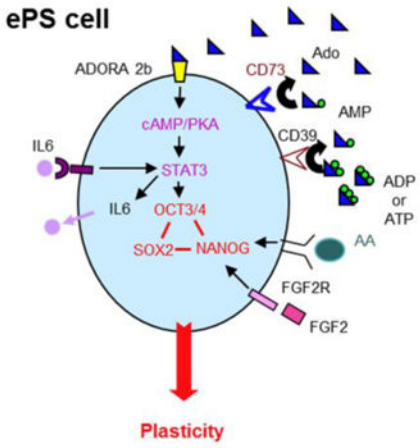

1. Introduction

Until recently, it was thought that cellular plasticity, i.e. the ability to form multiple somatic lineages, was restricted to a transient population of pluripotent cells within the blastocyst (embryonic stem cells) and an enduring population in the gonads (germ cells) [1]. A self-regulating positive feedback network, that includes expression of SON proteins, maintains a plastic state in these cells. However, we now know that acquisition of cell plasticity or a pluripotent state is not restricted to the above cells. Exogenous expression of key factors has been shown to induce the transition of fully differentiated cells to a pluripotent state [2]. Additionally, rare cells within adult tissues have also been documented to express SON and generate some, or all, somatic lineages [3-10]. In some instances, such cells are isolated from stressed tissues generating “multi-lineage differentiating stress-enduring” (MUSE) cells [11]. In other instances, such rare cells (endogenous Plastic Somatic (ePS) cells) have been found in a latent state in non-damaged tissues that can subsequently be activated to express SON [12]. The latter cells provide a unique opportunity to study the activation of SON and acquisition of cellular plasticity.

The recently described ePS cells exhibit three coordinated phenotypes that are regulated by repressed $p16^{INK4a}$: they (a) lack activation of cell cycle arrest when stressed, (b) express distinctive surface proteins whose expression (high CD73 and lack of CD90) enables their prospective enrichment and (c) exhibit enhanced expression of chromatin remodeling proteins [13], a state associated with acquisition of cellular plasticity and loss of differentiation properties [13-15]. The unexpected extensive lineage plasticity of ePS cells was characterized according to strict criteria previously outlined within the stem cell field [16]. *In vitro*, *in vivo*, teratoma assays and forensic analysis verified that ePS cells can give rise to a population of cells that generates all somatic lineages and retains a diploid karyotype [12]. Importantly, in contrast to other plastic cells that have the ability to differentiate into all three lineages, such as human embryonic stem cells (hESC) or induced pluripotent stem cells (iPSC), ePS cells are mortal, and retain low telomerase expression while expanding for an extensive, but finite, number of cell doublings before arresting in G1 (Table 1).

Expression of the surface marker CD73, a glycosyl phosphatidyl-inositol (GPI)-anchored ecto-5'-nucleotidase present in cell membrane lipid rafts [17, 18], is a key distinguishing marker of ePS cells. Expression of CD73, regulated by many stress-associated factors [19-21], has been widely implicated in cellular responses to stress and activating plasticity through catalyzing the generation of extracellular adenosine and control of adenosine signaling [22-24]. CD73 has been detected in many cell types where it participates in multiple functions including immune response and protection from ischemia and tissue damage [17]. Recently, CD73 has been identified as a critical reprogramming landmark in the generation of iPS cells [25]. This study examines the cellular and molecular mechanisms by which ePS cells attain a plastic state. We demonstrate that activation of SON is necessary for ePS cells to exhibit plasticity and we identify novel CD73-dependent signaling pathways that are key to the regulation of SON in these cells. In identifying these key stress-signaling cascades, we identify novel circuitry for accessing plasticity in rare human cells within the adult body and clues as to where activated ePS cells may be found *in vivo*.

2. Materials and methods

Information about reagents and tissue samples is shown in Tables S1 and S2.

2.1. Isolation and culture of ePS cells from human breast tissues

Acquisition of breast tissues from healthy women undergoing reduction mammoplasty after informed consent and the described experiments were approved by the UCSF Committee on Human Research under protocol 10-01532. Obtained information met Health Insurance Portability and Accountability Act guidelines. Tissues were devoid of visible disease, bacterial, fungal or viral contamination. ePS cells were isolated and cultured as described in [12]. Briefly, sorted $CD73^{+}CD90^{-}$ cells (10^{4} cells/well) were cultured on irradiated fibroblast cells (feeders) from 6.2-6.6 week placentas or expanded in a feeder-free medium (F-FM) [12]. The number of colonies over 100 μm or 400 μm in size growing on feeders was recorded. ePS cells within the R1 sorted subfraction were expanded in F-FM (α-MEM medium with glutamine supplemented with 15% (vol/vol) ES-FBS (Omega Scientific; FB-05), 18% (vol/vol) Chang B, and 2% (vol/vol) Chang C (Irvine Scientific; C-100 and C-106, respectively)) and passaged by trypsinization.

2.2. Cell culture and drug treatment

T47D breast cancer cells were maintained in DMEM+10%FBS, $CD73^{-}CD90^{+}$ cells in MEGM and ePS cells growing on feeders in mammary stem cell medium [12]. After a 3 day recovery post-sorting, ePS cells were treated with various drugs as described in the Figure legends and fed fresh medium every other day for 7 days. Cell growth was determined by daily cell counting for 4 days after initial plating of 1×10^{5} cells in triplicate. hESCs were propagated either on irradiated MEF feeders in Knockout Serum Replacement (KSR) medium or in feeder-free medium (F-FM) used to expand ePS cells.

2.3. Immunoblotting and Real-Time qPCR

Immunoblotting was carried out as described in [12]. Total RNA was isolated from cells and cDNA synthesized as previously described [26]. qPCR (Taqman) was performed in technical

triplicates using the standard curve method with gene-specific primer/probe sets and monitoring of glucuronidase B (GUSB) expression to normalize for variances in input cDNA.

2.4. FACS analysis

After epitope regeneration in MEGM + 2%FBS for 1-2 hours on ice, the single cell suspension was stained with a FITC-labeled anti-EpCAM antibody, fixed, permeabilized with a transcription factor buffer set, stained with an anti-OCT3/4 primary antibody and an Alexa 647 goat anti-rabbit secondary antibody and analyzed using a FACS Aria II cell sorter.

2.5. Apoptotic analysis

ePS cells growing on feeders were harvested and stained with a FITC-conjugated antibody against EpCAM and Alexa 647 conjugated Annexin V (Life Technologies; A23204) prior to FACS analysis.

2.6. Adipogenesis and osteogenesis differentiation assays

Adipogenesis: ePS cells or MSCs were differentiated using an adipogenesis differentiation kit. After 14 days, cells were stained with Oil Red O [12] or LipidTox following manufacturer's instructions (Life Technology; H34350) or analyzed by qRT-PCR. Osteogenesis: 10^4 cells seeded into 4-well Permanox slides were treated with or without 400 μm adenosine in the presence or absence of 12 μm APCP for 48h before applying osteogenic conditions (StemPro Osteogenesis Differentiation Kit; Gibco, Inc) for 21 days. Osteogenesis was assessed after cell fixation in 4% paraformaldehyde for 15 min and staining with 2% alizarin red solution (pH 4.1-4.3) for 20 min. Stained monolayers were visualized and recorded by phase microscopy.

