Adrenal progeny and factors of adrenal differentiation

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To

My wife Julia,

My two children Mirja and Jesper,

And my parents

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I also would like to express my fullest gratitude towards my program mentors in Cologne, Prof. Dr. H. Abken, Prof. Dr. J. Brüning, and, not to be forgotten, Prof. Dr. J. Campos-Ortega. They always had time and an open ear for me. They did not only guide my through the PhD-program but also gave very fruitful suggestions from the outside on how to strengthen the data and about their opinion on how to continue. Regarding organizational aspects, Prof. Dr. Campos-Ortega strongly supported my externship in Ann Arbor, Michigan at the beginning of the program. Prof. Dr. Abken was my main supporter, who helped me to overcome the obstacles for continuing the MD-PhD program under my new mentor, Prof. Dr. Beuschlein in Freiburg, when my study group moved away from Düsseldorf and changed its research interests. Special thanks also to Dr. D. Grosskopf-Kroiher and Prof. Dr. M. Paulsson, who initiated and kept alive this wonderful MD-PhD program. They took care of numerous personal organizational problems, and are not getting tired to bring together the MD-PhD-students as a team, supporting each other by knowledge and experience exchange.

Besides these professional supporters, I would like to thank my family and my parents for their loving support and their understanding for the countless hours that I was not available for them due to the research activities, which tended to not obey to the regular working hours. Without them, completion of this thesis would not have been possible either.

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Overview of thesis projects, collaborations, and publications

All steps, tasks and experiments necessary for planning, realization, completion, and publication of the projects, which all contribute to this thesis work were carried out by myself, unless stated otherwise in ‘contribution of coauthors or collaborators’ under each of the following projects. Felix Beuschlein, senior author in all publications, my mentor, and principle investigator of our study group is not mentioned separately, as he was closely involved in all parts and aspects of the projects, which were carried out under his permanent supervision.

Project 1: Identification and characterization of the adrenal side population

Publication: The side population phenomenon enriches for designated adrenocortical progenitor cells in mice. **Lichtenauer U**, Shapiro I, Sackmann S, Drouin J, Scheele J, Maneck M, Klein C, Beuschlein F. J Endocrinol. 2012 Dec;215(3):383-91. doi: 10.1530/JOE-12-0393. Epub 2012 Oct 5

Contribution of coauthors or collaborators:

Jaques Droin: principal investigator, Montreal Canada, provided us with Tpit-Knockout animals, which we bred and used for the investigation of the role of ACTH in side population cells.

Jürgen Scheele, principal investigator, Freiburg, provided us with Pbx1 haploinsufficent animals, which we bred and used to characterize the influence of Pbx1 haploinsufficiency on side population cells.

Christoph Klein, principal investigator and Matthias Maneck, PhD student, Institut für Pathologie, Regensburg, carried out Microarray analyses on side population vs. non-side population cells, and helped us interpreting the data and provided us with the heatmap (Figure 2A of original publication). His established platform is especially designed for small cell numbers.

Simone Sackmann: graduate student, supported me with organ harvesting and intraperitoneal ACTH applications.

Igor Shapiro, lab technician of our research group, took over most of the maintenance work such as tagging, tailing and genotyping of mice, passaging of cell culture cells and regular cell culture medium, exchange. He also supported me with the immunohistochemistry staining procedure.

All coauthors contributed to their part of the Material & Method section, read the draft, made suggestions to improve the manuscript and proof-read the final version before submission.

My contribution to this project:

Except for the contributions of the collaborators listed above, the whole project was planned, carried out and published by myself under the supervision of my mentor Felix Beuschlein, as mentioned in the preceding introductory statement of the overview. In detail, the acquisition of the mice strains needed for our study as well as most of the breeding was organized by me including taking care of the licensing for animal testing. In addition, I also established the PCR genotyping assay that was required. I adapted the side population staining procedure for adrenal cells and it is now the standard protocol used by our laboratory. Thereafter, I carried out adrenal harvesting, preparation of adrenal single cell suspensions and staining procedures for all subsequent FACS and flow cytometric analysis. All of these staining duties were performed under my direct supervision while they were carried out by a technician of the Core Facility in Freiburg. Except for maintenance work, I performed all cell culture experiments including the production of cell culture photographs used in publication. With the support of our lab technician, Igor Shapiro, I also carried out all immunohistochemistry experiments used in publication as well. I established and managed the communication contacts required for the project with all the collaborators involved. All data, including data from collaborators, was collected, interpreted, as well as prepared for publication by me under the supervision of my principal investigator. I wrote all drafts of the manuscript, incorporating comments and corrections from all the coauthors involved, and was responsible for submitting the final version of the manuscript as well as handling all revisions and aspects of the rebuttal process.

Project 2: Cell Fusion as a model to define pluripotent and committed adrenal progenitor cells

Publication: not published

Contribution of collaborators:

Keith Parker, principal investigator, Jean D. Wilson Center for Biomedical Research, Southwestern University, Texas, USA, provided us with Sf1-Cre animals.

Hans Jörg Fehling, principle investigator, Institut für Immunologie, Universitätsklinikum Ulm, provided us with tdRFP reporter mice.

Martin Zenke, principle Investigator, Institut für Biomedizinische Technologien, RWTH Aachen, provided us with Oct4-GFP animals.

Michele Boiani, PostDoc, Max Planck Institute for Developmental Biology in Münster, helped us during a short research stay with SF1-tdRFP blastocyst generation to establish a primary embryonic stem cell culture. Michele Boiani also started the embryonic body cultures.

Igor Shapiro, lab technician of our research group, was involved in mouse breeding and maintenance work including tagging and tailing, embryonic cell culture (medium change, feeder cell preparations, passaging of ES cells).

My contribution to this project:

Except for the contributions of the collaborators listed above, the whole project was planned, carried out by myself under the supervision of my mentor Felix Beuschlein, as mentioned in the preceding introductory statement of the overview. In detail, the acquisition of the mice strains needed for our study as well as most of the breeding was organized by me including taking care of the licensing for animal testing. In addition, I also established the PCR genotyping assay that was required. I established embryonic stem cell line cultures and feeder cell preparations in our laboratory. I initiated the collaboration with the Max Planck Institute (MPI) for Developmental Biology and organized short research visits to Münster, where the embryonic primary SF1-tdRFP cell line was established together with Michele Bojani from the MPI. I had to harvest murine adrenal glands for this project, in order to obtain adrenal single cell suspensions for the fusion experiments, which I carried out with the support of our lab technician, Igor Shapiro. I designed and processed all real-time PCR experiments of this project. I established and managed the communication contacts required for the project with

all the collaborators involved. All data, including data from collaborators, was collected and interpreted by me under the supervision of my principal investigator. This project was supported by a LMU FOEFOLE grant, for which I successfully applied. In consequence, I had to manage the associated administrative tasks as the responsible investigator.

Project 3: Defining regulators of adrenal organogenesis and steroidogenesis

Publication: **Lichtenauer UD**, Duchniewicz M, Kolanczyk M, Hoeflich A, Hahner S, Else T, Bicknell AB, Zemojtel T, Stallings NR, Schulte DM, Kamps MP, Hammer GD, Scheele JS, Beuschlein F. Pre-B-cell transcription factor 1 and steroidogenic factor 1 synergistically regulate adrenocortical growth and steroidogenesis. Endocrinology. 2007 Feb;148(2):693-704. Epub 2006 Nov 2.

Contribution of coauthors or collaborators:

Mark Kamps, principle investigator, Department of Pathology (M.P.K.), University of California San Diego, School of Medicine, La Jolla, California, initiated the project of generating Pbx1 haploinsufficient animals (Figure 1A of the original publication).

Jürgen Scheele, principal investigator, Department of Internal Medicine, Universitätsklinikum Freiburg, originally generated Pbx1 haploinsufficient animals together with Thomas Zemojtel and Matteusz Kolanczyk, PostDocs and provided us with Pbx1 animals (Figure 1B – D)

Dominik Schulte, graduate student of our research group, started breeding Pbx1 animals in our facility and found evidence for smaller adrenal glands in Pbx1 haploinsufficient animals compared to wild type mice in preliminary experiments, before he left our research group.

Stephanie Hahner, endocrine fellow, Department of Medicine, Uniklinik Würzburg, carried out the hormonal measurements in mouse serum.

Andrew Bicknell, principal investigator, School of Animal and Microbial Sciences, University of Reading, Reading, United Kingdom, carried out the Hox in-situ hybridization experiment (Figure 2 of the original publication).

Andreas Höflich, principal investigator, Genzentrum, LMU München, performed the realtime PCR analyses of IgF1, IgF binding protein 1 and IgF1-receptor (Figure 4E of original Pbx1 publication).

Gary Hammer, principal investigator and Tobias Else, PostDoc, Department of Internal Medicine, Ann Arbor, Michigan, USA, carried out Pbx1 und Sf1 immunoprecipitation (Figure 6E of original Pbx1 publication).

Nancy Stallings, graduate student, Department of Internal Medicine, University of Texan, Southwestern, Texas, USA, provided us with a Sf1 artificial promotor.

All coauthors contributed to their part of the Material & Method section, read the draft, made suggestions to improve the manuscript, and proof-read the final version before submission.

My contribution to this project:

Except for the contributions of the collaborators listed above, the whole project was planned, carried out and published by myself under the supervision of my mentor Felix Beuschlein, as mentioned in the preceding introductory statement of the overview. In detail, the acquisition of the mice strains needed for our study as well as most of the breeding was organized by me including taking care of the licensing for animal testing. In addition, I also established the PCR genotyping assay that was required. I carried out adrenal gland harvesting and measuring, paraffin embedding, DNA, RNA, Protein extraction, cDNA synthesis, and blood collections. I performed the hormone stimulatory and restrain stress experiments and carried out the immunohistochemistry and, immunoblotting, used in the publication. I established and managed the communication contacts required for the project with all the collaborators involved. All data, including data from collaborators, was collected and interpreted by me under the supervision of my principal investigator. I wrote the manuscript draft, kept in contact with the coauthors, and carried out corrections, before the final draft was submitted.

Project 4: Defining the role of side population cells in adrenocortical carcinomas

Publication: **Lichtenauer UD**, Shapiro I, Geiger K, Quinkler M, Fassnacht M, Nitschke R, Rückauer KD, Beuschlein F. Side population does not define stem cell-like cancer cells in the adrenocortical carcinoma cell line NCI h295R. Endocrinology. 2008 Mar;149(3):1314-22. Epub 2007 Dec 6.

Contribution of coauthors or collaborators:

Klaus Rückauer, attending, Department of Neurosurgery, Universitätsklinik Freiburg, Martin Fassnacht, fellow, Medizinische Klinik Würzburg, and M. Quinkler, fellow, Medizinishce Klinik, Charité, Berlin provided us with human adrenal tissues for our studies.

Technical on-site support was provided by Klaus Geiger, lab technician, Core Facility, Universitätsklinikum Freiburg, for FACS experiments, and by Roland Nitschke, biologist and head of the Life Imaging Center, Universität Freiburg for laser confocal microscopy.

Dirk Engelbert, graduate Student, Department of Internal Medicine, Universitätsklinikum Freiburg, helped us with the FACS analysis and data interpretation of the cell cycle study.

Igor Shapiro, lab technician of our research group, was involved in cell culture maintenance work, helped to establish adrenal primary cultures and was involved in the side population staining procedure.

All coauthors contributed to their part of the Material & Method section, read the draft, made suggestions to improve the manuscript, and proof-read the final version before submission.

My contributions to this project:

Except for the contributions of the collaborators listed above, the whole project was planned, carried out and published by myself under the supervision of my mentor Felix Beuschlein, as mentioned in the preceding introductory statement of the overview. In detail, I adapted the side population staining procedure to human and mice cancer cells, e.g. by employing confocal microscopy time course experiments, I prepared adrenal single cell suspensions from human adrenal tumor entities. Later, I prepared and supervised all FACS and cytometric investigations and performed all cell culture experiments with the cell line NCI-h295R including chemotherapy treatments and proliferation assays. I performed all real-time PCR analyses and produced all photographs used in publication. I established and managed the communication contacts required for the project with all the collaborators involved. All data, including data from collaborators, was collected, interpreted, as well as prepared for publication by me under the supervision of my principal investigator. I wrote all drafts of the manuscript, incorporating comments and corrections from all the coauthors involved, and was responsible for submitting the final version of the manuscript as well as handling all revisions and aspects of the rebuttal process.

