The side population phenomenon enriches for designated adrenocortical progenitor cells in mice

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Abstract

Somatic adrenal stem cells are believed to reside in the periphery of the adrenal cortex throughout life for organ maintenance. Herein, we used the side population (SP) phenomenon to enrich for these progenitors, which made up to 0.01–0.64% of the total cell count. Microarray analysis revealed an expression profile of SP cells, which clearly differed from that of non-SP cells. However, a promising adrenal specific stem cell marker could not be identified. *In vitro*, SP cells could be maintained in long-term culture, whereas non-SP cells did not proliferate. After 4 weeks of culturing, immunohistochemistry revealed the expression of steroidogenic enzymes such as 3 β -HSD, StAR, and P450SCC, suggesting spontaneous differentiation. Interestingly, the quantity of SP cells was significantly diminished in

Phx1 haploinsufficient mice, suggesting a stem cell deficit. By contrast, the subcapsular zone of ACTH-deficient *Tpit*^{−/−} mice was significantly wider compared with wild-type adrenals (*Tpit*^{−/−} 259 ± 10.7 vs *Tpit*^{+/−} 100 ± 12.3%; *P* < 0.01). Accordingly, the number of SP cells in these mice was significantly higher (*Tpit*^{−/−} 0.45 ± 0.16 vs *Tpit*^{+/−} 0.13 ± 0.04%; *P* < 0.004). ACTH treatment of these animals reverted the subcapsular zone width and the SP fraction back to normal (130 ± 10.2%; *P* = 0.33 and 0.09%), providing indirect evidence for a stem cell ‘arrest’ in *Tpit*^{−/−} mice and the role of ACTH in adrenocortical stem cell modulation and differentiation.

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Introduction

According to the migration theory, each zone of the adult adrenal cortex is derived from a common pool of stem cells located in the periphery of the cortex, which, for organ maintenance and replenishment, migrates centripetally and populates the inner cortical zones upon differentiation (Kim *et al.* 2009, Wood & Hammer 2011). In fact, an increasing body of evidence supports this notion: first, tritiated thymidine tracing studies reveals centripetal migration of adrenocortical cells from outer to inner layers (Taki & Nickerson 1985). Secondly, after enucleation of rat adrenals leaving behind the capsule only, the adrenal cortex regrows and re-forms all three adrenocortical zones (Engeland & Levay-Young 1999). Thirdly, unilateral adrenalectomy leads to compensatory adrenal growth of the contralateral side (Engeland *et al.* 2005) with most of proliferation activity taking place within the subcapsular zone (Beuschlein *et al.* 2002, Lichtenauer *et al.* 2007, Schulte *et al.* 2007). Fourthly, recent studies on Wnt (Kim *et al.* 2008) and Shh (Ching & Vilain 2009) signaling within the adrenal cortex have

demonstrated particular pathway activity in the subcapsular region. Furthermore, Gli1 tracing shows centripetal displacement throughout the adrenal cortex (Huang *et al.* 2010). In addition, capsular Gli1-positive, Sfl-negative cells gave rise to Sfl-positive, steroidogenic enzyme expressing cells throughout the adrenal cortex, providing genetic evidence that differentiated cells derive from progenitors that reside underneath the capsule (King *et al.* 2009). Despite these advances in adrenal developmental biology, both identification of adequate adrenal stem cell markers and isolation or enrichment of adrenal stem cells, allowing further in depth characterization, have been unsuccessful thus far.

There is convincing data that isolating the side population (SP), which is based on Hoechst 33342 dye exclusion, leads to an enrichment of progenitor or stem cells in the hematopoietic system (Goodell *et al.* 1996) and multiple other tissues, including skin, muscle, liver, brain, lung, testes, endometrium, heart, and pituitary (Kim & Morshead 2003, Montanaro *et al.* 2003, Wulf *et al.* 2003, Martin *et al.* 2004, Meeson *et al.* 2004, Majka *et al.* 2005, Umemoto *et al.* 2006, Xu *et al.* 2011, Gremaux *et al.* 2012, Sandstedt *et al.* 2012).