2.7. Embryoid body formation, cardiomyocyte differentiation and video documentation

ePS cells grown on feeders were manually dissected for embryoid body formation, subjected to cardiomyocyte differentiation and video-taped [12]. All videos time lapses were 10-15 s.

2.8. Plasmids and Retroviral Gene Transfer

Lentiviral plasmids expressing CD73-Flag or short hairpins for OCT3/4, SOX2, NANOG, STAT3 or CD73 and matched control plasmids were used to transfect packaging 293 cells. Lentiviral suspensions were used to transduce ePS cells cultured in F-FM as described in [26]. 72 h after initial transduction, cells were selected for 48 h with 1.5 μg/mL puromycin, expanded and induced with 1μg/ml doxycycline for 48 h to express the short hairpins.

2.9. Immunohistochemistry and Multiplex analysis

Serial 4 μm paraffin-embedded RM tissue sections were deparaffinized and rehydrated. One section was stained with hematoxylin and eosin (H&E) for histological evaluation and other sections subjected to multiplex immunostaining. All steps were performed at room temperature unless mentioned otherwise. Briefly, to reduce non-specific background staining due to endogenous peroxidase, sections were treated with 3% hydrogen peroxide for 10 min. For antigen retrieval, sections were microwaved for 10 min in citrate buffer (pH=6.0). For

double staining, sections were incubated simultaneously for 60 min with a mouse monoclonal anti-CD73 antibody diluted 1:2,000 and either a rabbit monoclonal anti-CD90 antibody diluted 1:100 or a rabbit monoclonal anti-EpCAM antibody diluted 1:100. Sections were washed then incubated for 30 min with anti-mouse IgGs coupled to horseradish peroxidase (HRP) and anti-rabbit IgGs coupled to alkaline phosphatase (AP) (Power-Stain^{TM} 1.0 double stain kit I). Sections were washed and developed for 5 min using diaminobenzidine (DAB) and Vector blue as HRP and AP substrates, respectively. For triple staining of SON, sections were incubated first with a rabbit monoclonal anti-SOX2 antibody diluted 1:50, then with anti-rabbit IgGs coupled to AP and finally revealed with Vector blue chromogen. Sections were microwaved for 5 min in citrate buffer (pH=6.0) and incubated simultaneously with a rabbit monoclonal anti-OCT 3/4 antibody diluted 1:800 and a mouse monoclonal anti-NANOG antibody diluted 1:6,000. Sections were washed then incubated for 30 min with Power-Stain^{TM} 1.0 double stain kit I. After 2 washes, sections were developed for 5 min using DAB and Vector Red as HRP and AP substrates, respectively, and counterstained with methyl green or nuclear fast red to visualize the nuclei. Multiplex analysis was carried out after image acquisition using a multispectral Nuance FX camera and unmixing of color images using the Nuance software (Perkin-Elmer). The analysis generated black and white images showing staining pattern for each marker separately as well as a merged composite image with pseudocolors assigned to each marker to facilitate visualization of multiplex staining pattern.

2.10. Statistical analysis

Two-sided t test assuming unequal variance was used to test the relations between mRNA expression of OCT3/4, NANOG, SOX2, ADORA1, ADORA2a, ADORA2b, ADORA3, LEPTIN, FABP4, and PPARγ or EpCAM^{+}OCT3/4^{+} cell percentages in ePS cells. For all comparisons, $P \leq 0.05$.

3. Results

3.1. Multiplex immunohistochemical analysis of CD73 and CD90 visualizes latent ePS in adult human breast tissue

We previously reported that ePS cells isolated on the basis of the CD73^{+}/CD90^{-} immunophenotype constitute a small fraction (~0.15%) of cells within human breast tissue (~3% of the CD73^{+}/CD90^{-} population). Significant evidence differentiates ePS cells from breast tissue-specific stem cells (documented to be EpCAM negative), as well as mesenchymal stem cells, adipocytes and fibroblasts (documented to be EpCAM negative and CD90 positive) [27, 28] (Table 2). Additionally, ePS cells express EpCAM and CD49f [12], indicating that they are not mature luminal or myoepithelial breast cells since these differentiated cells are CD49f negative and EpCAM negative, respectively [29, 30]. Notably, ePS cells, either when observed *in vivo* in healthy tissues or when initially isolated from those tissues, do not express SON proteins. However, when these cells are placed in feeder-free or feeder conditions, SON proteins are induced to levels equivalent to those observed in hESCs. Thus, ePS cells exist as undifferentiated cells in a latent state in healthy tissues and are activated by our culture conditions, providing us with an assay to study their acquisition of SON expression and plasticity.

Here, we sought to visualize the latent rare cells *in situ* by multiplex analysis of the $CD73^+CD90^-$ immunomarkers. Some of the reduction mammoplasty (RM) specimens utilized were characterized in our previous study for ePS cell lineage plasticity both *in vitro* and *in vivo* [12]. Multiplex immunohistochemical analysis allowed visualization of the four subpopulations (R1-R4) of cells based on their expression of CD73 and CD90 (Figs. 1A-B and S1A-B). In agreement with our reported flow cytometric analysis [12], the majority of breast cells were negative for both CD73 and CD90 expression (R3 population). Based on our analysis, latent ePS cells were expected to be rare and enriched in a population expressing CD73 and lacking CD90 and SON. $CD73^+CD90^-$ cells were more prevalent in the large ducts than in terminal alveoli (Fig. 1), with infrequent cells dispersed within the surrounding stroma. The frequency of $CD73^+CD90^-$ cells was ~ 3% (136/4610 cells) consistent with the frequency (~3%) of these cells measured by flow cytometry using the same markers [12]. A similar frequency was confirmed in 11 independent RM specimens (Table S1). Multiplex analysis of CD73 and EpCAM, an additional marker expressed on ePS cells, demonstrated that a subset of $CD73^+$ cells also express EpCAM. On the basis of the $CD73^+CD90^-$ $EpCAM^+$ immunophenotype, ePS cells can be differentiated from other cell types within the adult mammary gland (Table 2). Consistent with the proposed latent state of ePS cells, multiplex analysis of healthy breast tissue also failed to identify cells exhibiting coincident nuclear expression of SON (Fig. S1A-B), contrasting with a seminoma sample used as positive control (Fig. S1C).

3.2. SON signaling is necessary for ePS cell plasticity

Latent ePS cells can be activated to coincidently express nuclear SON and display lineage plasticity, providing a distinct state to allow us to investigate molecular mechanisms for induction of these two phenotypes in these rare cells. Once $CD73^+CD90^-$ (R1) populations (enriched for ePS cells) are directly sorted from human breast tissues and placed on irradiated placental fibroblast feeders, the ePS cells attach and form colonies. After 9-12 days in culture, they express pluripotency-associated proteins such as SON, Lin28 [12], Tra-1-60 and Tra-1-81 (Fig. S2A) at levels comparable to hESCs cells grown on feeders. $CD73^+CD90^-$ (R1) cells were also placed in F-FM conditions (Fig. S2A). These expanded ePS cells were genomic stable displaying a normal diploid phenotype (Fig. S1A). This complementary feeder-free approach allows us to specifically and sufficiently expand ePS cells to facilitate mRNA and biochemical analysis as well as perform genetic manipulations. Immunocytochemistry, qPCR, and Western blot (WB) analyses confirmed that the expression of SON was comparable in ePS cells and hESCs grown in F-FM conditions (Fig. S2B-D).