Abstract

Radioactive and transgenic tracing experiments indicate that the adult adrenal cortex is maintained by a common pool of stem cells, which reside in the periphery of the adrenal cortex in the subcapsular zone. For repopulation, adrenal progenitor cells migrate towards the organ center, where they differentiate within the different specialized adrenocortical zones and take over specific adrenocortical functions. However, isolation and detailed investigation of adrenal progenitor cells has been hampered by the lack of known marker genes. There is convincing data that utilizing the side population, which is based on Hoechst 33342 dye exclusion, leads to an enrichment of progenitor or stem cells in the hematopoietic system and multiple other tissues. In the absence of known stem cell marker genes, we employed the side population technique on adrenal cells. In contrast to non-side population cells, side population cells readily grew over several passages *in vitro*. Furthermore, after 4 weeks in culture, immunohistochemistry revealed steroidogenic enzyme expression, suggesting spontaneous differentiation. Microarray analysis was performed and showed that the two populations clearly differed on the mRNA level. However, none of the genes analyzed presented with a substantially higher expression level to be considered a valuable adrenal stem cell marker candidate.

Interestingly, the quantity of side population cells was significantly diminished in the context of Pbx1 haploinsufficiency. This transgenic mouse model is associated with smaller adrenal glands, a lower adrenal proliferation rate, and impaired adrenal function, suggesting a stem cell deficit in these animals. In contrast, $Tpit^{-/-}$ mice, which are ACTH-deficient, presented with a subcapsular zone width, which was significantly enlarged in comparison to wild type adrenals. Accordingly, the number of side population cells in these mice was significantly higher. ACTH treatment of these animals not only reverted the subcapsular zone width back to normal, but also resulted in a reduction of the side population fraction to a level

similar to that of wild type animals, providing indirect evidence for a stem cell ‘arrest’ in the state of ACTH deficiency. Overall, adrenal side population cells seem to be enriched with progenitor cells with a rather differentiated or designated phenotype.

To define the characteristics of multipotent versus somatic adrenal stem cells, a fusion model was established. Herein, PEG mediated cell fusion between murine adult adrenal and embryonic stem cells was performed, however, did not render the expected spectrum of progenitor cells.

The involvement of stem cell like cancer cells in adrenal tumorigenesis was assessed by identifying the side population in different human adrenal tumor entities and in the human adrenocortical carcinoma cell line NCI H295R. The latter were analyzed further and characterized, however, a tumor stem cell-like behavior could not be detected: NCI H295R side population cells had no greater contribution to tumor growth compared to the other cells and had no survival benefit upon exposure to chemotherapeutic substances, typically administered in this tumor entity. Therefore, identifying side population cells does not per se guarantee stemness, and should always result in further extensive investigations.

Zusammenfassung

In Untersuchungen mit Hilfe radioaktiver- und transgener Tracer konnte gezeigt werden, dass die adulte Nebenniere einen subcapsulären Stammzellpool aufweist, der für die Organplastizität hauptsächlich verantwortlich zu sein scheint. Für Regenerations- und Anpassungsvorgänge migrieren Zellen aus dem subcapsulären Bereich ins Organinnere, wo sie innerhalb der verschiedenen spezialisierten adrenocorticalen Zonen differenzieren und endokrine Organfunktionen übernehmen. Die Isolierung und Charakterisierung der Nebennierenstammzelle ist allerdings bisher nicht gelungen, nicht zuletzt aufgrund fehlender bekannter Nebennierenstammzellmarker. Es kann angenommen werden, dass durch die Gewinnung der „Side Population“ (SP) aus Knochenmark und vielen anderen Geweben adulte Stammzellen angereichert werden können. Wegen fehlender Stammzellmarker passten wir die Methode an Nebennierenzellen an und konnten zeigen, dass SP-Zellen – im Gegensatz zu nicht-SP Zellen –über viele Passagen hinweg *in vitro* kultiviert werden konnten. In SP-Zellkulturen konnte darüber hinaus nach 4 Wochen die Expression typischer Steroidenzyme immunhistologisch nachgewiesen werden, was auf eine spontane Differenzierung hindeutet. Auf der Suche nach weiteren, nebennierenspezifischeren Stammzellmarkern führten wir eine genomweite Microarray Analyse an SP und Nicht-SP-Zellen durch. Obwohl sich die beiden Populationen in Bezug auf ihr Expressionsmuster unterschieden, ließen sich keine Gene ermitteln, die als potentielle Nebennierenstammzellmarker in Frage gekommen wären.

Transgene Mäusen, die haploinsuffizient für den Transkriptionsfaktor Pbx1 sind, zeichnen sich u.a. durch kleinere Nebennieren mit einer geringeren Proliferationsrate und beeinträchtigter Nebennierenrindenfunktion aus. Interessanterweise fand sich in diesen Tieren ein deutlich reduzierter adrenaler SP-Zell Anteil, so dass hier möglicherweise ein Stammzelldefizit vorliegen könnte. Bei ACTH-defizienten Tpit$^{-/-}$ Mäusen hingegen war die subcapsuläre Zone im Vergleich zu Wildtyptieren deutlich verbreitert. Passenderweise war

der Anteil der adrenalen SP-Zellen in diesem Fall deutlich erhöht. Eine ACTH-Langzeittherapie der Tiere normalisierte nicht nur die Breite der subcapsulären Zone, sondern reduzierte auch die SP-Zellfraktion auf ein mit Wildtyptieren vergleichbares Niveau, was indirekt auf einen „Stamzellarrest“ in Tpit$^{-/-}$ Tieren hinweisen könnte. Aufgrund dieser Daten gehen wir zusammenfassend davon aus, dass es sich bei Nebennieren SP-Zellen am ehesten um schon recht differenzierte direkte oder designierte Vorläuferzellen handelt.

Um somatische Progenitorzellen und multipotente Stammzellen in der Nebenniere identifizieren, unterscheiden und charakterisieren zu können, wurde ein komplexes PEG-basiertes Fusionsmodell etabliert. Leider zeigten aber die resultierenden Zellen und Fusionshybride nicht das gewünschte Spektrum an unterschiedlich differenzierten Stammzellen.

Des Weiteren wollten wir wissen, ob SP-Zellen als Tumorstammzellen in humanen Nebennierentumoren und der adrenocorticalen Zelllinie NCI H295R eine Rolle spielen könnten. Insbesondere die Zelllinie wurde detailliert untersucht, stammzellartige Eigenschaften konnten jedoch, trotz eindeutig identifizierbarer SP-Zellen, nicht gefunden werden: NCI H295R SP Zellen wuchsen nicht schneller und hatten, inkubiert mit diversen gebräuchlichen Chemotherapeutika, keinen Überlebensvorteil gegenüber Nicht-SP-Zellen. Es muss geschlussfolgert werden, dass der Nachweis von SP-Zellen das Vorhandensein von Stammzelleigenschaften nicht verlässlich vorhersagen kann und die isolierten Zellen stets einer genaueren funktionellen Untersuchung unterzogen werden müssen.

Introduction/Background

Adrenal insufficiency and impaired steroidogenesis require life-long replacement therapies, and are associated with considerable morbidity [4,5]. Acute deficiency of glucocorticoids – 'Addison crisis' – is a common, dangerous threat for patients suffering from restricted adrenal function. Due to the rarity of the disease, those episodes often remain undiagnosed at first, leading to unnecessary and improper treatments, before the right diagnosis eventually is found. Furthermore, patients with adrenal insufficiency have a substantially lower quality of life compared to healthy controls [6]. For these patients, stem cell based treatments and replacement therapies should mean major advances. To achieve this goal, defining the adrenal stem cells, the stem cell niche and the regulatory mechanisms involved are prerequisites.

As the structure of the adrenal gland divides the adrenal cortex in three distinct zones, and adaptive processes seem originate exclusively from a common pool of stem cells residing underneath the adrenal capsule, the adrenal gland is predestinated as a model to study stem cell regulatory and differentiation mechanisms. Furthermore, the adrenal gland is hormonally active and part of the hypothalamus-pituitary-adrenal axis, which easily allows monitoring of cell function by measuring hormone concentrations and their influence on the regulatory system. Studying the adrenal gland thus offers advantages over other organ systems in stem cell research field, despite the fact, that adrenal diseases are rare and research ambitions in this area are limited.

1. Embryonal development:

During organogenesis, the adrenogenital primordium is formed, when coelomic epithelial cells migrate into the neighboring mesenchyme [7,8]. The adrenogenital primordium eventually separates, and the cranial migrating cells differentiate into adrenal precursor cells, forming the fetal zone of the adrenal cortex (human gestational week 4). Soon thereafter, cortisol production under ACTH regulation begins (human gestational week 8 – 9) [9]. The definitive zone of the adrenal cortex is build up, once mesothelium mesenchyme migrate into the mesenchyme and surround the forming cortex (human gestational week 22 – 24) [10]. Temporarily, a third zone, called transitional zone, develops between the fetal and definitive zone after midgestation. Simultaneously, early cells of the sympathic nervous system, originating from the neural crest, migrate into the center of the developing adrenal gland where they differentiate into chromaffine adrenal medullary cells [11]. In the second trimester, the adrenal fetal zone makes up 80 – 90 % of the total adrenocortical cell mass, however vanishes quickly shortly after birth by differentiation and apoptosis. Zona glomerulosa and Zona fasciculata are now present, but the buildup of the Zona reticularis will take another 3 years of time. The zonation process of the adrenal cortex with its 3 distinct zones will not come to completion until shortly before puberty. It results in the well-known classical adrenocortical structure with an outer Zona glomerulosa, where mineralocorticoid production takes place, a Zona fasciculate with its glucocorticoid production, and the Zona reticularis with DHEA synthesis (Figure 1).

2. Somatic stem cells

Organ plasticity persists throughout life in the adrenal gland, necessary for adaption processes and organ maintenance. According to the 'migration theory' these tasks are

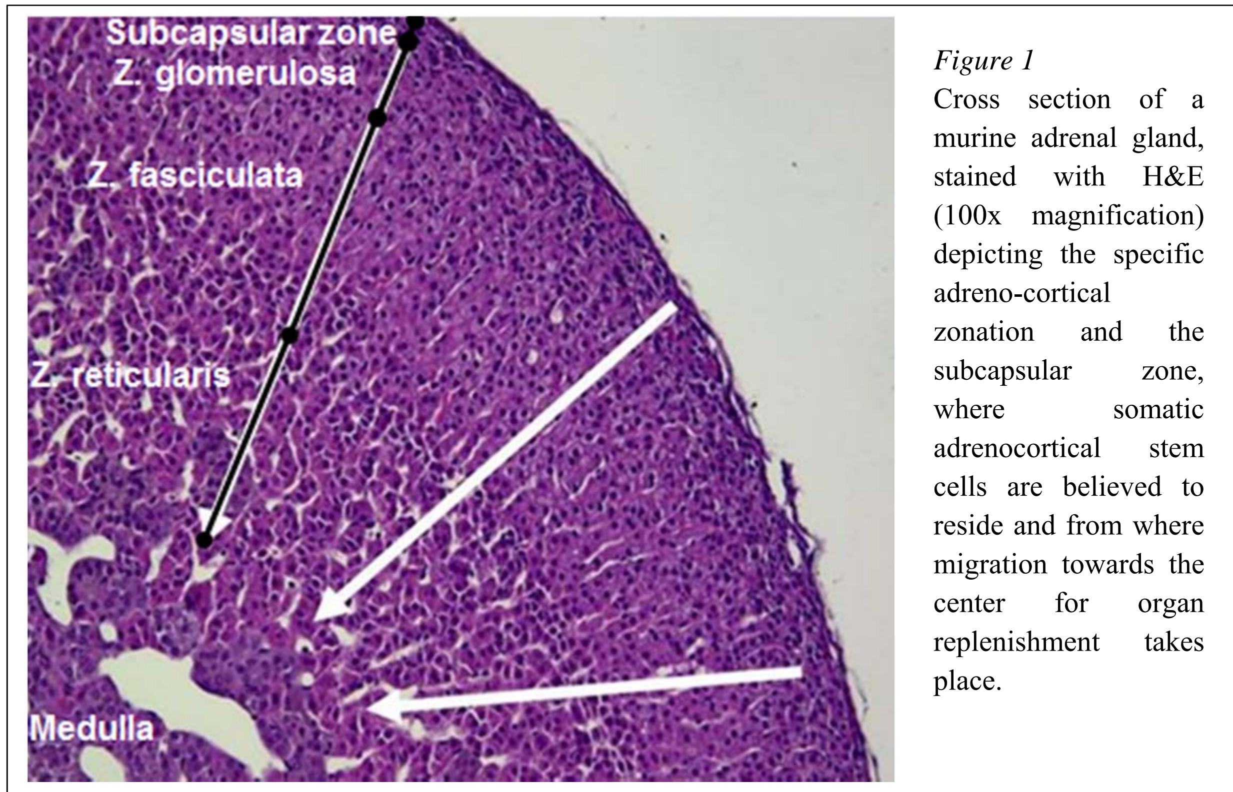

Figure 1
Cross section of a murine adrenal gland, stained with H&E (100x magnification) depicting the specific adreno-cortical zonation and the subcapsular zone, where somatic adrenocortical stem cells are believed to reside and from where migration towards the center for organ replenishment takes place.

believed to be carried by a somatic stem cell pool, which has its niche directly underneath the adrenal capsule (Figure 1). From here, the cells migrate radially towards the center of the adrenal gland and differentiate within the zone at need [3]. Somatic stem cells generally are believed to proliferate by symmetric and asymmetric cell division allowing the adjustment of the total number of persisting somatic stem cells as well as the number of differentiating cells (Figure 2A).