This phenomenon is believed to be attributed to membrane-based pumps, in particular ABCG2 and MDR1 that are mainly expressed in stem and progenitor cells. The presence of these pumps renders cells capable of Hoechst dye exclusion, resulting in a distinct small population stained less intense 'by the side' upon FACS analysis (Goodell *et al.* 1996, Zhou *et al.* 2001). Interestingly, disruption of the sonic hedgehog pathway by cyclopamine has led to significant reduction of SP cells (Bar *et al.* 2007), and ABCG2 has been proposed as a regulator of sonic hedgehog signaling (Balbuena *et al.* 2011), further indicating an interaction between stem cell properties and the SP phenomenon.

Herein, in the absence of well-characterized adrenal stem cell marker genes, we employed the SP technique on mouse adrenals and investigated its suitability for enriching adrenocortical progenitor cells and for identifying adrenal specific stem cell markers. SP cells isolated were analyzed regarding their growth and self-renewal potential, expression pattern, and potential regulatory mechanisms.

Materials and Methods

Animal models

All experiments involving animals were performed in accordance with institutionally approved and current animal care guidelines. Animals were maintained under standard conditions of temperature (22 °C) and lighting (12 h light: 12 h darkness) and with food and water given *ad libitum*. Eight- to 24-week-old Bl/6 wild-type animals (C57Bl/6, Jackson Laboratory, Bar Harbor, ME, USA) were used for adrenal single-cell suspensions. Generation of *Pbx1*^{-/-} (Luo *et al.* 1994) and *Tpit*^{-/-} (synonym: *Tbx19*^{-/-}) animals (Pulichino *et al.* 2003) has been described elsewhere. *Pbx1*^{+/-} haploinsufficient and wild-type mice were obtained by breeding *Pbx1*^{+/-} mice with Bl/6 wild-type animals. *Tpit*^{+/-} animals, which have been shown to be indistinguishable from wild-type littermates (Pulichino *et al.* 2003), were used as controls for *Tpit*^{-/-} mice. For ACTH stimulation experiments, *Tpit*^{-/-} mice received 30 µg per injection of ACTH1–24 in a slow release formula (Synacthen depot, Novartis) i.p. once daily for 10 days. Thereafter, animals were killed and adrenals harvested and prepared for FACS analysis, as described below.

Adrenal preparation, single-cell suspension, primary cell culture, and culture conditions

For primary cultures of mouse adrenals, organs were cleaned of surrounding fat, connective tissue, and large blood vessels. Thereafter, tissue samples were minced into pieces smaller than 0.5 mm using a razor blade. Minced samples were transferred into 50 ml Falcon tubes, spun down at 600 g for 5 min, and rinsed twice with fresh PBS. Digestion was performed with 1 mg collagenase II (Biochrom, Berlin,

Germany) per milliliter of PBS at 37 °C for 50 min in a shaking water bath. Cell suspension was pipetted up and down at least twice during the incubation time. After digestion, pure FCS was added to a minimum concentration of 10% in order to inactivate the collagenase, followed by a centrifugation step as described earlier. Cell pellets were resuspended in erythrocyte lysis buffer and incubated for 7 min at room temperature. After another centrifugation step, cells were resuspended in 5–10 ml culture medium depending on the expected cell count (DMEM/F12 with 10% FCS, 3.1 g/l glucose, 15 mM HEPES, and 10 µl/ml Pen/Strep, all from Gibco) and sequentially filtered through a 100 and 70 µm nylon mesh respectively. Cells were counted using a Neubauer counting chamber and further processed.

Hoechst staining and FACS sorting

Murine single-cell suspensions were transferred into DMEM high-glucose medium (Gibco) containing 2% FCS and 10 mM HEPES and stained with the fluorescent dye Hoechst 33342 (Sigma) at a concentration of 5 µg/ml at 37 °C for 90 min as described (Goodell *et al.* 2005). After the staining procedure, propidium iodide (2 µg/ml; Sigma) was added to the samples for identification and exclusion of dead cells. Cell analysis and sorting were performed on a triple-laser cell sorter (MoFlo, Dako (formerly Cytomation), Fort Collins, CO, USA). The Hoechst dye was excited at 350 nm using an argon ion laser. Emission wavelengths were detected at 450 nm using a 450/20 bandpass filter and above 675 nm using a 675LP filter. The SP was defined as described (Goodell *et al.* 2005), including the verification procedure with verapamil (Sigma), with the only difference that special care was taken to exclusively isolate the tip of the SP cell fraction.