The pluripotent state of hESCs is dependent upon the expression and activity of SON [31-33]. Therefore, we next asked if the expression of each component of SON within this mutually regulated feedback circuit was also necessary for ePS cell plasticity [34-36]. ePS cells, freshly isolated from breast tissue, were cultured in F-FM conditions and transduced with a shRNA construct against OCT3/4, SOX2 or NANOG. Both qPCR (Fig. S3A) and WB (Fig. S3B) analyses confirmed efficient knockdown of each gene compared to control cells transduced with a scrambled shRNA vector. Additionally, we observed that knockdown of any one of the three SON factors promoted the downregulation of the other two

untargeted SON factors (Fig. S3A and B). ePS cells silenced for OCT3/4, SOX2 or NANOG were all similarly defective in the ability to differentiate into adipocytes compared to control ePS cells, as demonstrated by the lack of accumulation of Oil Red O-positive (top panels) or LipidTox-positive (bottom panels) lipid droplets (Fig. S3C). Consistent with the functional staining, ePS cells silenced for OCT3/4, SOX2 or NANOG all failed to express molecular markers of adipocyte differentiation fatty acid binding protein 4 (FABP4), LEPTIN and the transcription factor PPARγ, (Fig. S3D). Together, these data indicate that SON expression is required for ePS cell plasticity, just as it is for hESCs.

3.3. CD73-mediated generation of adenosine is necessary for SON expression and plasticity in ePS cells

We sought to determine whether CD73, a key immunomarker used to enrich for ePS cells [12], plays a mechanistic role in the acquisition of SON expression and plasticity. Exposure of ePS cells cultured on feeders to 6 μM of adenosine 5'-(α, β-methylene) diphosphate (APCP), an inhibitor of CD73 enzymatic activity, for 1 or 3 weeks dramatically decreased the size and number of ePS colonies (Fig. 2A). Immunocytochemical analysis of ePS colonies treated with APCP revealed a dramatic reduction in SON expression compared to vehicle-treated cells and hESCs (Figs. 2B and S4A). Consistent with this, exposure of SON-expressing ePS cells cultured in F-FM to APCP for 48 hours resulted in a 40-60% reduction of SON mRNA expression (Fig. 2C) and a dramatic decrease in protein expression (Fig. S4B) compared to vehicle-treated cells. As expected, APCP treatment did not affect CD73 protein expression (Fig. S4B). Doxycycline-inducible shRNA knockdown of CD73 in ePS cells cultured in F-FM validated our results with APCP. Upon doxycycline addition, ePS cells exhibited a 3-fold reduction in CD73 mRNA (Fig. S4C) as well as a reduction in SON mRNA (Fig. 2D) and protein (Fig. S4D) compared to ePS cells transduced with scrambled shRNA. When cultured under differentiation conditions conducive to adipogenesis, both APCP-treated and shCD73-knockdown (+doxycycline) cells failed to accumulate Oil Red O-positive lipid droplets (Fig. 2E and S4E) and expressed lower levels of FABP4, LEPTIN and PPARγ (Fig. S4F) compared to control cells. Additionally, APCP-exposed ePS colonies failed to generate embryoid bodies (EB) and beating cardiomyocytes compared to control cells (Fig. 2F and Movies S1-2). APCP-mediated inhibition of CD73 activity did not affect ePS cell growth or survival in F-FM (Fig. S4G). However, upon APCP treatment, ePS colonies cultured on feeders exhibited an increase in apoptosis over time as demonstrated by $EpCAM^{+}ANNEXIN\ V^{+}$ staining (Fig. S4H).

Having established a role for CD73 in SON expression and ePS plasticity, we next investigated the role of adenosine (the enzymatic product of CD73 activity). Exposure of shCD73-ePS cells in F-FM conditions to 200 μM adenosine rescued OCT3/4 mRNA expression, which was increased 2-fold compared to untreated shCD73-ePS cells (Fig. S4I). More importantly, addition of adenosine was sufficient to rescue both adipogenic and osteogenic differentiation in APCP-treated ePS cells without affecting differentiation of ePS cells in the absence of APCP (Fig. S4J). As an additional control, we investigated whether adenosine was sufficient to induce SON expression in the $CD73^{-}/CD90^{+}$ subpopulation lacking ePS cells, the majority of which were of myoepithelial lineage. $CD73^{-}/CD90^{+}$ cells failed to express SON when exposed to 200 μM or 500 μM adenosine for up to 96 h (Fig.

S4K), even in conditions that supported both myoepithelial (MEGM) and ePS (F-FM) cell expansion. Furthermore, ectopic expression of CD73 in T47D breast cancer cells, which do not normally express CD73, failed to upregulate OCT3/4 protein (Fig. S4L). These data indicate that CD73-mediated adenosine production is required but not sufficient for ePS colony growth on feeders, SON expression and cellular plasticity.

3.4. Adenosine receptor ADORA2b activates an intracellular signaling cascade necessary for SON expression in ePS cells

Adenosine signaling is dependent on four G protein-coupled receptors (GPCR), ADORA1, ADORA2a, ADORA2b, and ADORA3 [37]. Only ADORA2b expression was observed in ePS cells by qPCR analysis (Fig. 3A). Inhibition of ADORA2b, either specifically (MRS1754 (MRS)) or in addition to ADORA2a (DMPX, 3,7-Dimethyl-1-propargylxanthine), decreased ePS colony formation on feeders in comparison to vehicle-treated cells, similar to the effect of APCP (Fig. 3B). Treatment of ePS cells grown under F-FM conditions with DMPX or MRS resulted in a decrease in the percentage of cells expressing OCT3/4 protein (Fig. 3C) and SON mRNA (Figs. 3D and S5A). In contrast, cells exposed to inhibitors of ADORA1 or ADORA3 (8-phenyltheophylline (8-PT) and VUF-5574 (VUF), respectively) formed ePS colonies on feeders (Fig. 3B) and expressed SON mRNA in F-FM culture conditions (Figs. 3D and S5A). ePS cells cultured in MRS failed to form EBs and to differentiate into beating cardiomyocytes compared to vehicle-treated cells or cells cultured in the presence of 8-PT or VUF (Fig. 3E and Movies S3-5). In a complementary approach, ePS cells were transduced with a doxycycline-inducible shRNA against ADORA2b in F-FM conditions. Silencing ADORA2b (+doxycycline) (Fig. S5B) resulted in decreased SON mRNA (Fig. S5C) and prevented adipogenesis, as demonstrated by reduced expression of FABP4, LEPTIN, and PPARγ (Fig. 3F). These results indicate that ADORA2b receptor function is necessary for ePS cells to regulate SON expression and cellular plasticity.