Several observations support the migration theory concept:

- In tritiated thymidine tracing studies, centripetal migration of adrenocortical cells from outer to inner layers was found[12].
- LacZ expression under a 21-hydroxylase[13] and a side chain cleavage enzyme promotor [14] in transgenic animals revealed a adrenocortical

staining pattern, suggesting that cells from inner adrenocortical zones originate from the subcapsular region, and that repopulation takes place in a 'radially variegated pattern', as the cells in each 'radial stripe' have a common clonal origin.

- The adrenocortical cortex reformed to some extend after enucleation of rat adrenals leaving behind the adrenal capsule only [15,16].
- unilateral adrenalectomy led to compensatory adrenal growth on the contralateral side [17]. Most of the proliferation activity was taking place within the subcapsular zone [2,18].
- Stem cell typical signaling, such as active Wnt- and Shh- pathways, were virtually exclusively present in the subcapsular zone. When silenced, proper adrenal development was disrupted [19-21]
- Genetically marked, originally subcapsular Gli1 positive cells, were found differentiated and steroidogenic enzyme expressing scattered throughout the adrenal cortex[20].

There is now evidence, that Gli1 positive, Sf1 negative cells might represent long-lived non-steroidogenic adrenocortical subcapsular progenitor cells, while Gli1 positive, Sf1 positive cells are more differentiated and centripetally displaced throughout the adrenal cortex [22].

Several marker genes could be identified for the definite zone (NovH, metallopanstimulin, CD56) and the fetal zone (P-Glycoprotein, low density lipoprotein (LDL) receptor) during embryogenesis [23,24], and, with limitations, for the Zona glomerulosa in the adult adrenal gland (Dab2, CD56) [25,26].

Despite all these advances in adrenal developmental biology, the identification of adequate adrenal stem cell markers allowing the isolation or enrichment of adrenal stem cells for further in depth characterization had been unsuccessful thus far. As the fetal zone marker P-Glycoprotein is a product of the multidrug resistance gene 1, this finding helped to set base for the side population studies described below.

3. Adrenal stem cell regulation

Similar to marker genes, a few factors driving and regulating adrenal differentiation have been identified thus far. Besides its well accepted role as a regulator of P450 enzymes of steroidogenesis [27], the transcription factor and putative orphan nuclear receptor Sf1, was found mandatory for the initiation of the adrenal primordium and its further development, as Sf1 knock-out mice present with a complete agenesis of adrenal glands and gonads [28,29]. Dax1, another member of the orphan nuclear receptor superfamily, suppresses SF1mediated transactivation, however, shows a similar cellular distribution in highly proliferating areas of the adrenal cortex [30,31]. In humans, DAX1 dysfunction leads to X-linked adrenal hypoplasia [32,33]. Other transcription factors involved in adrenal development include pre-B cell leukemia transcription factor 1 (Pbx1) [34], Wilms tumor 1 (Wt1) [35,36], and CBP/p300-interacting transactivator with ED-rich tail 2 (Cited2) [36,37]. The latter acts as a Wt1 cofactor and boosters Sf1 transcription. Recently, the importance of secreted signaling mechanisms, such as the canonical Wnt [19,38] and Shh [20-22] signaling pathways, was described. In accordance with the orphan nuclear receptors, these factors are also indispensable for proper adrenal development (Table 1; for review: [39]).

Mutant Gene human (mouse)	Function	Phenotype human (mouse KO)	Adrenal morphology human (mouse KO)	Reference
SF1 (Sf1)	Transcription factor, NHR superfamily	XY sex reversal (Lethal, gonadal aplasia and dysgenesis of ventromedial hypothalamus)	Adrenal insufficiency, adrenal hypoplasia (aplasia)	[29,40-42]
DAX1 (Dax1)	Transcription factor, NHR superfamily	XY sex reversal (Male infertility)	Adrenal hypoplasia, persistence of fetal zone, adrenal insufficiency (no X-zone regression)	[43,44]
(Pbx1)	Transcription factor	(lethal, adrenal agenesis)	(Adrenal aplasia)	[34]
(Cited2)	Transcription factor, cofactor	(Cardiac malformations, neural crest defects)	(Adrenal aplasia)	[37]
(Shh)	Morphogen		thin capsule and small cortex, reduced adrenocortical proliferation	[20,22]

Table 1: Overview of factors involved in adrenal development (adopted from Else et al.[45])

However, the individual roles of these factors for adrenal differentiation, or initiation of zonation, or regression of the fetal zone, are still not well understood.

4. The cancer stem cell concept

It is intriguing to speculate that a small subset of cancerously transformed progenitor cells could be responsible for most of the tumor growth in a given tumor entity. As this progenitor could give rise to a variety of differentiated tumor daughter cells with diverse phenotypes, present therapeutic approaches aiming at tumor mass reduction to the greatest possible extent will fail to achieve long-term remissions, as residing malignant progenitors will eventually lead to recurrence of disease (Figure 2B). Furthermore, as progenitor cells seem to be equipped with cell membrane based pumps capable of cytotoxic drug exclusion, stem cell-like cancer cells are proposed to have the ability to withstand higher concentrations of cytotoxic agents used in standard chemotherapeutical regimens [46-48].

Besides a cancerously transformed progenitor cell, alternatively, a differentiated cell could regain stem cell characteristics with the same clinical and biological consequences [49].

This concept, which has first been established in hematological malignancies such as acute myeloid leukemia, was first described in solid breast cancer tumors by Al Hajj et al. Herein, $CD44^{pos}$ and $CD24^{neg}$ cells, which accounted for approximately only 0.1 % of the total tumor population, were able to regrow tumor in breast pads of SCID mice, while all other tumor cells transplanted in equal numbers on the contralateral side did not have that potential [50]. By now, tumor cells with stem-cell-like properties have now been described for a variety of solid tumors including, neuroblastomas [51], different brain tumors [52,53], head and neck squamous cell carcinomas [54], colon carcinomas [55], and pancreatic cancer [56]. Depending on the tumor type, different stem cell associated markers were utilized for the isolation of stem cell-like tumor cells. Common markers besides CD44/CD24 include CD133 (prominin), c-kit, Sca-1, sphere forming ability and the side population phenomenon.

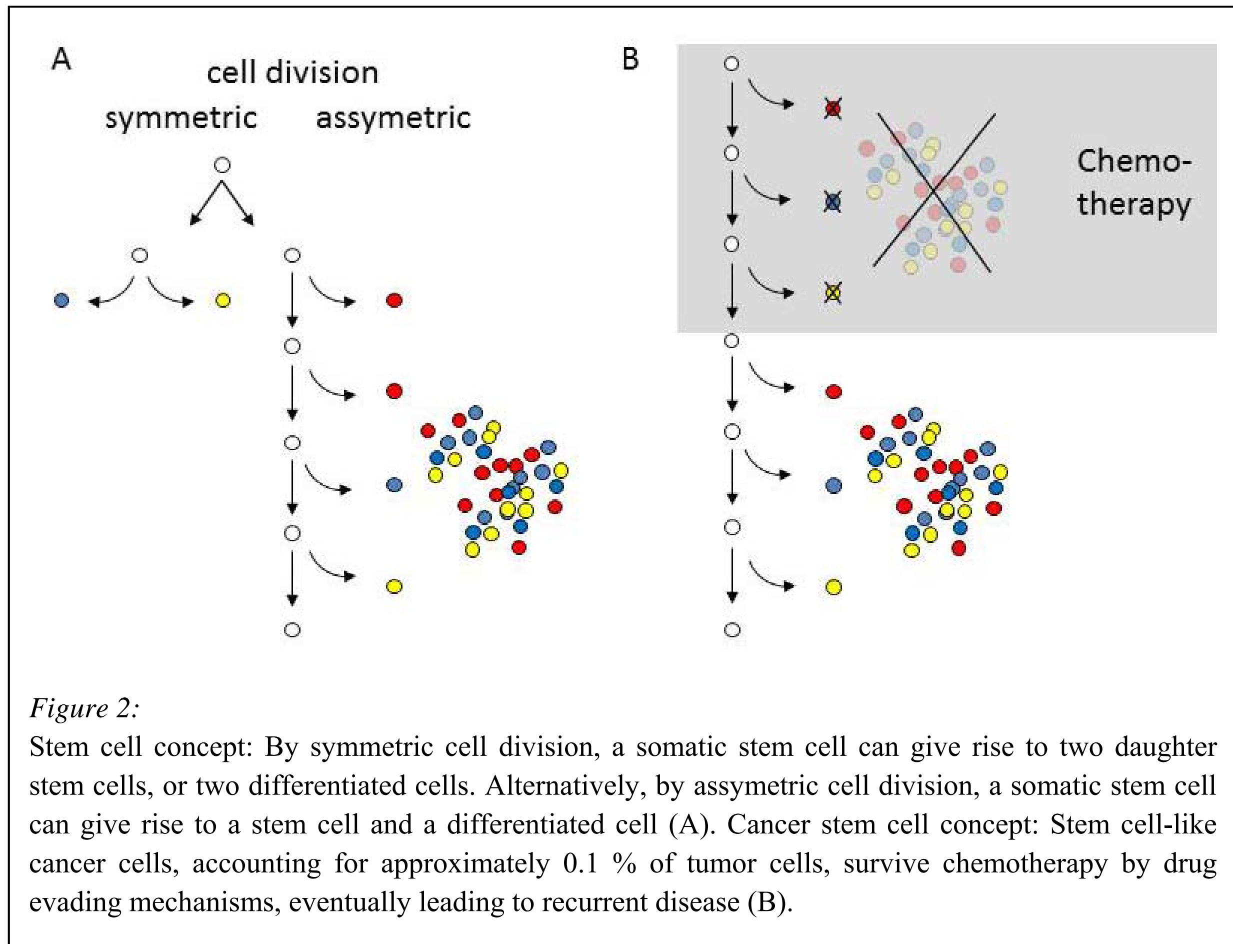

Figure 2:
Stem cell concept: By symmetric cell division, a somatic stem cell can give rise to two daughter stem cells, or two differentiated cells. Alternatively, by assymetric cell division, a somatic stem cell can give rise to a stem cell and a differentiated cell (A). Cancer stem cell concept: Stem cell-like cancer cells, accounting for approximately 0.1 % of tumor cells, survive chemotherapy by drug evading mechanisms, eventually leading to recurrent disease (B).

General accepted criteria for a stem cell-like cancer cell include [49,57-59]:

- ability to self-renew
- ability to re-grow the original tumor (e.g. in xenograft *in vivo* models) with
- recapitulation of most or all more differentiated cell types within an individual tumor
- extensive proliferative capacity

Specifically targeting stem cell-like cancer cells could potentially eradicate the cellular origin of a stem cell driven tumor and cure the disease, even when the rest – in this case the majority – of tumor cells are left in place. This pathophysiological new concept has aroused considerable clinical interest. However, most isolation methods used today lead to cancer stem cell enriched heterogeneous and impure cell populations, limiting the advances in

specifically targeting stem cell-like cancer cells. However, even if this obstacle can be overcome, and a cancer stem cell treatment was found, it remains unclear what consequences such a treatment would mean for physiologic stem cells in other organ systems.