Microarray analysis

Microarray analysis was performed for SP vs non-SP adrenal cells of eight male Bl/6 wt mice. To ensure optimal cell quality and purity, FACS sort gates were set restrictively at the most representative areas of SP and non-SP populations. Taken into account the limited number of SP cells resulting from such stringent selection criteria, gene array analysis was performed on a platform, designed for very small cell numbers (Klein *et al.* 2005).

Data preprocessing was done in R using the limma package (Smyth & Speed 2003). Raw probe intensities were background corrected by applying the 'normexp' method. Only cy5 intensities were used. Loess normalization was used in M vs A plots of individual cy5 intensities of transcripts in one array and the median cy5 intensity across all arrays of the same transcript. Log2 ratios were calculated from the normalized intensities and quantile normalization was applied for normalization across arrays. All further analysis is based on normalized log-ratios.

The expression of genes was compared between the SP and non-SP samples by applying regularized linear models in a paired setting as implemented in the limma package

(Smyth 2004). Genes were considered to be significantly differentially expressed if their adjusted *P* value (corrected for multiple testing as proposed by Benjamini and Hochberg) was ≤ 0.05 . Each group contained eight samples.

cDNA synthesis and semiquantitative MC-2 receptor PCR

RNA from cell preparations was extracted using the Qiagen RNA mini kit (Qiagen) following the instructions of the manufacturer. cDNA was transcribed using a RT kit (Promega) and 1.0 µg total RNA. For PCR (Promega Taq), 10 pmol of each primer (MC-2 receptor: F, CTGCC-ACGAGGCTTAAGATAAC and R, GCCTGTCAAG-CATTAGTGACAA; nucleotides 663–1150; annealing temperature 58 °C) were used. PCR conditions were denaturated at 94 °C for 2 min, followed by 30 cycles of amplification (each consisting of denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, and extension for 40 s at 72 °C). A final extension for 5 min at 72 °C was included. The product was run and analyzed on a 1% agarose gel.

Culture conditions for adrenal SP cells

SP and non-SP cells were sorted directly into separate wells of 24-well plates (Sarstedt, Nürnbrecht, Germany). When the cells reached 80% confluency, cells were trypsinized and subsequently replated in 12-well plates (BD Falcon, Franklin Lakes, NJ, USA), six-well plates, and 10 cm dishes (Sarstedt) respectively. All cells were cultivated under the same culture conditions mentioned earlier and pictures were taken using a standard microscope (Leica DMRB).

Tissue staining and immunohistochemistry

Adrenal SP and non-SP cells were grown on coverslips (Becton Dickinson, Franklin Lakes, NJ, USA) for 1 week and fixated with PFA for at least 1 h. Tissue slides and coverslips were subsequently investigated by hematoxylin and eosin (H&E) staining. For immunohistochemical analysis, coverslips were blocked with 0.3% H₂O₂ in methanol for 10 min and incubated with blocking buffer for 15 min. Anti-P450Sc α , Star, and 3 β -Hsd primary rabbit antibodies (generously provided by Dr Walter Miller, UCSF, CA, USA) were incubated overnight at 4 °C after dilution in blocking buffer containing 3% BSA (Sigma), 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and 0.5% Tween 20 (Calbiochem, San Diego, CA, USA). After rinsing for 15 min in PBS, secondary antibody (goat anti-rabbit biotinylated IgG; Vector Laboratories, Burlingame, CA, USA) was applied for 30 min at RT. Bound primary antibody was detected using the VECTASTAIN Elite ABC Kit (Vector Laboratories) according to the manufacturer's protocol.