3.5. Identification of PKA/STAT3 signaling downstream of ADORA2b in regulating SON expression in ePS cells

In ESCs, GPCR activity has been hypothesized to regulate SON expression and pluripotency through activation of cAMP/cAMP-dependent protein kinase (PKA) and downstream STAT3 signaling [38, 39]. ADORA2b is coupled to the stimulatory G-protein subunit that promotes cAMP production and PKA activation [40]. To determine if ADORA2b regulates SON expression through the downstream activation of the PKA and STAT3 signaling pathways, ePS cells were incubated with increasing concentrations of H89, a PKA inhibitor, or Stattic, a STAT3 inhibitor. As previously demonstrated, adenosine (200μM) enhanced SON expression in ePS cells at both mRNA (Fig 4A and S6A) and protein (Fig. 4B) levels. In this experiment, both H89 and Stattic reduced SON mRNA (Fig 4A and S6A) and protein (Fig. 4B) levels in the absence and presence of adenosine. Validating this result, ePS cells silenced for STAT3 with inducible shRNAs in F-FM conditions (Fig. 4C) also exhibited a decrease in SON protein (Fig. 4C) and mRNA (Fig. S6B).

We next examined if the PKA and STAT3 signaling pathways were epistatic or parallel downstream of adenosine signaling. ePS cells were treated with H89 and Stattic, either alone

or together in the presence of 200μM adenosine. Both the single inhibitor and combination groups exhibited a 40% reduction in OCT3/4 expression suggesting that PKA and STAT3 were part of a single pathway downstream of adenosine-induced ADORA2b activation (Fig. 4D). Consistent with this, inhibition of PKA resulted in a dramatic decrease in STAT3 phosphorylation (Fig. 4B), suggesting that PKA was upstream of STAT3 signaling.

To validate a functional link between PKA activity and SON expression, we activated PKA by treating ePS cells with 8-Br-cAMP (a non-hydrolyzable analog of cAMP) in the absence and presence of the ADORA2b-specific inhibitor MRS1754. Treatment with 8-Br-cAMP led to a 70% increase in SON mRNA (Figs. 4E and S6C) as well as increased OCT3/4 protein and STAT3 phosphorylation (Fig. 4F) compared to ePS cells treated with MRS1754 alone. These results demonstrated that in ePS cells with compromised ADORA2b activity, stimulation of the PKA/STAT3 pathway was sufficient to rescue SON expression.

We next probed the role of JAK and JNK, two kinases responsible for STAT3 activation in many cellular systems, in the response of ePS cells to adenosine. ePS cells, grown in F-FM conditions, were treated with the stable adenosine analogue 5′-(N-Ethylcarboxamido) adenosine (NECA). NECA increased JAK2 phosphorylation, but not that of JNK (Fig. S6D). Notably, phosphorylation of JAK2 in ePS cells treated with NECA was blocked by increasing concentrations of the PKA inhibitor H89 (Fig. S6D). Treatment of ePS cells with Forskolin (a PKA activator) also increased STAT3 phosphorylation and SON protein to the levels observed in NECA-treated cells. Treatment with Ruxolitinib (a specific inhibitor of JAK kinase) reduced both STAT3 phosphorylation and SON expression (Fig. S6E). Thus, in ePS cells, adenosine signaling through ADORA2b activates SON expression and cellular plasticity via an intracellular signaling cascade characterized by sequential activation of PKA, JAK and STAT3.

3.6. Increased IL6 production in response to adenosine signaling promotes autocrine activation of SON and plasticity in ePS cells

Interleukin 6 (IL6) potently activates JAK/STAT3 signaling through the cell surface type I cytokine receptor [41]. Mining of the ALEXA-Seq database [42] revealed that IL6 transcripts were expressed at high levels in sorted breast $CD73^{+}$ (R1 plus R2) cells compared to other mammary cell populations and hESCs (Fig. S6F). In F-FM conditions, treatment of ePS cells with adenosine increased IL6 mRNA (Fig. 4G), while abrogation of the intracellular signaling pathway downstream of adenosine signaling in ePS cells, by treatment with the PKA inhibitor H89, prevented IL6 upregulation by adenosine (Fig. 4G). The expression of LIF, another member of the IL6 superfamily widely involved in regulating SON expression in pluripotent stem cells [43, 44], was unaltered in response to adenosine treatment (Fig. 4G). Our results suggest that adenosine signaling increased IL6 expression in a PKA-dependent manner. To validate this observation, we treated ePS cells cultured in F-FM with specific ADORA inhibitors and assessed IL6 expression by qPCR analysis. Pharmacological inhibition of ADORA2b by MRS and DMPX reduced IL6 expression whereas inhibition of ADORA1 and ADORA3 by 8-PT and VUF, respectively, did not (Fig. 4H). As described earlier, ADORA2b expression was downregulated using an inducible shRNA vector targeting ADORA2b. This knockdown of ADORA2b (+doxycycline)

similarly decreased IL6 levels in the conditioned medium (CM) when measured by ELISA 72 hours after addition of fresh medium (Fig. 4H).

We next examined if CD73 was required for IL-6 expression. In ePS cells cultured in F-FM conditions, pharmacological inhibition of CD73 activity by APCP reduced IL6 mRNA, an effect that was blunted upon addition of adenosine (Fig. 4I). Knockdown of CD73 (Fig. 4I) and STAT3 (Fig. S6G) also decreased IL6 mRNA. These results strongly support that STAT3 activation downstream of adenosine signaling drives an autocrine positive-feedback loop through IL6. Consistent with a role for IL-6 in ePS cells, addition of exogenous IL6 to the medium of ePS cells cultured on feeders increased both the size and frequency of ePS colonies (Fig. S6H). Flow cytometry analysis revealed an increased expression of OCT3/4 protein in EpCAM-positive ePS cells (Fig. S6H-I). Reinforcing the importance of the identified positive feedback loop in control of SON expression, we observed that the addition of IL6 rescued SON protein expression in APCP-treated cells in which CD73 activity was compromised (Fig. S6J). Our observations demonstrate that generation of adenosine by CD73 contributes to SON expression in ePS cells through the autocrine activation of STAT3 by IL6 (Fig. 6D).

3.7. The canonical FGF2 and ACTIVIN A signaling pathways are also essential for SON expression in ePS cells

Several extracellular factors critical to the maintenance of SON expression have been documented in hESCs, including FGF2 and ACTIVIN A, a member of the TGF-β superfamily [45-47]. The medium utilized for culturing human ePS cells on irradiated placental fibroblasts contains supplemental FGF2, suggesting that this factor may also be important for SON expression and plasticity in ePS cells. To test this hypothesis, ePS cells cultured under F-FM conditions were treated with increasing concentrations of the FGFR-specific inhibitor PD173074 and probed for SON protein expression. This treatment significantly reduced SON expression (Fig. 5A), suggesting a role for FGF2 signaling in ePS cell plasticity. Additionally, in F-FM conditions, treatment of ePS cells with Follistatin, a specific inhibitor for ACTIVIN A, reduced SON mRNA (Fig. S7A). Conversely, treatment of ePS cells with 5 ng of ACTIVIN A enhanced SON mRNA (Figs. 5B and S7B) and protein (Fig. 5C). In contrast, treatment of ePS cells either with TGF-β or with a neutralizing antibody against TGF-β did not affect SON expression (Fig. S7A). Our results are consistent with previous reports that ACTIVIN A and TGF-β fulfill different functions despite their structural similarities and ability to activate SMAD signaling [48, 49]. These data demonstrate that FGF2 and ACTIVIN A, identified as important for maintaining expression of SON in hESCs, also contributed to expression of SON in ePS cells.