Own Contributions

1. Adrenal side population enriches for adrenal progenitor cells [3]

One approach for progenitor or stem cell enrichment is the side population isolation method. It is based on Hoechst 33342 dye exclusion mediated through cell membrane based pumps, resulting in a distinct small cell population stained less intense accounting for approximately 0.1 % of the total cell number 'by the side' upon FACS analysis with an ultraviolet laser [60,61]. The responsible membrane based pumps seem to be primarily encoded by the multidrug resistance genes Abcg2 and Mdr1, since suppression of these genes leads to fading or disappearance of the side population. Stem cells and premature cells are known to express multidrug resistance genes on their cell surface, allowing the side population phenomenon to be utilized for stem cell enrichment. This was tried first successfully with mouse bone marrow cells [61]. Later, based on this method, potential progenitors could successfully be isolated from multiple other tissues, including skin, muscle, liver, brain, lung, testes, endometrium, heart, and pituitary [62-71]. Several observations made investigating the side population in the adrenal gland particularly interesting:

- Disruption of the sonic hedgehog pathway – which was found an important regulator of adrenocortical development – by cyclopamine, led to a significant reduction of side population cells [72]
- Abcg2 has been proposed a regulator of sonic hedgehog signaling, [73] further indicating that cells isolated by Hoechst 33342 exclusion are involved in stem cell and progenitor cell signaling.
- MDR1 was found to be a marker for the adrenal fetal zone

Hence, in the absence of adrenal stem cell markers, we adopted the side population protocol according to our needs to investigate the suitability of this procedure to enrich for murine adrenal progenitor cells.

Side population cells could readily be detected in mouse adrenals and accounted for 0.01 – 0.64 % of the total cell population (Figure 3A). As expected for a proposed cell population with stem cell like properties, side population cells continued to grow over multiple passages and over a period of several months, whereas non-side population cells exposed to the same culture conditions adhered to the culture plate but never grew to a

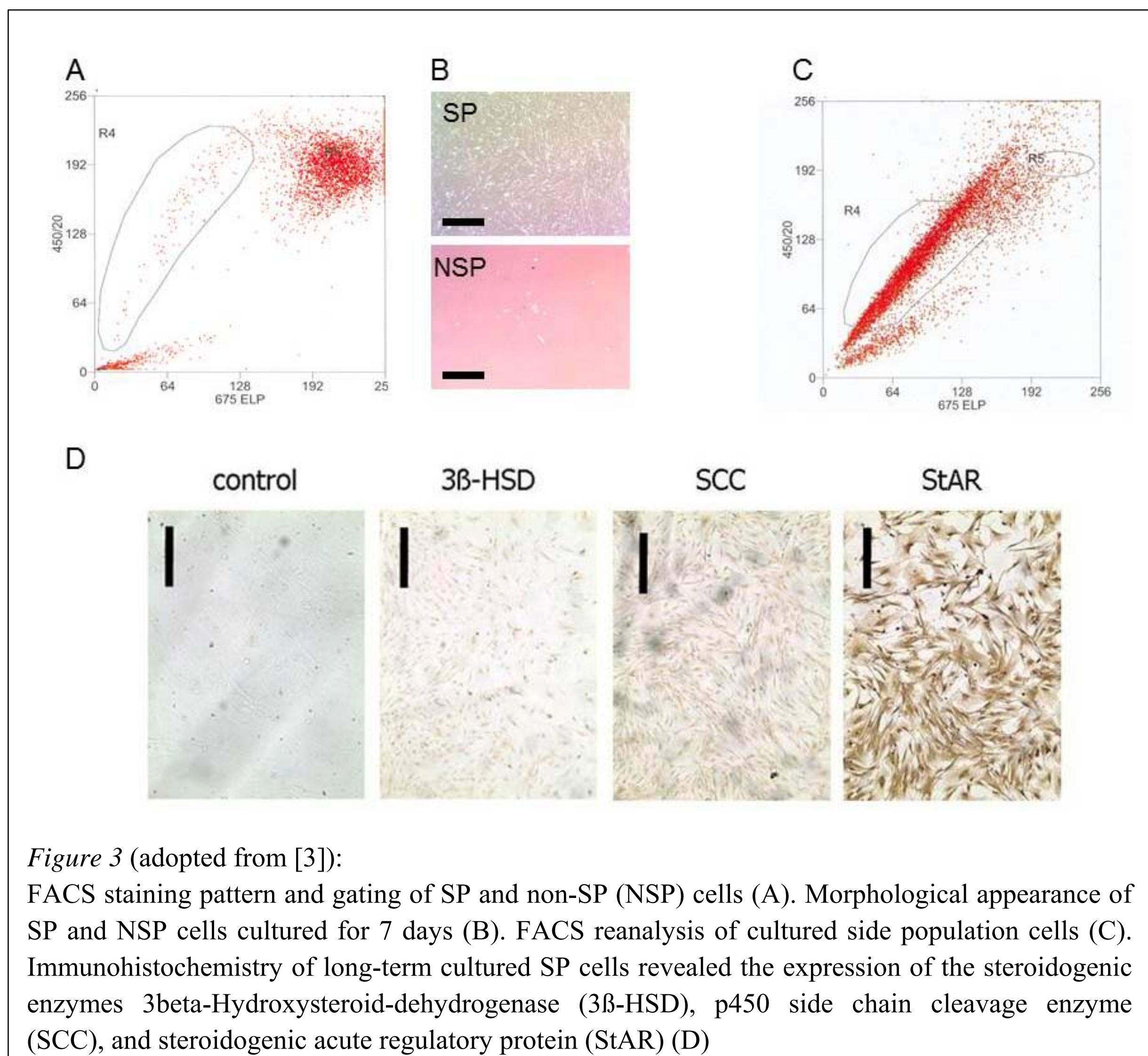

Figure 3 (adopted from [3]):
FACS staining pattern and gating of SP and non-SP (NSP) cells (A). Morphological appearance of SP and NSP cells cultured for 7 days (B). FACS reanalysis of cultured side population cells (C). Immunohistochemistry of long-term cultured SP cells revealed the expression of the steroidogenic enzymes 3beta-Hydroxysteroid-dehydrogenase (3ß-HSD), p450 side chain cleavage enzyme (SCC), and steroidogenic acute regulatory protein (StAR) (D)

relevant density. Consistently, passaging of non-side population cells was not possible, demonstrating self-renewal and proliferative capacity for murine adrenal side population cells only (Figure 3B). Re-staining with Hoechst 33342 dye and Re-FACS analysis of long-term cultured side population cells resulted – except for an expected higher proportion of side population cells – in a FACS pattern similar to the original FACS sort, suggesting asymmetric cell division (Figure 3C).

Immunohistochemistry of long-term cultured side population cells grown on cover slips in a standard adrenal culture medium revealed the expression of a variety of adrenocortical markers and typical steroidogenic enzymes indicating spontaneous differentiation *in vitro* (Figure 3D). We were not able to detect significant amounts of glucocorticoid concentrations in the culture medium, an observation we routinely find in adrenal primary cultures grown *in vitro* for a couple of weeks. Besides this exception, the established stem cell characteristics self-renewal, asymmetric cell division and differentiation were met by murine adrenal side population cells.

2. Potential regulators of the adrenal side population [3]

For studying potential regulators of adrenal side population cells, we took advantage of two available transgenic mouse models.

Pbx1 is an important transcription factor for proper adrenal development, maybe even with a regulatory function upstream of Sf1. Both factors seem to work synergistically. Homozygous knock-out of Pbx1 leads to bilateral adrenal aplasia and is lethal. Adrenals in the context of Pbx1 haploinsufficiency are smaller in size, present with a lower proliferation rate (Figure 4), and impaired adrenal function, demonstrated by higher basal ACTH levels and lower glucocorticoid output upon restrained stress experiments [2].

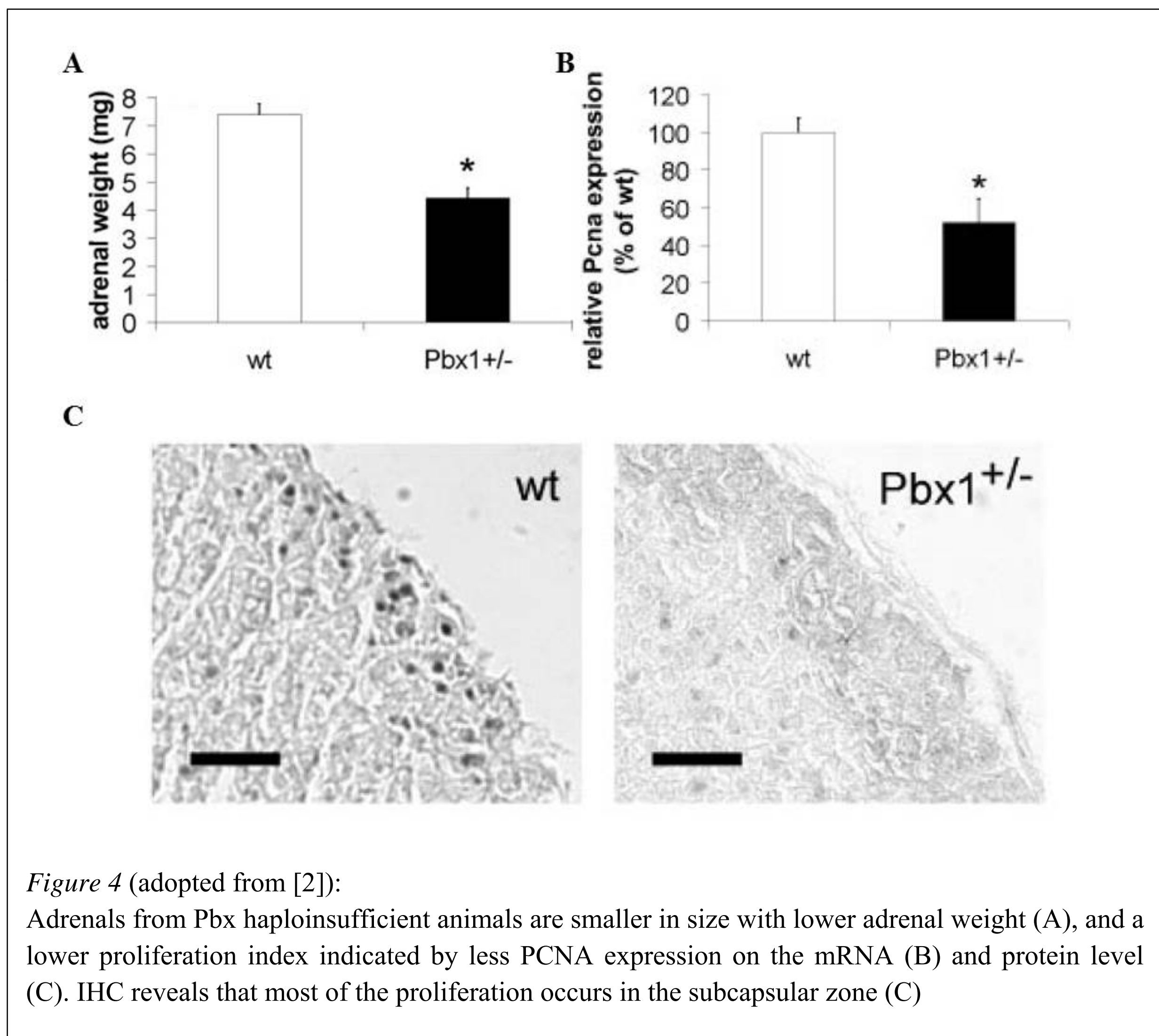

Figure 4 (adopted from [2]):
Adrenals from Pbx haploinsufficient animals are smaller in size with lower adrenal weight (A), and a lower proliferation index indicated by less PCNA expression on the mRNA (B) and protein level (C). IHC reveals that most of the proliferation occurs in the subcapsular zone (C)