For localization studies, adrenals from C57Bl/6 adult wild-type mice were cut into half and incubated according to the SP staining protocol. Thereafter, adrenals were shock frozen in liquid nitrogen and sliced using a cryomicrotome. After 5 min of

PFA fixation and PBS washing, Yo-Pro-1 Iodine (Molecular Probes, Eugene, OR, USA) was added in a concentration of 10 nmol/ml for 3 h. Slides were subsequently analyzed using a standard fluorescence microscope (Leica DM2500).

Measurement of subcapsular zone width in Tpit animals

For morphological evaluation of adrenals from Tpit animals, sections were dehydrated, embedded in paraffin, sectioned, and stained with H&E following standard procedures. 3 β -HSD immunohistochemistry as described earlier and H&E-stained adrenal sections from heterozygous *Tpit*^{+/−} and *Tpit*^{−/−} mice were examined with a standard light microscope using 400× magnification and pictures were taken. The area beneath the organ capsule containing 3 β -HSD-negative cells was quantified using Image J Software (NIH, Bethesda, MD, USA) and compared between the groups. Morphometric analysis for quantification of the subcapsular zone width was performed on at least three individual animals per genotype as described earlier (Lichtenauer *et al.* 2007).

Statistical analysis

Statistical analyses of the microarray are described separately. All other results are expressed as mean $\pm$ S.E.M. Statistical comparisons were analyzed on the basis of Student's *t* and ANOVA tests using StatView 5 (SAS Institute, Inc., Cary, NC, USA). Statistical significance was defined as $P < 0.05$ and is indicated as an asterisk (*) in the figures.

Results

Identification of the adrenal SP

After successful identification of SP cells in murine bone marrow specimen (Fig. 1A) in accordance with the staining procedure originally described by Goodell *et al.* (2005), we adopted the technique for murine adrenocortical cell preparations. The adrenal SP fraction made up to 0.01–0.64% (mean $0.23 \pm 0.04\%$) of the total cell population and was reliably and reproducibly detectable (Fig. 1B). Unspecific staining was excluded by co-incubating with verapamil, which by blocking the responsible cell membrane channels prevents Hoechst dye exclusion and leads to a disappearance of cells within the predefined FACS gate (Fig. 1C). Double nuclear staining of wild-type adrenal glands with Hoechst 33342, according to the SP staining protocol, and YoPro revealed several cells in the periphery of the adrenal cortex that were clearly YoPro positive, but not, or barely positive for Hoechst 33342, possibly resembling adrenal SP cells (Fig. 1D).

Characterization of the adrenal SP

Microarray analysis was carried out with very stringent selection criteria for SP and non-SP cells. The expression

Figure 1 Side population (SP) staining. FACS analysis of Hoechst 33342-stained cells and identification of SP cells in wild-type murine bone marrow used as a positive control (A) and in wild-type adrenal glands (B and C). The proportion of SP cells was largely diminished upon co-incubation with verapamil (C). YoPro and Hoechst 33342 double staining renders several Hoechst-negative, YoPro-positive cells in the periphery of the adrenal cortex (D). Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0393>.

profile of the two cell populations was clearly different (Fig. 2A) and a classifier could be trained that separated the two groups without error. However, none of the genes analyzed was differentially expressed in a manner to be

considered a marker with potential value for detection of adrenocortical cells with stem cell properties. Nevertheless, *Fgf2* and *Gli1*, but also *Gli3*, *Shh*, and β -catenin tended to be upregulated, while *SF1* tended to be downregulated in

Figure 2 Characterization of adrenal side population (SP) cells. Heatmap of microarray analysis of SP vs non-SP cells with evident differential gene expression between the two cell populations (A). Semiquantitative PCR analysis reveals MC2 receptor expression in adrenal SP cells; adr., wild-type adrenal; liver, MC2 receptor-negative control tissue; H₂O, negative water control (B). FACS-sorted murine adrenal glands upon Hoechst 33342 staining after long-term *in vitro* culture indication proliferation of SP cells only (C). FACS re-analysis (D) of cultured cells and immunohistochemical staining for StAR, P450SCC, and 3 β -HSD (and control staining lacking primary antibody) of *in vitro*-cultivated SP cells (E). Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0393>.