3.8. The signaling circuitry identified in ePS cells in feeder-free conditions is recapitulated in feeder conditions

The detailed characterization of signaling pathways regulating SON expression in ePS cells described above often necessitated their growth under feeder-free conditions (F-FM). We validated these findings using the feeder culture system (Fig. S2A), an important tool to access ePS cell plasticity. The CD73+/CD90-subpopulation was freshly isolated from RM tissues and seeded on irradiated placental fibroblast feeders. The ePS cells were allowed to

attach and grow over 3 days before being exposed to selected pathway inhibitors for 7 days. Inhibitors targeting specifically ADORA2b, STAT3, ACTIVIN A or the FGF2 receptor all dramatically reduced formation of ePS colonies and the percentage of cells ($EpCAM^+$) expressing OCT3/4 compared to vehicle-treated control cells (Fig. 5D). Thus, the molecular circuitry identified in ePS cells grown in F-FM is also operable in ePS cells growing as colonies on feeders.

3.9. CD73 and ADORA2b are required for SON expression in ePS cells, but not hESCs

In this study, we demonstrated that CD73 and ADORA2b signaling are required for the expression of SON and cellular plasticity in ePS cells. To date, neither CD73 nor ADORA2b have been reported to play a role in the maintenance of SON expression in hESCs. Flow cytometric analysis of CD73 and OCT3/4 revealed that <1% of hESCs express CD73 protein, while the vast majority of hESCs (>97%) expressed the core pluripotency factor OCT3/4 (Fig. 2B and 6A). Additionally, qPCR analysis revealed that hESCs expressed low levels of ADORA family adenosine receptors. Notably, ADORA2b expression in hESCs was 15-fold lower than in ePS cells (Fig. 6B). Importantly, whereas SON expression was downregulated at the protein level in ePS cells treated with the CD73 inhibitor APCP for 48 hours in F-FM conditions, APCP had no effect on SON expression in hESCs, even after 7 days of treatment (Fig. 6C). Additionally, when hESCs were cultured on feeders and exposed to inhibitors targeting CD73, ADORA1, ADORA2 or AdDORA3 activity for 7 days, immunocytochemical analysis revealed no significant change in OCT3/4 or NANOG expression in all treated groups (Fig. S8A), further demonstrating that CD73 and adenosine signaling were dispensable for maintenance of SON expression in hESCs. Similarly, exposure of hESC to either H89 or Stattic (inhibitors of the PKA and STAT3 pathways, respectively) did not significantly alter OCT3/4 or NANOG expression when measured by immunocytochemistry (Fig. S8B) or the percentage of cells ($EpCAM^+$) expressing OCT3/4 when measured by flow cytometry (Fig. S8C). This demonstrates that the PKA/STAT3-dependent signaling pathway necessary for SON expression in ePS cells is not required for SON expression in hESCs. Exposure of hESCs cultured on feeders to either PD173014 or Follistatin (inhibitors of the FGF receptor and ACTIVIN A, respectively) for 7 days resulted in a significant reduction in OCT3/4 or NANOG expression when measured by immunocytochemistry (Fig. S8B) and in the percentage of cells ($EpCAM^+$) expressing OCT3/4 when measured by flow cytometry (Fig. S8C). Similarly we found that both PD173014 (Fig. 5A) and Follistatin (Fig. S7A) inhibited SON expression in ePS cells, demonstrating that the ACTIVIN A and FGF2 pathways are required for SON expression in both ePS cells and hESC. In summary, our study reveals that ePS cells utilize a unique combination of signaling pathways to access SON expression when compared to hESCs.

4. Discussion

In the present study, we identified four extracellular factors (adenosine, IL-6, FGF2 and ACTIVIN A) that are required for SON expression and the extensive lineage plasticity characteristic of ePS cells. The activated ePS cell state we have described shares many characteristics with pluripotent hESCs. Both ePS cells and hESCs express high levels of canonical pluripotency markers [SON, Lin28 [12], TRA-1-60 and TRA-1-81 (Fig. S2A)],

respond to similar cues for differentiation into functional ectodermal, mesodermal and endodermal derivatives, and require stable expression of SON for plasticity (Fig. S3 and [34-36]). However, for ePS colonies to access the SON machinery, they require the expression and activity of the cell surface protein CD73. CD73 is required for the production of extracellular adenosine, which binds to the adenosine receptor ADORA2b in ePS cells and activates STAT3 and IL6. The importance of STAT3 signaling and its complex relationships with IL6 and LIF signaling in control of SON expression have been previously documented in various human and mouse stem cell models [50, 51]. However, the present study identifies for the first time a key functional link between upstream CD73/adenosine/ADORA2b signaling and this downstream signaling cascade independent of LIF in ePS cells. hESCs do not express CD73 and, unlike FGF2 and ACTIVIN A, adenosine signaling is not required for the maintenance of SON expression in hESCs. The data presented here indicate that CD73, working through adenosine, is necessary, but not sufficient, for SON expression and ePS plasticity.

The canonical mechanisms controlling SON expression in embryonic stem cells (ESCs) have been extensively investigated [33, 52]. Although some aspects of this control are not fully elucidated, it is clear that environmental signals play a key role in ESC biology through action of specific growth factors [53, 54]. ESCs can be cultured either on feeder layers or in feeder-free systems. In their most potent state, the ground state, mouse ESCs require Leukemia Inhibitory Factor (LIF)/STAT3 and Bone Morphogenic Protein (BMP4) signals either secreted by feeder cells or present in the culture medium [43, 55]. As mouse ESCs acquire distinct epigenetic marks of a primed and more restricted state, equivalent to the state exhibited by human ESCs, now ACTIVIN A and FGF2 signals are necessary to maintain a primed state of pluripotency [46, 47]. These environmental signals have been shown to directly control the expression of SON and maintain the proper expression of genes required for self-renewal and repression of differentiation of these cells [31-33] or for cell reprogramming [56-58].

The requirement for CD73 signaling, and the fact that CD73 expression is controlled by stress signals, suggests that, in rare cells, activation of SON and plasticity may be linked to physiological conditions of cellular damage [19-21]. Recent studies in several tissues indicate that two distinct stem cell populations exist: one population is responsible for maintaining tissue homeostasis while the other is responsible for wound healing [59-61]. For instance, the intestine differentially uses an actively cycling stem cell population for tissue homeostasis and a distinct quiescent, damaged-induced stem cell population for tissue damage repair [61], each generating the same range of tissue-specific differentiated cells. Additionally, cells with stem-like properties and multi-lineage potential have been reported to participate in stress responses and have been described within human breast tissue and milk [62, 63], skin [64] and at multiple other tissue sites. These multiple cell types are easily distinguished from ePS cells by several criteria. MUSE (Multilineage-differentiating stress-enduring) cells, MSC (mesenchymal stem cells), VSEL (Very Small Embryonic-Like) cells, MIAMI (Marrow-Isolated Adult Multi-lineage Inducible), ADSC (adipose-derived stem cells) and MAPC (Multipotent Adult Progenitor Cells) all express CD90, whereas ePS cells do not [3-10]. Additionally, ePS cells express EpCAM and can generate teratomas, whereas MUSE and MSCs fail to do so [12]. Lastly, knockdown of OCT3/4 in MSCs does not affect

differentiation into adipocytes [8], while our data demonstrate that knockdown of OCT3/4 ablates adipocyte differentiation in ePS cells (Fig. S3). Thus, ePS cells seem to represent a novel cell state that has not been previously described. Interestingly, recent reports demonstrate the expression of CD73 as a landmark in the reprogramming of iPS cells [25], thus extending the relevance of our findings in ePS cells.