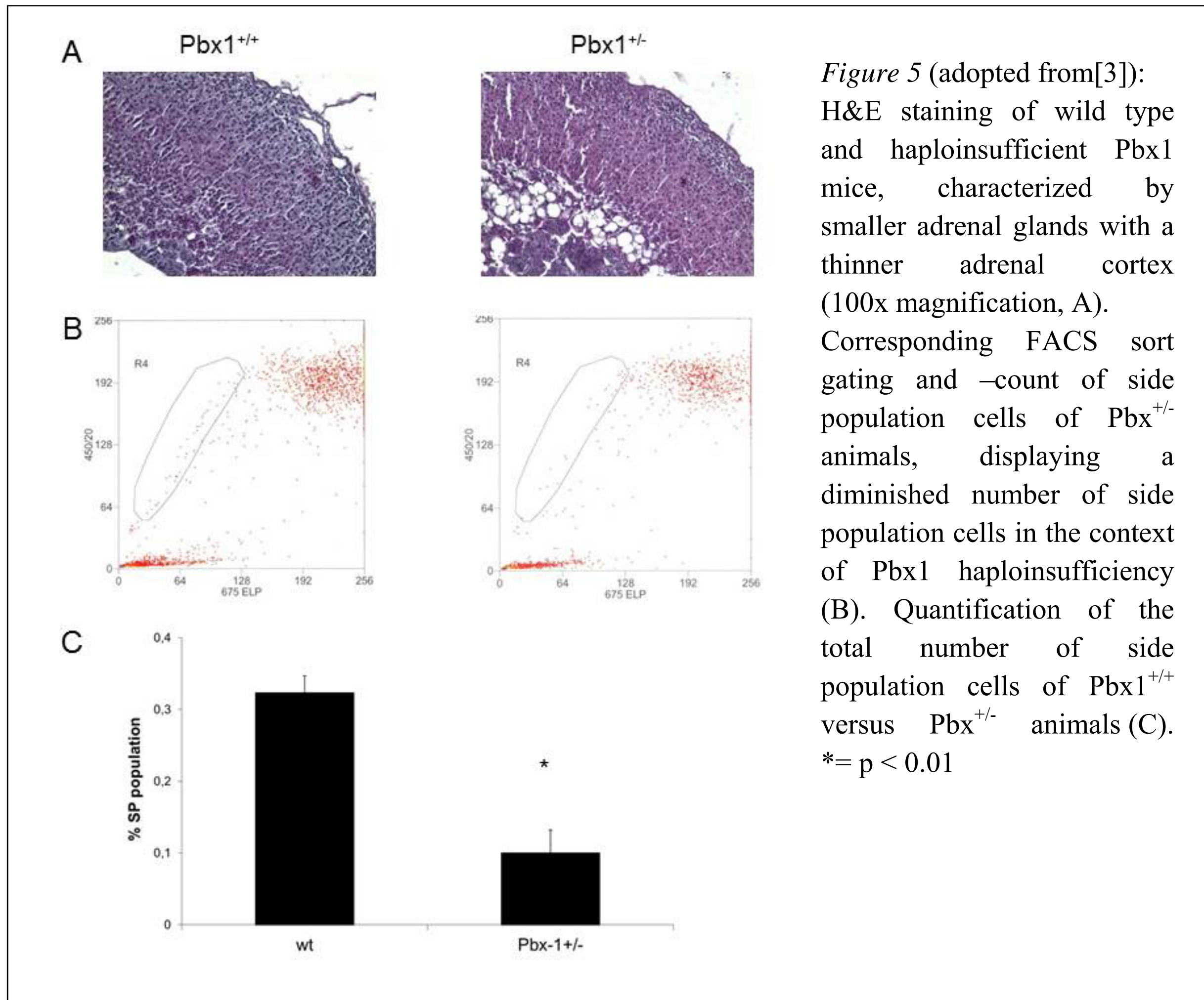

Figure 5 (adopted from[3]): H&E staining of wild type and haploinsufficient Pbx1 mice, characterized by smaller adrenal glands with a thinner adrenal cortex (100x magnification, A). Corresponding FACS sort gating and –count of side population cells of $Pbx^{+/-}$ animals, displaying a diminished number of side population cells in the context of Pbx1 haploinsufficiency (B). Quantification of the total number of side population cells of $Pbx1^{+/+}$ versus $Pbx^{+/-}$ animals (C). *= $p < 0.01$

In contrast to this observation, $Tpit^{-/-}$ mice present with an adrenal subcapsular zone which is substantially thinker compared to wild type animals. These mice are ACTH-deficient and the wider adrenal subcapsular zone is similar to that of ACTH receptor knock-out mice [74], strongly indication that disrupted ACTH signaling leads to those morphological changes.

As described typical for the subcapsular zone, and in contrast to the rest of the adrenal cortex, the enlarged subcapsular region of $Tpit^{-/-}$ animals did not stain positive for steroidogenic enzymes upon immunohistochemistry, which is in accordance with the theory of a residing stem cell pool in the periphery of the adrenal cortex. Interestingly, the larger subcapsular zone coincides with a significant higher amount of side population cells in these

adrenals compared to wild type adrenals. Furthermore, ACTH application *in vivo* over 7 days not only reverted the zone width back to normal, but is paralleled by a normalization of the number of side population cells in these animals (Figure 6). These data might indicate a 'stem cell arrest' with impaired differentiation capabilities in the context of ACTH deficiency.

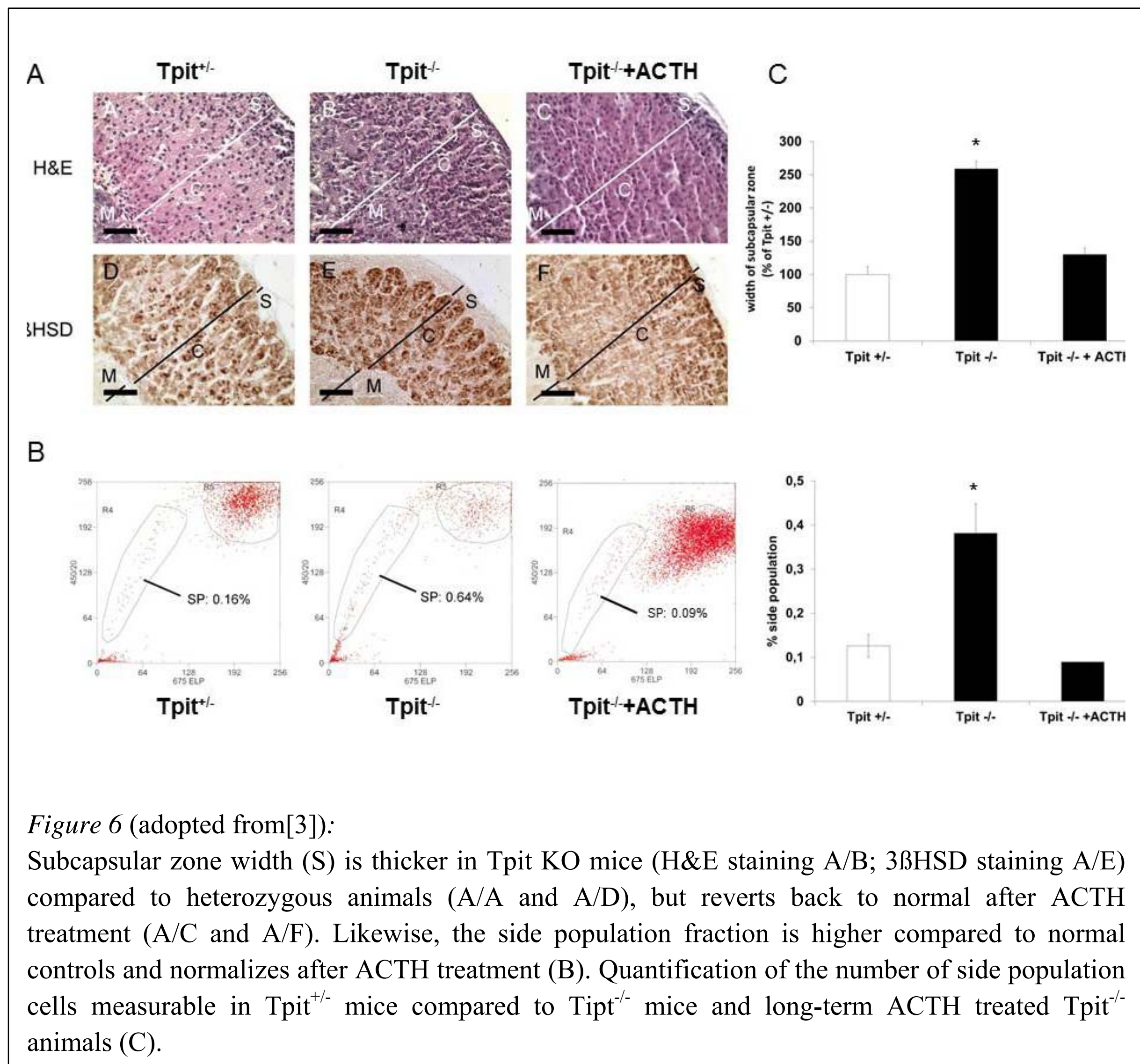

Figure 6 (adopted from[3])*:*
Subcapsular zone width (S) is thicker in Tpit KO mice (H&E staining A/B; 3ßHSD staining A/E) compared to heterozygous animals (A/A and A/D), but reverts back to normal after ACTH treatment (A/C and A/F). Likewise, the side population fraction is higher compared to normal controls and normalizes after ACTH treatment (B). Quantification of the number of side population cells measurable in $Tpit^{+/-}$ mice compared to $Tipt^{-/-}$ mice and long-term ACTH treated $Tpit^{-/-}$ animals (C).

Keeping this interpretation in mind, the higher ACTH levels detected in Pbx1 haploinsufficient animals could have driven a higher amount of subcapsular adrenal progenitor cells towards differentiation, reducing the number of dormant progenitors in the subcapsular zone. In conclusion, ACTH has to be considered an important regulator of

adrenocortical stem cell maintenance and fate in addition to its well defined action on steroidogenesis.

As the ability to exclude Hoechst 33342 dye is not exclusively found in progenitor cells, the side population has to be regarded as a heterogeneous collection of cells. Although enriching progenitor cells is possible, side population cells cannot compensate for adequate adrenal specific stem cell markers. Therefore, whole genome microarray analysis was performed on side population versus non-side population cells to elucidate relevant gene expression differences between the two cell types. Since the number of harvestable adrenal side population cells per mouse is limited, microarray analysis was performed on a platform optimized for small cell numbers [75]. Expectedly, the expression patterns of the two populations clearly differed. Genes associated with stem cell signaling tended to be higher expressed, while Sf1 tended to be lower expressed in side population cells. However relevant divergence could only be found for sets of genes and not for individual genes. Unfortunately, even within the gene sets, no interesting candidates for a potential stem cell marker could be identified [3].

3. Defining pluripotent and committed adrenal stem cells

Since microarray analysis did not reveal relevant adrenal stem cell markers, alternative strategies were being sought. Starting in the late 1990's, fusion of somatic cells or somatic stem cells with embryonic stem (ES) cells of both mouse and human origin arouse great attention, as fusion hybrids were found to be pluripotent [76,77]. It has been proposed that the somatic fusion partner undergoes reprogramming during the fusion process, in that regulatory programs of gene expression change along with alterations of DNA methylation, leading to a dedifferentiation of the somatic cell towards a lesser differentiated state. Do et al. even demonstrated a complete erasure of the 'somatic memory' of adult neurospheres upon cell fusion [78]. In consequence, fusion hybrids were found to have an epigenetic status and phenotype similar to that of embryonic stem cells: Genes associated with pluripotency were hypomethylated, the hybrid cells displayed prolonged self-renewal ability, differentiated into derivatives of the three germ layers and even contributed to chimeras [79,80]. Despite these conformities with embryonic stem cells, fusion hybrids usually remain tetra- or polyploid, although spontaneous reduction to regular diploidity has been observed [81]. However, this reduction process is considered a very rare event. Furthermore, expression profiling led to the notion that fusion hybrids remain cells with features distinct different from both of the originating cell populations [82]. Similarly, later studies demonstrated, that cell fusion does not inevitably mean a unidirectional dedifferentiation process, but that, depending on the experimental settings, characteristics of the somatic fusion partner can be acquired by fusion hybrids during the fusion process as well [83-85].

In consequence, cell fusion appeared to be an ideal method to achieve a whole spectrum of different adrenal cell types, ranging from pluripotent, more embryonic cells to dedicated somatic adrenal progenitors. Serving as a model for adrenal cells in various differentiation stages, we postulated that in depth characterization of fluorescence labeled

resulting fusion hybrids, clonally expanded, should allow the identification of genes, relevant for governing adrenal precursors or regulating differentiation.