Table 1 Microarray analysis. The table displays the most differentially expressed genes (adrenal SP vs non-SP cells). The most upregulated genes in SP cells are in normal font; the most downregulated genes in SP cells compared with non-SP cells are presented in boldface. In addition, the expression profile of genes associated with the adrenal subcapsular stem cell niche is depicted in (B)

LogFC	t	P value	Adj. P value	Gene id	Gene symbol	Description	Chromosome	Ref. Seq.
A								
2-07380	4-69188	0-00034	0-33554	ENSMUSG00000018932	Map2k3	Mitogen-activated protein kinase kinase 3	11:60748250-60769002	NM_008928
2-02257	4-73195	0-00031	0-33554	ENSMUSG000000019558	Slc6a8	Solute carrier family 6 (neurotransmitter transporter, creatine), member 8	X:69925870-69935222	NM_133987
1-93096	4-77483	0-00029	0-33554	ENSMUSG000000046561	Arsj	Arylsulfatase J	3:126355874-126432396	NM_173451
1-87056	4-57722	0-00042	0-33554	ENSMUSG000000059610	Olfir222	Olfactory receptor 222	11:59386977-59387933	-
1-85065	4-68976	0-00034	0-33554	ENSMUSG000000032387	Rbpms2	RNA binding protein with multiple splicing 2	9:65427654-65458534	-
1-82150	5-15199	0-00014	0-33554	ENSMUSG000000020828	Pld2	Phospholipase D2	11:70356359-70374305	NM_008876
1-78441	4-58903	0-00041	0-33554	ENSMUSG000000056486	Chn1	Chimerin (chimaerin) 1	2:73411584-73575937	NM_029716
1-75747	4-94551	0-00021	0-33554	ENSMUSG00000001739	Cldn15	Claudin 15	5:137252496-137260467	NM_021719
1-72152	4-67669	0-00035	0-33554	ENSMUSG000000033147	Slc22a15	Solute carrier family 22 (organic anion/cation transporter), member 15	3:101988015-102021742	NM_177729
1-70936	4-98094	0-00020	0-33554	ENSMUSG000000043943	Naalad2	N-acetylated alpha-linked acidic dipeptidase 2	9:18073423-18138540	NM_028279
1-64649	4-97038	0-00020	0-33554	ENSMUSG000000028532	Cachd1	Cache domain containing 1	4:100274611-100527152	NM_198037
1-46285	5-10281	0-00016	0-33554	ENSMUSG000000031073	Fgf15	Fibroblast growth factor 15	7:144705922-144710343	NM_008003
1-45748	4-76068	0-00030	0-33554	ENSMUSG000000000386	Nx1	Nyxovirus (influenza virus) resistance 1	16:97553052-97562402	-
1-27693	4-79900	0-00028	0-33554	ENSMUSG000000038975	Rabggb	RAB geranylgeranyl transferase, b subunit	3:153844673-153849697	NM_011231
1-34927	4-53578	0-00046	0-33554	ENSMUSG000000003541	Ier3	Immediate early response 3	17:35429737-35430969	NM_133662
1-41691	4-72057	0-00032	0-33554	ENSMUSG000000022370	Mrp13	Mitochondrial ribosomal protein L13	15:55364178-55387401	NM_026759
1-62572	4-73127	0-00031	0-33554	ENSMUSG000000075320	Scn3a	Sodium channel, voltage-gated, type III, alpha	2:65262303-65267867	XM_141275
1-67273	5-37513	0-00009	0-33554	ENSMUSG000000035042	Ccl5	Chemokine (C-C motif) ligand 5	11:83341978-83346713	-
1-68805	4-92496	0-00022	0-33554	ENSMUSG000000048534	Amica1	Adhesion molecule interacts with CXADR antigen 1	9:44830178-44858846	NM_001005421
1-72793	4-75173	0-00030	0-33554	ENSMUSG000000035042	Ccl5	Chemokine (C-C motif) ligand 5	11:83341978-83346713	NM_013653
1-75461	5-15518	0-00014	0-33554	ENSMUSG000000028358	Zfp618	Zinc finger protein 618	4:62451971-62620203	XM_143826
1-89923	4-89286	0-00023	0-33554	ENSMUSG000000031431	Tsc22d3	TSC22 domain family 3	X:135885901-135889549	NM_010286
1-93204	4-58435	0-00041	0-33554	ENSMUSG000000024610	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex)	18:60929217-60948821	NM_010545
2-15674	4-77307	0-00029	0-33554	ENSMUSG000000069515	Lyzs	Lysozyme	10:116681441-116686397	NM_017372
2-90727	5-30296	0-00011	0-33554	ENSMUSG000000036905	Ctqb	Complement component 1, q subcomponent, beta polypeptide	4:136152221-136158253	NM_009777
B								
1-199378	3-19805	0-00579	0-74890	ENSMUSG000000037225	Fgf2	Fibroblast growth factor 2	3:37503099-37596346	NM_008006
0-758613	2-21262	0-04234	0-82450	ENSMUSG000000025407	Gli1	GLI-Kruppel family member GLI1	10:126732838-126744538	NM_010296
0-563167	1-35901	0-19362	0-87592	ENSMUSG000000021318	Gli3	GLI-Kruppel family member GLI3	13:15254867-15517860	NM_008130
0-540747	1-56642	0-13746	0-85982	ENSMUSG00000002633	Shh	Sonic hedgehog	5:28787602-28797888	NM_009170
0-193668	0-66687	0-51468	0-93993	ENSMUSG00000006932	Ctnnb1	Catenin (cadherin-associated protein), beta 1	9:120782173-120809205	NM_007614
0-10593	0-34904	0-73177	0-97162	ENSMUSG000000024949	Sfi1	Splicing factor 1	19:6363945-6376425	NM_011750