This study exhibits numerous strengths: use of multiple human tissue samples (Table S1), multiple endpoints demonstrating ePS cellular plasticity, complementary analyses under both feeder and F-FM conditions and genetic and pharmacological manipulation of signaling pathways of interest. The complementary analyses under both feeder and F-FM conditions are important since growing any cell population under different culture conditions may alter gene expression, mechano-regulatory conditions or functional phenotypes. The analysis of 10 distinct human tissue samples for analysis of SON expression assures us that we are studying a general property of these cells and not a rare mutant phenotype. Most importantly for these studies, the role of stress signaling pathways in creating a niche for the activation of cellular plasticity provides predictions of where and when these cells may be found *in vivo*. Additionally, the in depth biochemical characterization of SON activation and complex regulation through CD73 circuitry provides insights that will allow control of these cells. Finally, comparison with hESCs demonstrates that not all cells control access to plasticity in the same manner. We will take advantage of our identification of key signaling pathways for acquisition of cellular plasticity in ePS cells *in vitro* to detect these cells *in vivo* and to inquire if they contribute to function or dysfunction *in vivo*.

We hypothesize that activation of plasticity in ePS cells in damaged tissues might contribute to widespread tissue repair and regeneration of functional tissues. Physiological observations may suggest why access to these plasticity circuits exists in rare cells and why they would be conserved throughout evolution. An extensive literature describes the widespread plasticity of cellular populations exposed to adverse growth conditions or injury [65-67]. The ability of pre-existing, undifferentiated latent cells to activate non-mutagenic phenotypic plasticity and explore phenotypic space could be advantageous to accomplish wound healing, differentiation, specialization or adaptation to conditions of stress. Future experiments with genetically engineered mice and stressed human tissues will be used to test these hypotheses.

5. Conclusions

- The CD73+CD90- population that enriches for ePS cells can be visualized in healthy human breast tissues by multiplex IHC.
- ePS cells plasticity is controlled by SON transcriptional network.
- Extracellular adenosine generated by CD73 can regulate SON expression and cellular plasticity of ePS cells by activating the PKA-dependent autocrine IL6/Stat3 pathway through the ADORA 2b receptor.
- The canonical Activin A and FGF2 pathways that are important for SON expression in human ESCs are also essential for SON expression in ePS cells.

- CD73/Adenosine signaling is a novel pathway that is essential for SON expression in human ePS cells but not in human ESCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Multiplex imaging identifies $CD73^{+}/CD90^{-}$ (R1) cells that enrich for ePS cells in human breast tissue
2. The CD73/Adenosine/AdoRa2b/Stat3 circuitry is required for SON expression in ePS cells but not in hESCs
3. Activin A and FGF2 pathways are also essential for SON expression in ePS cells
4. ePS cells enter a plastic state through a novel circuitry not utilized by hESCs

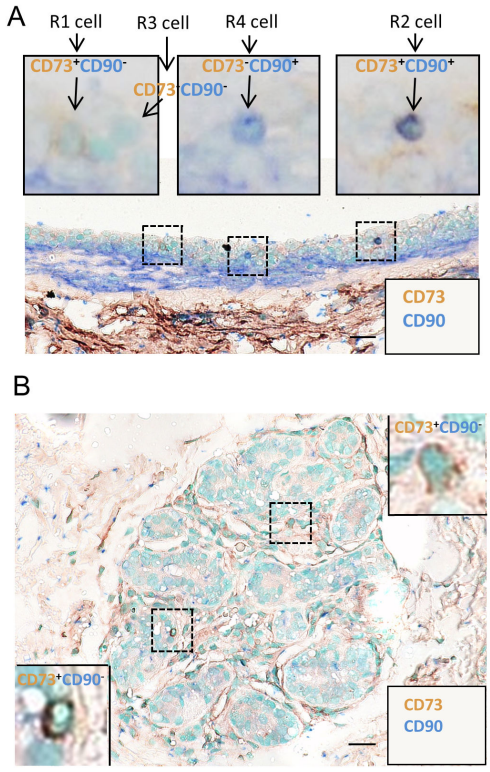

Fig. 1. Detection of candidate ePS cells *in vivo*. A

RM085 stained simultaneously with a monoclonal anti-CD73 antibody detected with a secondary antibody coupled to horseradish peroxidase and the chromogen, 3,3'-Diaminobenzidine (DAB) (brown) and with a polyclonal anti-CD90 antibody detected with a secondary antibody coupled to alkaline phosphatase and the chromogen, Vector blue (blue). Insets: examples of $CD73^{+}CD90^{-}$ (R1), $CD73^{+}CD90^{+}$ (R2), $CD73^{-}$ $CD90^{-}$ (R3) and $CD73^{-}CD90^{+}$ (R4) cells. **B.** RM179 stained as described for RM085 in (A). Scale bars: 20μm for A and B.