We adopted the well-established polyethylenglycol (PEG) mediated cell fusion method in our laboratory. In our model, adult adrenocortical cells from green-fluorescence-protein (GFP)-labeled Oct4 mice (fluorescence not expressed) were fused with embryonic stem cells from td(red fluorescence protein)RFP-labeled SF1 mice (fluorescence not expressed). While the adult adrenal cells were obtained by applying a single cell suspension protocol well established in our laboratory, the embryonic stem cells were harvested from embryonic primary cultures from crossbred SF1-Cre and tdRFP reporter mice (Figure 7).

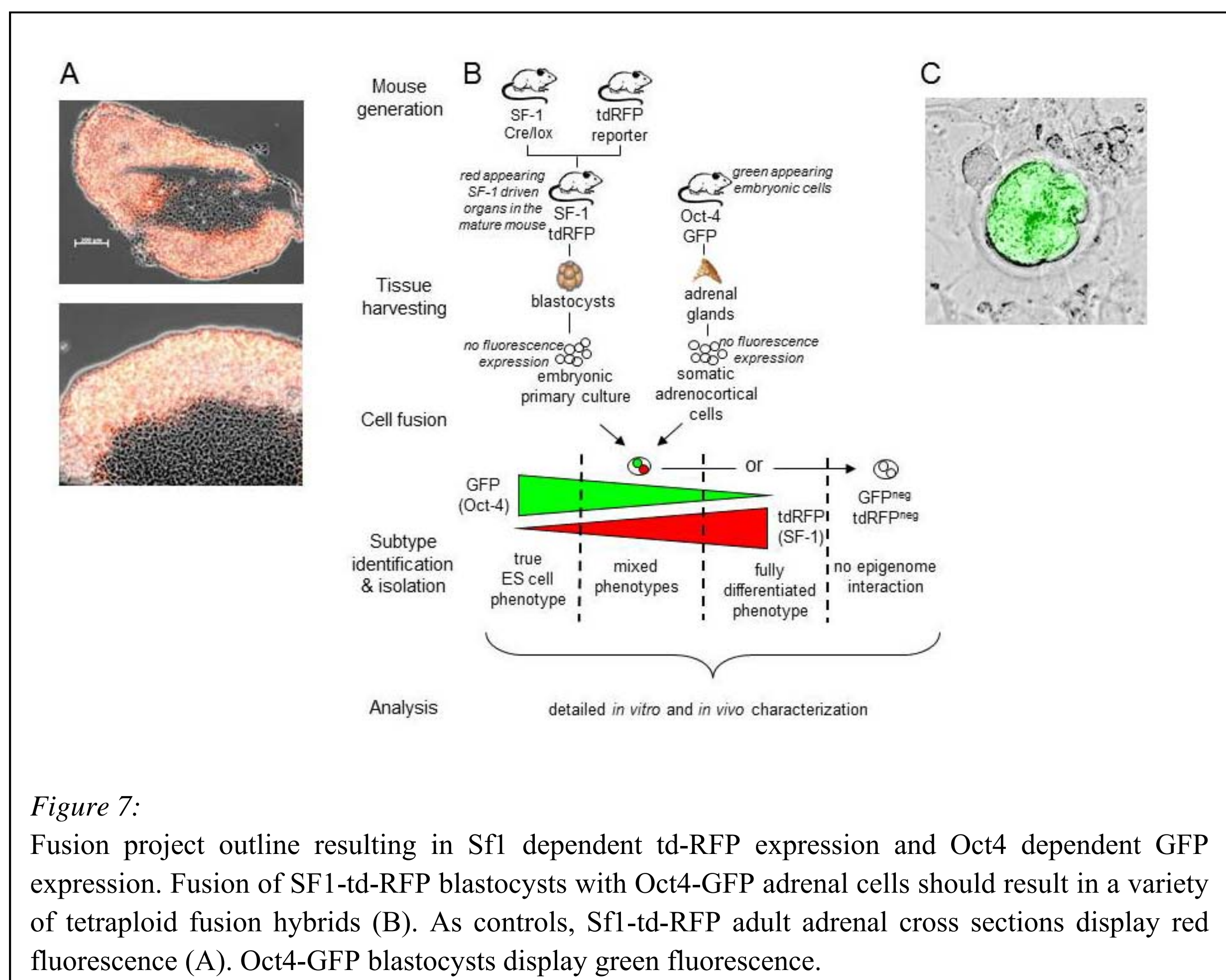

Figure 7:
Fusion project outline resulting in Sf1 dependent td-RFP expression and Oct4 dependent GFP expression. Fusion of SF1-td-RFP blastocysts with Oct4-GFP adrenal cells should result in a variety of tetraploid fusion hybrids (B). As controls, Sf1-td-RFP adult adrenal cross sections display red fluorescence (A). Oct4-GFP blastocysts display green fluorescence.

Fusion hybrids should either express green fluorescence, once Oct4 transcription is activated and the cell was reprogrammed to a rather embryonic state, or should express red fluorescence, if SF1 is expressed, an essential step for initiating differentiation towards the steroidogenic lineage. Alternatively, both fluorescences could become induced in cells with intermediate phenotypes. If no fusion has taken place, no fluorescence is detectable as neither the adult Oct-4/GFP adrenal cells nor the embryonic SF-1/tdRFP cells will have the ability to induce expression of the transgenes. According to the expertise of Hans Schöler's laboratory at the Max Planck Institute in Münster, GFP positive reprogrammed cells can be expected approximately 2 – 3 days after cell fusion [83]. However, out of 10 highly technically demanding cell fusion experiments, we were able to detect dim tdRFP expression only in one culture dish after 14 days, indicating differentiation towards the steroidogenic lineage.

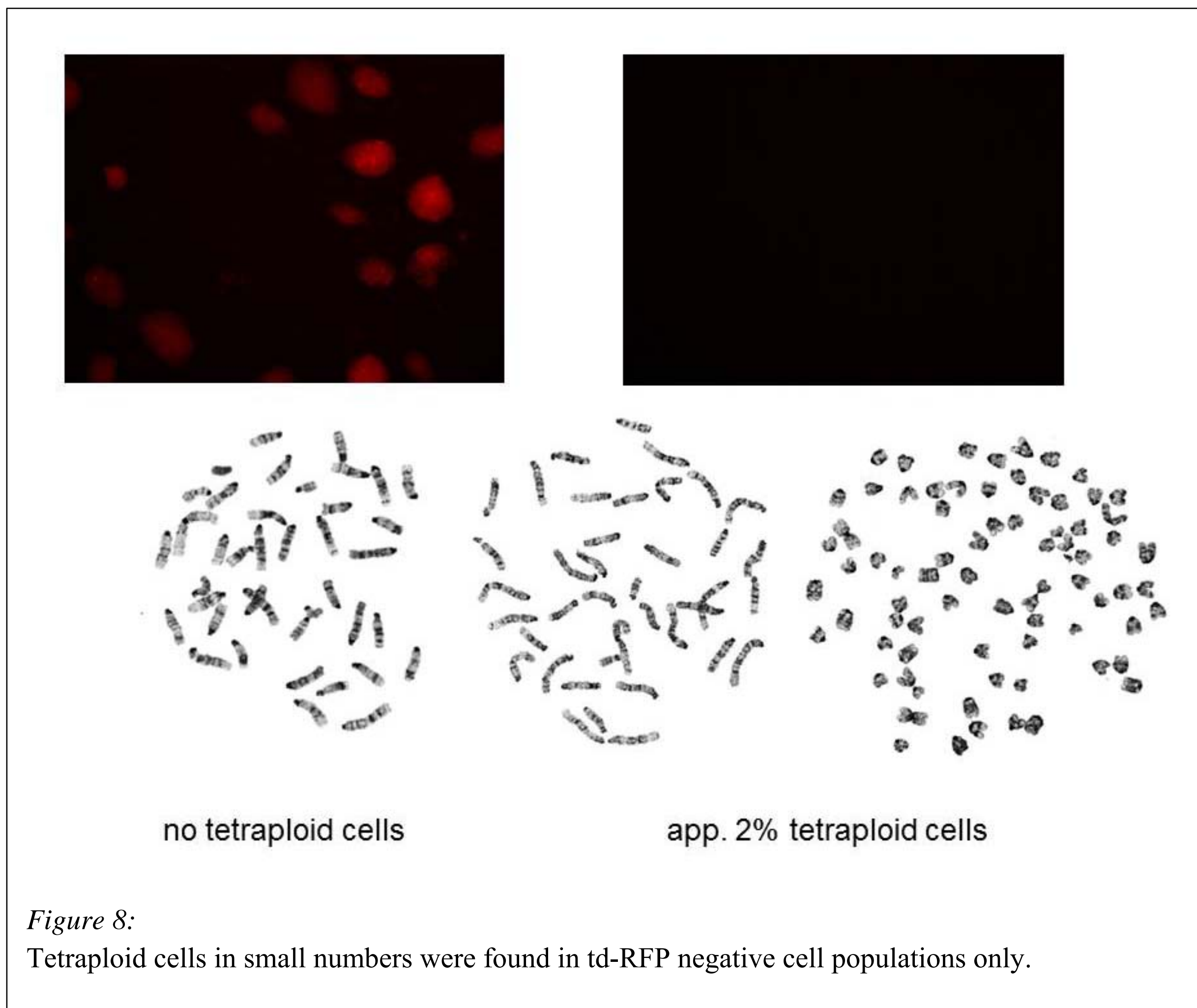

Figure 8:
Tetraploid cells in small numbers were found in td-RFP negative cell populations only.

Prolonged culturing intensified the detectable fluorescence over time. Surprisingly, tetraploidity was found in 2 % of cells in the fluorescence negative control group only (Figure 8).

Embryonic bodies are routinely used as a model for unspecific spontaneous differentiation of embryonic stem cells. Embryonic bodies derived from Sf1-tdRFP embryonic stem cells revealed increasing fluorescence intensity over time. Accordingly, Sf1 mRNA expression significantly increased in accordance to tdRFP fluorescence intensity (Figure 9).

Taken together, the fusion project did not render the desired spectrum of progenitor fusion hybrids. In contrast to the findings described in the literature, reprogrammed cells could not be detected. Technical and procedural difficulties have been tried to overcome by carrying out the fusion experiments partially in the Max Planck Institute for Developmental Biology in Münster, where a research group routinely fuses embryonic stem cells with neural progenitor cells. Even in their hands, better results were not obtainable, possibly because the fluorescence model is not working as it should, or because the cells used are not suitable for cell fusion experiments. Whatsoever, as the Sf1-tdRFP construct reliably turned red upon differentiation, this construct appears to be valuable to be utilized in other differentiation studies.