SP cells. Genes with the highest expression differences between the two groups are depicted in Table 1 (normal font: upregulated in SP; bold fontface: downregulated in SP, in accordance with heatmap). Semiquantitative analysis revealed the expression of the MC-2 receptor in adrenal SP cells (Fig. 2B).

After FACS sorting and plating of murine adrenal cells in 24-well plates, SP cells repeatedly grew *in vitro* in an adherent manner over several passages, while non-SP cells adhered, but did not display any growth potential (Fig. 2C). FACS re-analysis of cultured SP cells demonstrated the presence of a mixture of both SP (18.65%; Fig. 2D) and non-SP cells upon culturing. Immunohistochemistry performed on SP cell cultures grown on coverslips for further 4 weeks revealed the expression of several steroidogenic enzymes such as 3 β -HSD, p450SCC, and StAR on the protein level (Fig. 2E). Immunohistochemical analysis of non-SP cells was not possible, as these cells did not replicate.

SP in *Pbx1* haploinsufficient mice

Pbx1 – in synergy with *Sfl* – plays a critical role for proper adrenal development (Zubair *et al.* 2006, Lichtenauer *et al.* 2007). *Pbx1* haploinsufficient mice have been demonstrated to have smaller adrenals and a lower proliferation rate of adrenocortical cells. We, therefore, compared the proportion of SP cells from normal murine adrenals with those of *Pbx1* haploinsufficient animals. Interestingly, FACS analyses consistently revealed a significant lower number of SP cells in *Pbx1*^{+/-} adrenals (0.10 $\pm$ 0.04%) in comparison with wild-type animals (0.32 $\pm$ 0.04%, *P* < 0.01; Fig. 3).

SP in *Tpit* knockout mice

Tpit (*Tbx19*) knockout animals have been used as a model of secondary adrenal insufficiency (Pulichino *et al.* 2003). Interestingly, in the absence of pituitary ACTH, *Tpit*^{-/-} animals displayed a significantly enlarged subcapsular zone in comparison with *Tpit*^{+/-} controls (259.3 $\pm$ 10.7 vs 100.0 $\pm$ 12.3%, *P* = 0.02; Fig. 4A). Likewise, the fraction of SP cells in those mice was significantly higher compared with wild-type animals (0.38 $\pm$ 0.07 vs 0.13 $\pm$ 0.02%, *P* < 0.01; Fig. 4B). I.p. application of ACTH in *Tpit*^{-/-} mice over a period of 10 days resulted in a normalization of the subcapsular zone width (130.1 $\pm$ 10.2%, *P* = 0.24; Fig. 4A) as well as the number of SP cells detectable (0.09%; Fig. 4B).