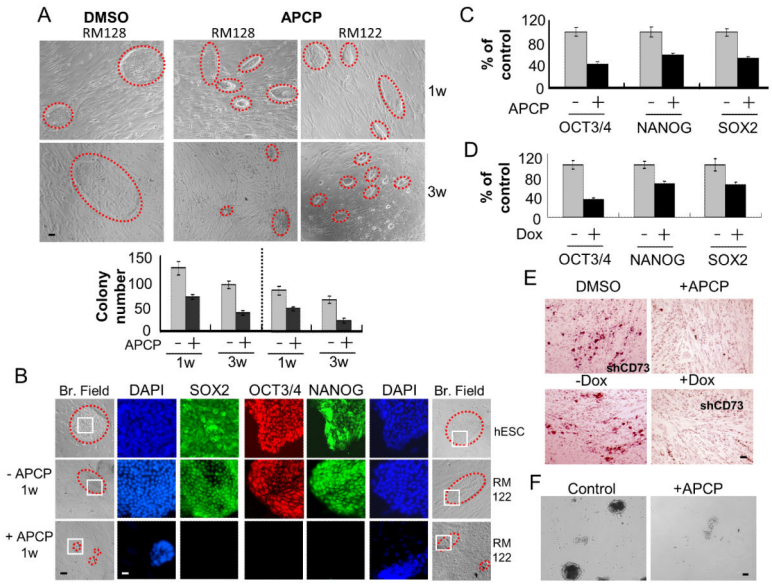

Fig. 2. CD73 is necessary for the plastic state of ePS cells

ePS colonies from RM122 and RM128 were treated with vehicle control (DMSO) or APCP and assessed for **A.** colony size (outlined in red dashed lines) and morphology (top panel) and number (bottom panel) after 1 week (w) or 3 w of treatment, n=2 and n=3, respectively; **B.** SON protein expression after 48h of treatment by immunocytochemistry along with hESC colonies (outlined in red dashed lines in corresponding bright field images) Scale bars: 10μm (n=2); Nuclei were stained with DAPI. Nuclear localization of SON in the areas outlined in white squares is shown at a higher magnification. Scale bars: 40μm. ePS cells cultured in F-FM and treated or not with APCP for 48 h were assessed for **C.** SON transcripts by qRT-PCR (n=2). Lentivirus carrying an inducible pTripZ shCD73 or scrambled shRNA were transduced into ePS cells from RM 122 or RM128 expanded for 21 days. Infected cells, selected with puromycin prior to and after doxycycline (Dox) induction, were assessed for **D.** SON transcripts by qRT-PCR. (n=3). **E.** Vehicle (DMSO)-treated, APCP-treated, CD73 knock-down (+Dox) and control (−Dox) ePS cells from RM122 or RM128 were subjected to adipogenic differentiation for 18 days and stained with Oil Red O (n=2). Scale bars: 10μm. **F.** Generation of embryoid bodies in ePS cells treated or not with APCP for 7 days (n=1 each). Scale bars: 10μm. Data from RM122 are shown in B, E and F.

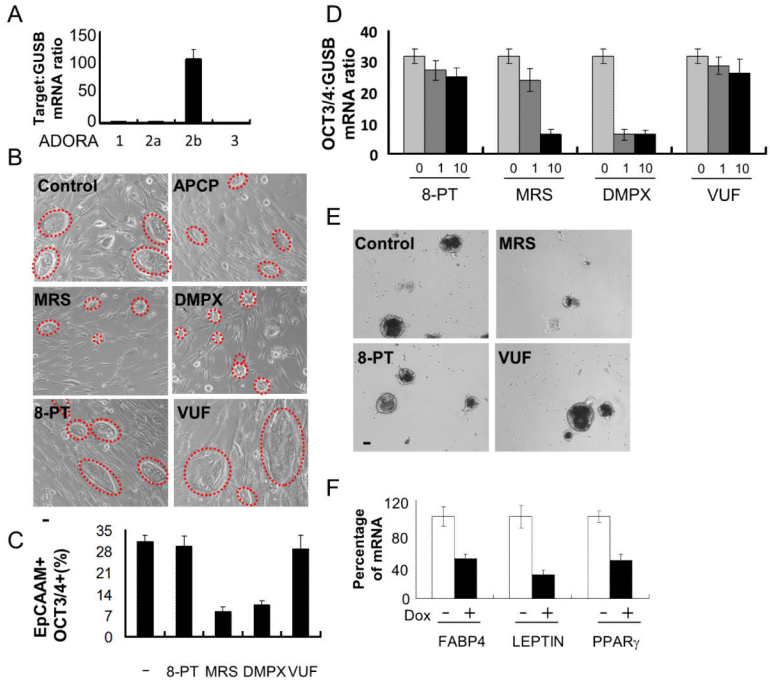

Fig. 3. ADORA2b receptor signaling mediates adenosine functions in ePS cells

ePS cells from RM136 and RM142, grown in F-FM (panels A, D and F) or on feeders (panels B, C and E), were assessed for **A.** ADORA1, ADORA2a, ADORA2b and ADORA3 transcript expression by qRT-PCR (n=3 each); **B.** size and morphology of ePS colonies (outlined with red dashed lines) treated or not with APCP and/or adenosine receptor-specific inhibitors (8-PT, MRS, DMPX or VUF; 10μm) for 7 days (n=2 each). Data from RM136 are shown. Scale bars: 10μm.; **C.** $EpCAM^+$ $OCT3/4^+$ cell fractions by FACS analysis. (n=2 each); **D.** OCT3/4 transcript expression by qRT-PCR in ePS cells treated or not with 8-PT, MRS, DMPX and VUF for 48 h (n=3 and n=6, respectively); **E.** generation of embryoid bodies treated or not with 8-PT, MRS and VUF for 7 days (n=2). Scale bars: 10μm.; **F**. adipogenesis at 14 days in ePS cells transduced with or without shADORA2b and induced or not with Doxycycline. Comparison of transcript expression of adipogenic differentiation markers FABP4, LEPTIN and PPARγ by qRT-PCR (n=3 each). Panels B and E show data from RM136.