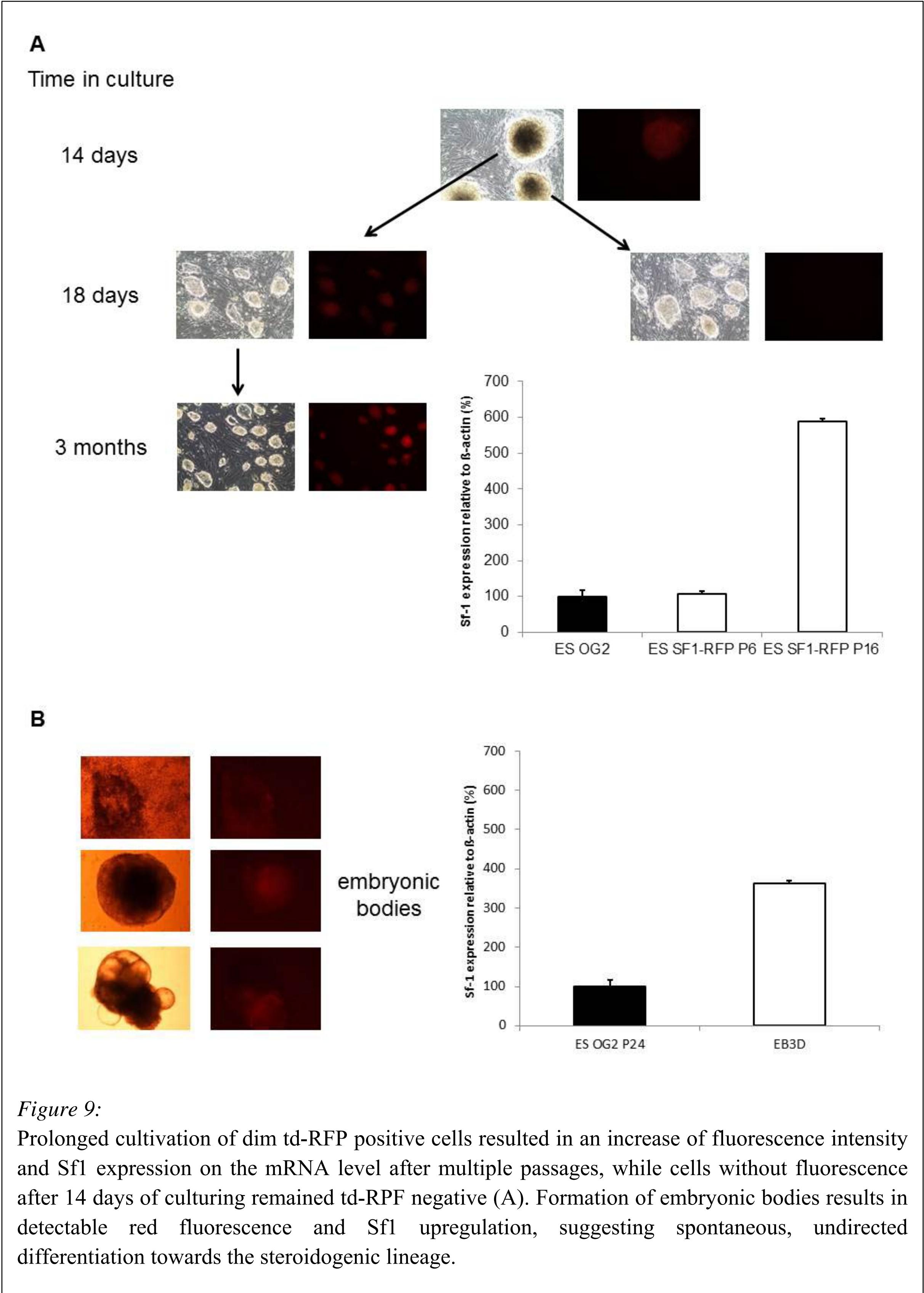

Figure 9:
Prolonged cultivation of dim td-RFP positive cells resulted in an increase of fluorescence intensity and Sf1 expression on the mRNA level after multiple passages, while cells without fluorescence after 14 days of culturing remained td-RPF negative (A). Formation of embryonic bodies results in detectable red fluorescence and Sf1 upregulation, suggesting spontaneous, undirected differentiation towards the steroidogenic lineage.

4. Stem cell-like tumor cells in adrenocortical carcinomas [1]

Adrenocortical carcinomas are rare, but highly malignant tumors of the adrenal cortex, which respond poorly to standard chemotherapeutic substances [86]. Cellular heterogeneity of the tumor and the presence of multi-drug resistance genes, which encode for membrane based pumps that actively expel the cytotoxic drugs are being discussed as two underlying mechanisms for this clinical observation [87]. P-glycoprotein was found a marker for the adrenal definitive zone [24] and represents a prototype member of the ATP-binding cassette (ABC) transporter family – especially Multi-Drug-Resistance-Gene 1 (MDR1) [61] and Breast Cancer Resistance Gene 1 (ABCG2) [60], which are ATP-dependent membrane proteins predominantly expressed in excretory organs. Since the Hoechst efflux capacity of side population cells is based on these pumps [88], the identification of the side population in adrenocortical tumors should allow an enrichment of stem cell-like tumor cells and thus the study of relevance of these cells in adrenocortical malignancies

The side population was first described for the isolation and characterization of stem cell-like cancer cells in neuroblastoma tumors and corresponding cell lines by Hirschmann-Jax et al [51]. Herein, similar to stem cell-like tumor cells isolated on the basis of cell surface markers, side population tumor cells were found to proliferate, self-renew, and withstand chemotherapy substances better than non-side population cells. Later, studies have extended these findings to primary cultures for example of gastrointestinal cancers [89,90], and ovarian cancer [91]. Interestingly, besides neuroblastoma cell lines, the isolation of tumor-like stem cells based on the Hoechst 33342 (side population) exclusion method could also be demonstrated for a variety of other tumor cell lines such as glioma [92], breast [93], thyroid cancer [94], and melanoma [95] cell lines. This is remarkable, keeping in mind that cell lines are believed to be prototypes for monoclonal cell expansion.

After optimizing the protocol for adrenal tumor primary cultures, we could demonstrate the presence of side population cells in a variety of different human adrenal tissues including adrenocortical carcinomas, adenomas with different clinically relevant hormonal excesses, and different human and mouse adrenocortical carcinoma cell lines. The side population fraction tended to be larger the more aggressive the tumor entity was. For in depth analyses and in order to obtain comparable results, we decided to thoroughly investigate the well-established and widely used adrenocortical cancer cell line NCI H295R [96,97], as the obtainable number of fresh adrenal cancers is very limited due to the rarity of the disease.

In contrast to our previous observations with regular adrenal side population cells and in contrast to the findings published by other groups, non-side population cells from NCI H295R cells proliferated at a similar rate as side population cells when co-cultured in equal cell numbers (Figure 10).

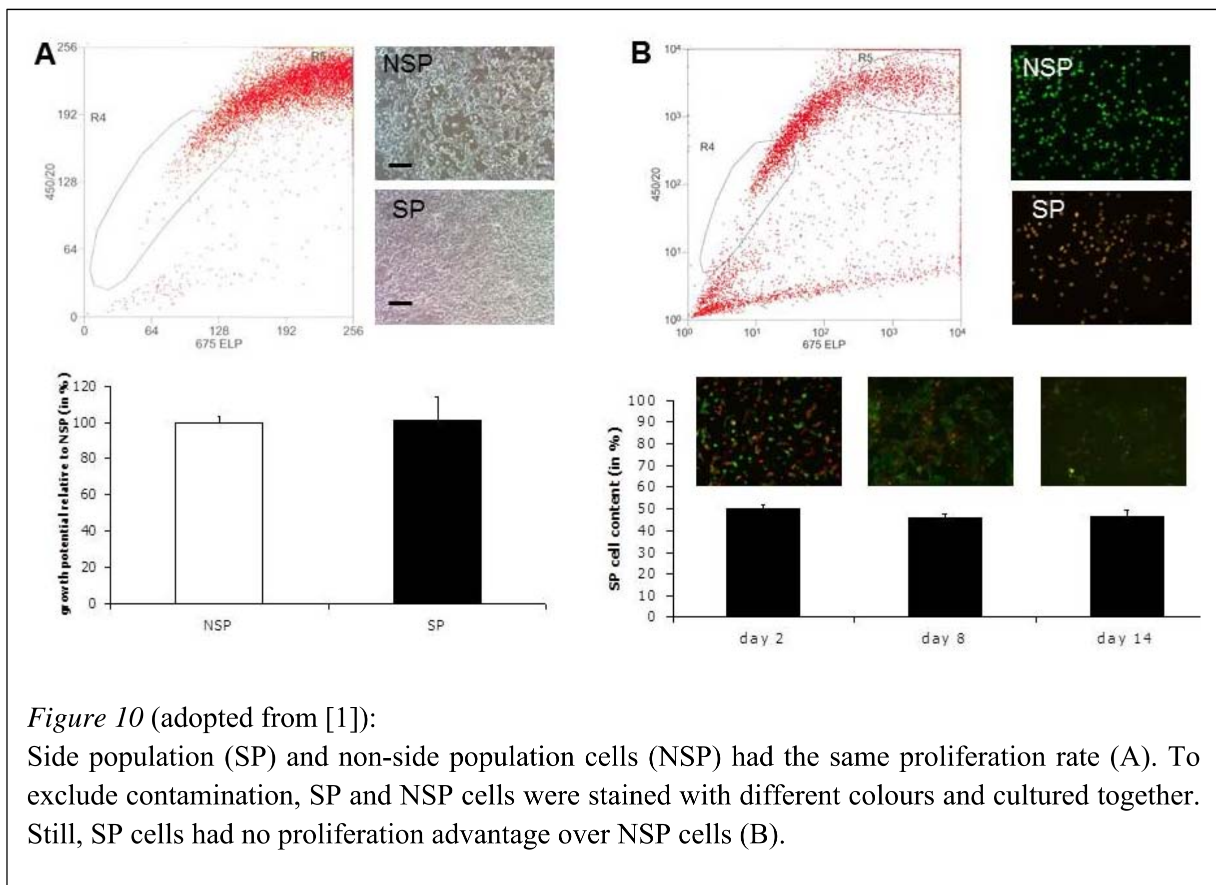

Figure 10 (adopted from [1]):
Side population (SP) and non-side population cells (NSP) had the same proliferation rate (A). To exclude contamination, SP and NSP cells were stained with different colours and cultured together. Still, SP cells had no proliferation advantage over NSP cells (B).

Interestingly, although – as expected – the fraction of side population cells was higher in FACS re-sorted side population cultures, side populations could consistently be detected in cultured non-side population cells as well, which is incompatible with the side population stem cell theory (Figure 11).

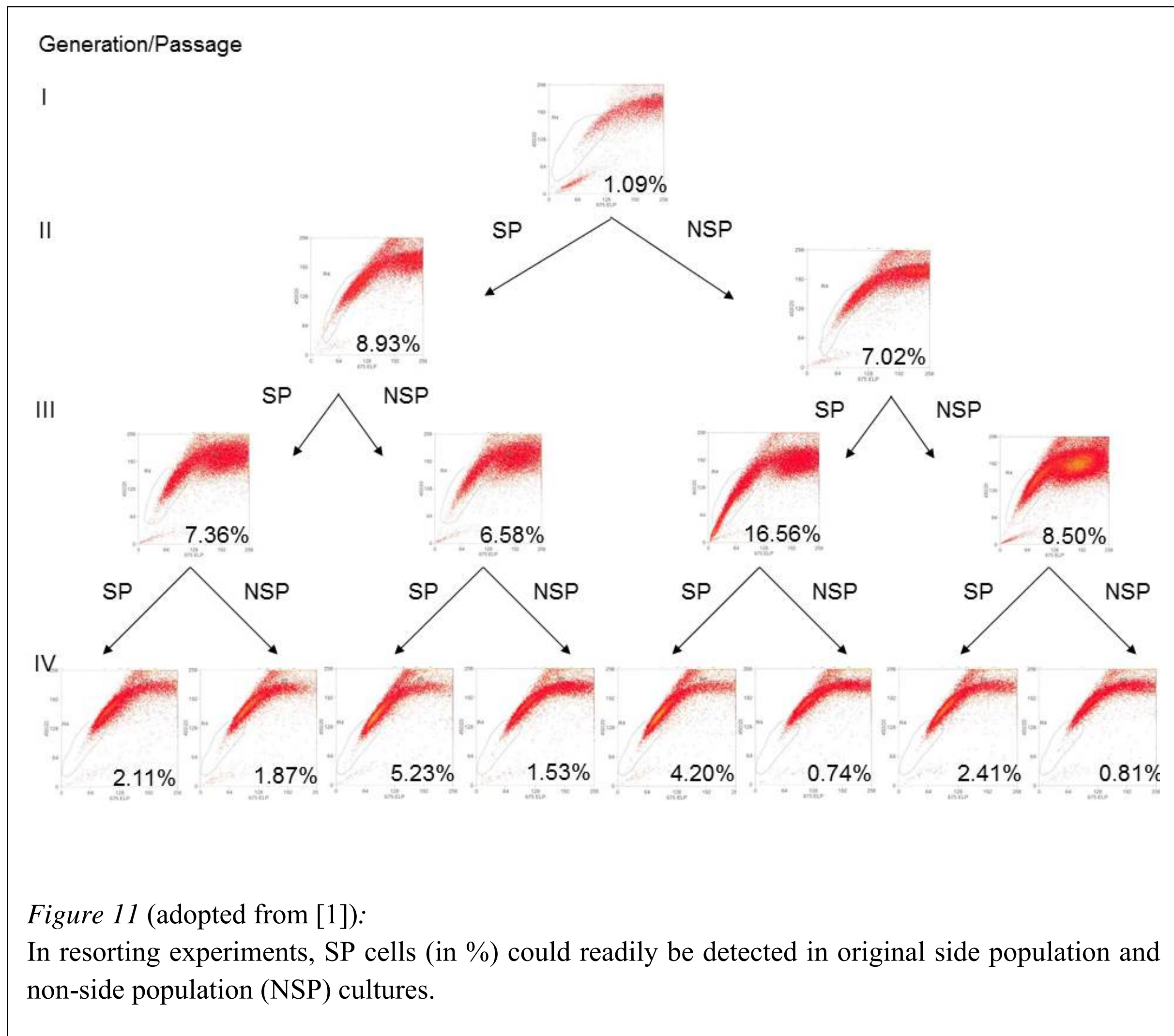

Figure 11 (adopted from [1]):
In resorting experiments, SP cells (in %) could readily be detected in original side population and non-side population (NSP) cultures.