Discussion

Although great advances have been achieved in localizing and characterizing the adrenal stem cell niche and molecular pathways involved, it was not yet possible to identify suitable adrenal specific stem cell markers. Taken into account that these designated progenitors are not expected to express the generally accepted embryonic markers of pluripotent cells,

Figure 3 Side population (SP) cells in the context of *Pbx1* haploinsufficiency. Morphological examination upon H&E staining (A), representative FACS analysis (B), and quantification of SP cells (C) in wild-type animals (*Pbx1*^{+/+}) and heterozygous littermates (*Pbx1*^{+/-}), indicating a significantly smaller proportion of SP cells in the context of *Pbx1* haploinsufficiency. **P* < 0.05. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0393>.

there is a great variety of different progenitor marker candidates to be considered. This is further complicated by the fact that these genes are often differently expressed in different tissues. Furthermore, in the adrenal gland, even other genes might be of importance. In the absence of known adrenal specific stem cell markers, we took advantage of the fact that the SP phenomenon was found suitable to enrich for progenitor cells in a variety of different organs. Thereby, we aimed at investigating the suitability of the SP procedure for the enrichment and characterization of murine adrenocortical progenitor cells, possibly resulting in the identification of tissue-specific adrenal progenitor markers.

After establishing the SP technique on adrenocortical tumor cells (Lichtenauer *et al.* 2008), SP cells could be readily detected in mouse adrenals that made up to 0.01–0.64% of the total cell population. Furthermore, co-incubation of

Figure 4 Side population (SP) in *Tpit* knockout mice. *Tpit*^{-/-} mice present with a significantly enlarged subcapsular zone, which is devoid of 3β-HSD immunoreactivity. Subcapsular zone width of this can be normalized by ACTH treatment (A). These phenotypic alterations are paralleled by concomitant changes in the proportion of adrenal SP cells (B). **P* < 0.05. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0393>.

murine adrenal cells with the calcium channel blocker verapamil rendered SP cells undetectable upon FACS analysis. Adrenal SP cells, therefore, fulfilled in number and appearance the validation criteria established for other tissue preparations (Goodell *et al.* 2005). Interestingly, double staining of frozen adrenal sections with YoPro revealed several Hoechst-negative cells in the periphery of the adrenal cortex, possibly resembling the localization of the adrenal SP cells.

Although the SP method is well established for detecting and enriching progenitor cells or stem cells from the hematopoietic system and a variety of other tissues, great caution has to be taken in interpreting the results, as the positive identification of SP cells does not inevitably guarantee stemness. In fact, we could demonstrate earlier that SP cells within the adrenocortical tumor cell line NCIh295R have similar functional properties in comparison with non-SP cells (Lichtenauer *et al.* 2008). Thus, further thorough and tissue-specific evaluations should be pursued for the identification of alternative reliable stem cell markers.

In consequence, we employed whole genome microarray analysis on SP vs non-SP cells to discover potential adrenal specific stem cell markers. Very stringent selection criteria were applied to ensure the best possible separation of the two populations. Owing to the limited number of SP cells available, microarray analysis was performed on a platform optimized for small cell numbers (Klein *et al.* 2005). Although the expression profile of SP vs non-SP cells clearly differed, subtle expression changes could be found for sets of genes only, but not for individual genes, a mandatory prerequisite for the positive identification of suitable adrenal progenitor markers. However, genes known to be relevant for adrenal

stem cell maintenance, especially *Egf2* and *Gli1*, but also *Shh* and β -catenin, tended to be upregulated, while *Sf1*, as the major transcription factor for adrenal differentiation, seemed to be downregulated in SP cells. The finding that these genes were not significantly altered in SP cells could be attributed to the fact that SP cells still represent a rather heterogeneous cell population, with progenitor cells – although enriched – still being a minority, resulting in less differential expression of SP cells compared with non-SP cells. Along the same line, novel adrenal progenitor markers might have been underscored and overlooked upon microarray data interpretation.