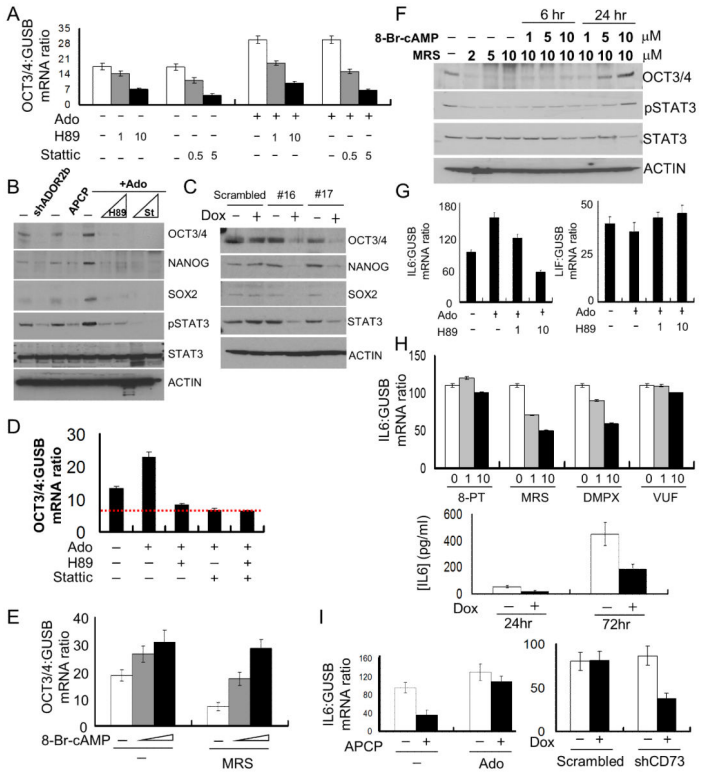

Fig. 4. The PKA/STAT3 signaling pathway mediates ADORA2b function in ePS cells

ePS cells from RM136 and RM159, grown in F-FM, were assessed for **A.** OCT3/4 transcripts by qRT-PCR in ePS cells treated or not with adenosine (Ado) in presence or absence of the PKA inhibitor H89 or the STAT3 inhibitor Stattic. (n=3 each). **B.** SON and STAT3 protein expression and phosphorylation status of STAT3 (p-STAT3) by WB in ePS cells with or without knock down of ADORA2b (shDORA2b), or treated with or without APCP for 48 h or treated with Ado for 48 h, in presence or absence of H89 (1 or 5μM) or Stattic (0.2 and 1μM) (n=1 and n=2, respectively). **C.** SON and STAT3 protein expression by WB after transduction of lentivirus carrying inducible shRNAs against STAT3 (#16 or #17) or a matched scrambled shRNA and induction by Doxycyclin for 48h (n=2 each). **D.** OCT3/4 transcripts by qRT-PCR in untreated ePS cells and ePS cells treated with Ado in presence or absence of H89 or Stattic, alone or various inhibitor combinations. Red dashed line: OCT3/4 expression in presence of H89, Stattic alone or their combination (n=3 each). **E.** OCT3/4 transcripts by qRT-PCR in MRS-treated or untreated ePS cells in presence or absence of the PKA activator 8-Br-cAMP (n=3 each). **F.** OCT3/4 protein expression and STAT3 phosphorylation status (phospho-STAT3) by WB in ePS cells treated or not with different concentrations of MRS-treated in presence or absence of different concentrations of 8-Br-cAMP (n=1 and n=2, respectively). **G.** IL6 and LIF transcripts by qRT-PCR in untreated or adenosine-treated ePS cells in presence or absence of H89 (n=3 each). **H.** IL6 transcripts by qRT-PCR in ePS cells treated or not with 10μm of 8-PT, MRS, DMPX and VUF (top panel). IL6 protein concentration by ELISA in CM collected from ePS cells with or without knock-down of ADORA2b (bottom panel) (n=2 each) **I.** IL6 transcripts by qRT-PCR in ePS cells treated or not with APCP in presence or absence of Ado or in ePS cells

transduced with a CD73 shRNA or a scrambled shRNA (n=3 each). In Panels B, C and F loading control: ACTIN and data from RM136 are shown.

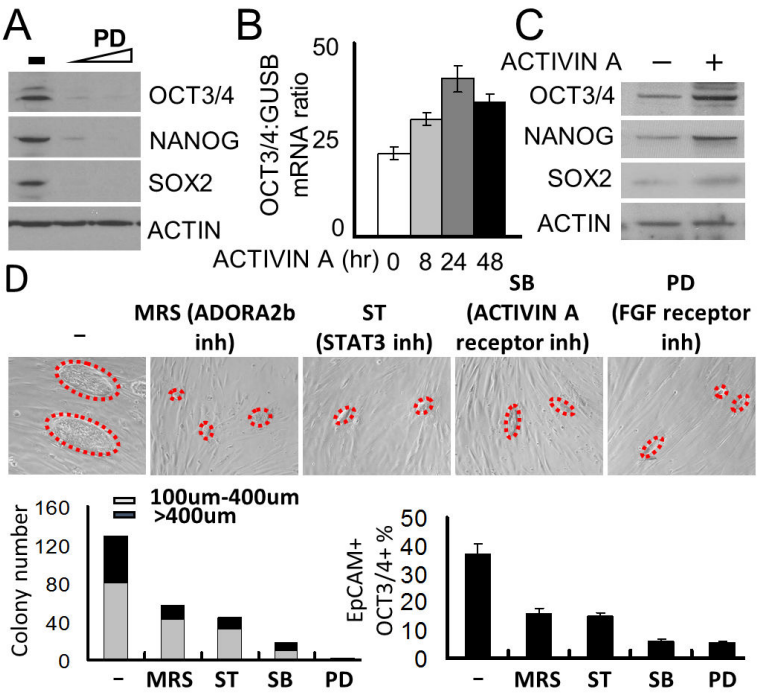

Fig. 5. FGF2 and ACTIVIN A pathways are essential for SON expression in ePS cells

ePS cells from RM165 or RM209, grown in F-FM, were assessed for **A.** SON protein expression by WB in ePS cells treated with PD173074 (FGFR inh, 50nM and 500nM) for 48h. (n=1 and n=2, respectively). Loading control: ACTIN. Data from RM209 are shown; **B.**OCT3/4 transcript expression by qRT-PCR (**B**) or protein expression by WB (**C**) in presence or absence of 5 ng ACTIVIN A for the indicated times (n=2 and n=1, respectively). Data from RM165 are shown. ePS colonies from RM172 and RM183, grown on feeders and treated or not with the indicated inhibitors for 7 days starting on day 3 after plating, were assessed for: **D.** size and number of ePS colonies (outlined with red dashed lines in corresponding bright field images - **bottom left**). Scale bars: 10μm. Data from RM172 shown. $EpCAM^{+}OCT3/4^{+}$ cell fractions by FACS analysis (**bottom right**) (n=2 and n=2, respectively).

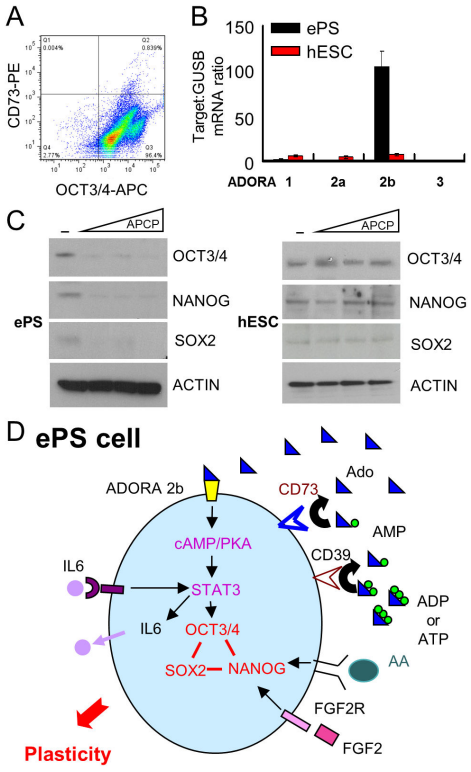

Fig. 6. Differential role of CD73 and ADORA2b in ePS cells compared to hESCs

hESCs (H7), grown on feeders, were assessed for **A.** OCT3/4 (x-axis) and CD73 (y-axis) expression by FACS analysis (n=3). **B.** adenosine receptor ADORA1, 2a, 2b and 3 transcript expression in ePS cells from RM142 or RM177 (n=3 each) by qRT-PCR. **C.** SON protein expression in ePS cells from RM142 or RM177 (n=3 each) by WB,in the presence or absence of increasing concentrations of the CD73 enzymatic activity inhibitor APCP. Data from RM142 are shown. **D.** Schematic representation summarizing the four extracellular factors (adenosine, IL6, STAT3 and FGF2) essential for SON expression and cell plasticity in ePS cells.

Table 1

Phenotypes that distinguish ePS cells from pluripotent ESC and iPSC as well as cancer cells

CHARACTERISTICS	***CELL TYPES***				
	ePS	**hESC**	**iPSC**	**pre-cancer**	**cancer**
Diploid karyotype	+	+	+	+/−	−
Benign teratomas	+	+	+	−	−
Pluripotency markers	+	+	+	−	+/−
Functional breast tissue	+	not determined	not determined	−	−
Immortality	−	+	+	+/−	+
High telomerase activity	−	+	+	50%	+
Malignant lesions	−	+/−	+/−	−	+

Table 2

Markers that distinguish ePS cells from differentiated cells

MARKERS	***CELL TYPES***							
	ePS	**luminal**	**myo-epithelial**	**MSCs**	**adipocytes**	**fibroblasts**	**MaSC**	**bone marrow-derived SC**
CD90	−	−	−	+	+	+	+	+
EpCAM	+	+	−	−	−	−	−	not determined
CD49f	+	−	+	+	−	−	+	+
CD73	+	−	−	+	−	−	−	not determined