The potential of adrenal SP cells to proliferate was assessed upon FACS sorting and *in vitro* cultivation in standard adrenal cell culture medium. As expected for a proposed cell population with stem cell-like properties, SP cells continued to grow over multiple passages within a period of several months, whereas non-SP cells could not be maintained following the same culturing conditions. This experiment foremost demonstrates the self-renewing capabilities of adrenal SP cells. In addition, the appearance of non-SP cells during long-term culturing also suggests asymmetric cell division which, together with the low proliferative potential of non-SP cells, might have resulted in the observed ratio of distinct cell populations.

When grown on a coverslip and subjected to immunohistochemistry, adrenal SP cells stained positive for a variety of adrenocortical markers such as steroidogenic enzymes, indicating spontaneous differentiation under *in vitro* culture conditions. Owing to the limited number of SP cells obtainable from five pooled mice adrenals (maximum of 5000 cells), *in vitro* expansion was a prerequisite for

immunohistochemistry, so that the analysis of earlier time points was technically not possible. We were not able to detect any relevant expression of embryonic stem cell markers (Oct4 and Sox2) on the basis of real-time PCR analysis (data not shown). This was not unexpected, as our cells were never exposed to media supplements or culture conditions routinely used for maintenance of embryonic stem cells, allowing a selection bias toward a less differentiated phenotype. Furthermore, well-characterized somatic cell populations, such as mesenchymal stem cells, also do not spontaneously express embryonic marker genes (Baddoo *et al.* 2003, Pochampally *et al.* 2004).

To investigate potential regulators of adrenocortical SP cells, we analyzed *Pbx1* haploinsufficient and *Tpit* knockout mice – the former characterized in detail by our group elsewhere (Lichtenauer *et al.* 2007). Adrenals in the context of *Pbx1* haploinsufficiency are smaller with concomitant lower proliferation and impaired adrenal function (Lichtenauer *et al.* 2007). Interestingly, compared with wild-type animals, the number of SP cells in *Pbx1*^{+/-} mice was significantly smaller, providing indirect evidence for a stem cell deficit in the context of *Pbx1* haploinsufficiency. By contrast, *Tpit*^{-/-} mice, which are ACTH deficient, present with an adrenal subcapsular zone, which is substantially wider in comparison with wild-type adrenals. This morphological feature has been reported for animals with disrupted ACTH signaling, i.e. ACTH receptor knockout mice (Chida *et al.* 2007), which also display an enlargement of the subcapsular zone. Based on the immunohistochemical analysis in *Tpit*^{-/-} mice, this zone is mainly populated by non-steroidogenic cells. Interestingly, this morphological feature is paralleled by a significantly higher number of SP cells in adrenals of *Tpit*^{-/-} mice in comparison with their wild-type littermates. Furthermore, ACTH treatment of knockout animals not only reverted the subcapsular zone width back to normal but also resulted in a reduction of the SP fraction to a level similar to that of wild-type animals. In contrast, higher levels of ACTH in *Pbx1*^{+/-} mice (Lichtenauer *et al.* 2007) might be involved in driving the adrenal stem cell pool toward differentiation reducing the number of dormant progenitors in the subcapsular zone. Taken together, these findings provide indirect evidence that ACTH, in addition to its well-known action on steroidogenesis, might also modulate adrenocortical stem cell maintenance and fate. In accordance with this theory, we found the MC2 receptor expressed in adrenal SP cells.

In conclusion, adrenal SP cells are likely to be located within the subcapsular stem cell zone, they are able to proliferate and self-renew. The expression profile, which is similar to non-SP, mainly population cells, suggests an enrichment of rather designated direct adrenocortical progenitors. Along the same line, the detection of typical steroidogenic enzymes in long-term *in vitro* cultured SP cells indicates spontaneous differentiation. Finally, ACTH is suggested to play an important role as a regulator for adrenal SP and progenitor cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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