This finding was surprising, especially since great care was taken to ensure that all established criteria to identify a valid side population were fulfilled:

- Disappearance of the side population upon FACS analysis after pre-incubation with the calcium channel blocker verapamil
- Optimization of the Hoechst 33342 staining procedure by confocal laser microscopy and FACS time course experiments

- Lower staining intensity of side population cells compared to non-side population cells was verified using confocal laser microscopy. Accordingly, MDR1 and ABCG2 were significantly higher expressed on the mRNA level in side population cells
- For comparative experiments, side populations cells from the outer tip of the side population fraction were utilized only, to ensure the highest purity of side population cells possible [98,99]
- Compared to non-side population cells, side population cells expressed genes responsible for steroidogenesis on a significantly lower level in accordance with the presumed more undifferentiated state.

Possible explanations for the same proliferation rate of side population and non-side population cultures include cell cycle dependent expression or activity of the membrane based pumps, or epithelial mesenchymal transition. Cell cycle differences between the two populations could not be found. Epithelial-mesenchymal transition is generally accepted as an important mechanism for tumor infiltration and metastasis. Although the original model proposes a unidirectional change towards a lesser differentiated mesenchymal phenotype, there is evidence suggesting the presence of incomplete epithelial-mesenchymal transition as well as a reversion of this transition [100]. However, differences in epithelial or mesenchymal marker expression levels between side population and non-side population cells could not be found. Clinically most relevant for stem cell-like tumor cells is certainly their ability to discharge or repel cytotoxic substances by membrane based pumps. As expected, we found ABCG2 and MDR1, the genes encoding for these pumps, substantially higher expressed in NCI H295R side population cells. Despite this equipment, with the exception of a mild effect in an etoposide environment, a relevant overall survival benefit compared to non-side

population cells could not be observed (Figure 12).

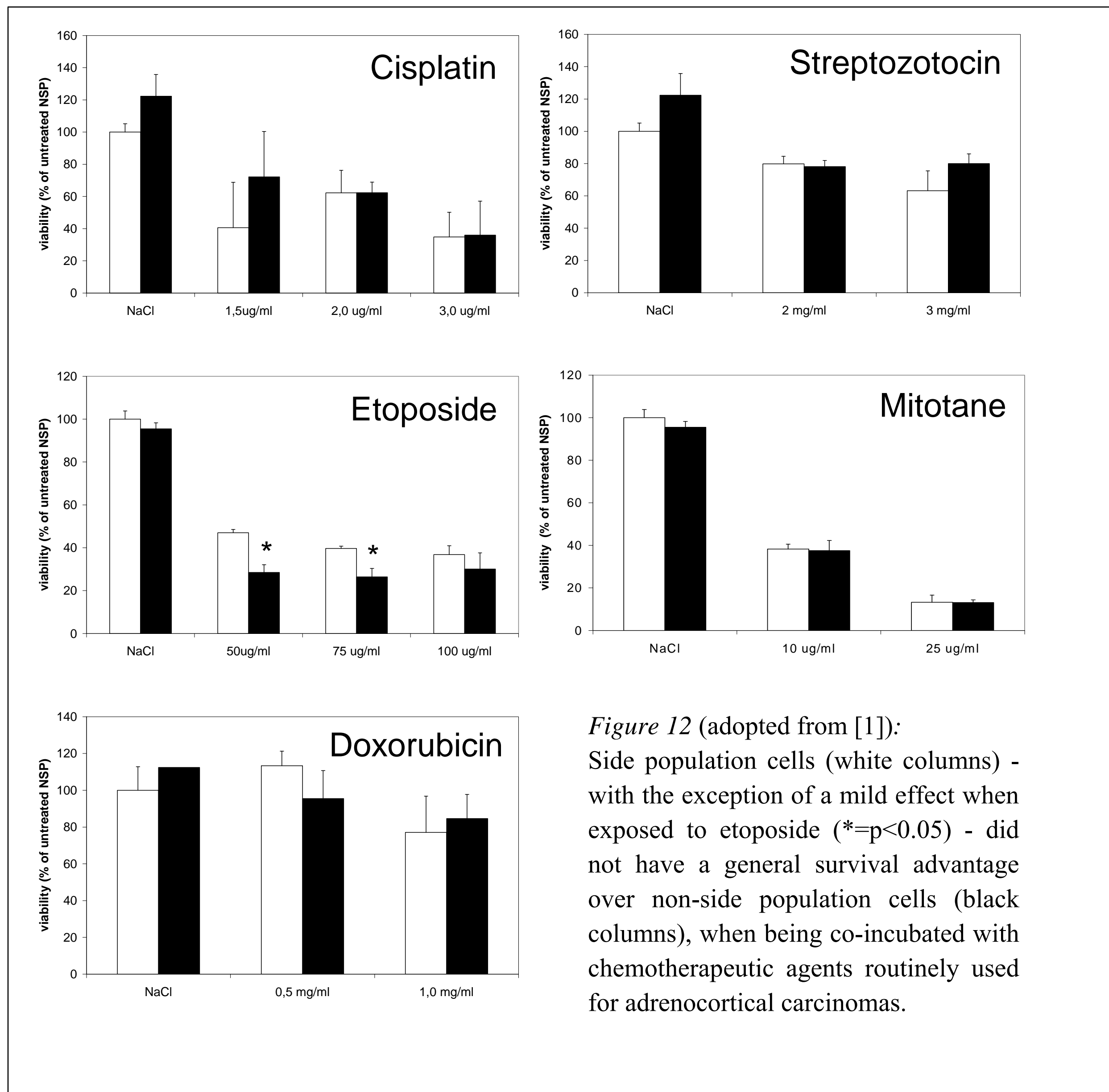

Figure 12 (adopted from [1])*:*
Side population cells (white columns) - with the exception of a mild effect when exposed to etoposide (*=$p<0.05$) - did not have a general survival advantage over non-side population cells (black columns), when being co-incubated with chemotherapeutic agents routinely used for adrenocortical carcinomas.

Since MTT measurements were performed, we cannot rule out that a small number of cells with stem cell characteristics in the side population dishes would have survived prolonged culturing.

In summary, side population cells of NCI H295R cells were indistinguishable in terms of viability, cell proliferation and cytotoxic resistance from non-side population cells, although a valid side population was clearly detectable and isolatable. It remains unclear,

whether these findings are attributed to this particular cell line, or if side population cells do not play a relevant pathophysiological role in adrenocortical carcinomas.

Nevertheless, this study arrestingly demonstrates that the mere identification of side population cells, even when all selection criteria were stringently fulfilled, does not guarantee ‘stemness’ per se. These cells need to be further evaluated and characterized functionally, to validate presumed progenitor capabilities.

Summary & Outlook

Despite the advances in stem cell biology, the endocrine stem cell field is still evolving, and besides the fact, that somatic stem cells seem to persist throughout life in the endocrine system, little is known about their nature and the niche in which they reside. The extent of plasticity of these cells and the mechanisms of their differentiation are uncertain, although some important factors have now been described. One of the reasons for the slow progress in adrenal stem cell research can be attributed to the rarity of adrenal diseases and the lack of appropriate tissue material. Nevertheless, it is generally accepted that current treatment regimens, no matter if substitution of adrenal insufficiency or adrenal cancer therapies, are insufficient for affected patients. This is the driving force to continue with adrenal stem cell research beside the goal to improve and extent the knowledge of physiology and mechanisms and factors in this field.

Applying the side population technique is an interesting approach to enrich progenitor cells, especially in the absence of well characterized marker genes. Earlier published observations on how adrenocortical cells are equipped together with the proposed mechanism of the side population phenomenon, made this approach the first choice to study. Expectedly, adrenal side population cells could be isolated, which fulfilled the general stem cell criteria self-renewal, proliferation and differentiation. Unfortunately, further investigations including whole genome microarray analysis, did not result in the detection of more adrenal specific stem cell markers. Our study however demonstrated regulatory effects for the transcription factor Pbx1 and for ACTH on side population cells, and therefore presumably also for adrenal progenitors.

Defining one or several tissue specific stem cell markers is a prerequisite for substantiated stem cell research. The fusion project was another approach to achieve this goal.

PEG-mediated cell fusion was applied to generate different types of progenitor cells ranging from embryonic-like pluripotent cells to designated adrenal progenitors. Unfortunately, despite several approaches, the desired fusion hybrids could not be obtained. As tetraploidity is inevitable, fusion hybrids would be an inferior choice for cell replacement treatment strategies. After all, continuing this project would have been unreasonable. Nevertheless, the project allowed the establishment of embryonic stem cell techniques in our lab und bore Sf1-tdRFP cells, which can be utilized as differentiation markers for further studies.

While the fusion project was underway, Yamanaka's land-mark paper was published, allowing cellular reprogramming back to the embryonic stage by applying 4 key factors, Oct4, Sox2, c-Myc, and Klf4, resulting in so called induced pluripotent stem cells [101]. Although this method is slower and not more efficient than stem cell fusion, resulting cells are more homogeneous and have regular ploidity [83]. Although not yet published, there is little doubt that reprogramming adrenal cells is possible, when this or other reprogramming protocols are applied. Recently, induced pluripotent stem cells could be transformed or differentiated to some extend into steroid producing cells by virally transfecting Sf1 [102]. Even so, we are not pursuing projects involving induced pluripotent stem cells as major obstacles of modern embryonic stem cell research, such as the dependence on viral vectors for efficient reprogramming, teratoma formation, or incomplete differentiation have yet to be overcome.

A more promising strategy in this context could be a limited reprogramming towards a designated progenitor, instead of reprogramming the cells completely towards the embryonic stem cell state. This more direct route has now been described for hepatocytes [103,104], cardiomyocytes [105], and neurons [106]. Newer data even suggests, that, in contrast to transdifferentation, a conversion of differentiated cells into a desired target tissue via designated progenitors by reprogramming could be possible *in vivo*. In this study, exocrine pancreatic cells were converted into functional ß-cells [107]. Although this concept could be

adapted to our needs by e.g. co-transfecting Sf1 and Oct4, there is no way around clearly defining and characterizing the adrenal somatic stem cell.

Recently, sonic hedgehog signaling studies on murine adrenal glands has led to the notion, that adrenal progenitor cells in the subcapsular region could be divided into long-term and short-term progenitor cells, defined as $Sf1^{pos}$ and $Gli1^{pos}$ cells being long-term, and $Sf1^{pos}$ $Gli1^{neg}$ cells being short term progenitors. Although these markers are not suitable for cell isolation, this study means a step forward in understanding and defining the adrenal stem cell. It would be e.g. interesting to see, if Gli1 or Ptch are significantly expressed in adrenal side population cells.

Although the initial enthusiasm regarding stem cell like-cancer cells seems to fade, this theory has become an accepted concept in modern tumor pathophysiology. The relevance and the extent of these subpopulations of tumor cells in endocrine cancer entities have yet to be clarified. We addressed this issue by characterizing the side population, which could be reliably detected in numerous human adrenal tumor entities and in the cell line NCI H295R. We could exclude that side population cells play a relevant role as ‘cancer stem cells’ in this standard model for adrenocortical cancer. This project in addition impressively demonstrated that one has to be cautious declaring a certain cell type as stem cells utilizing an established stem cell isolation method, without further characterizing these cells.

After all, the projects certainly not always rendered the anticipated results, and cleaving to the projects, trying to find the reasons and mechanisms for divergent data, was a challenge. Nonetheless, it has been great pleasure to work for several years intensively in the field of endocrinology, where stem cell research has been just emerging. A whole variety of topics and lab techniques from embryonic stem cells to stem cell-like cancer cells were covered. One of our ongoing projects focuses on the screening of different types of pheochromocytomas and paragangliomas for the expression of a variety of typical stem cell

markers. Tissue-array analyses on several hundred samples collected Europe-wide should allow to reliably answer the question whether and which stem cell markers are significantly expressed on the protein level in this tumor entity. Further studies will aim to investigate their involvement in tumorigenesis.

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Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
ACTH	adrenocorticotropic hormone
C-kit	tyrosine-protein kinase Kit (CD117),
	mast/stem cell growth factor receptor (SCFR)
Dab2	disabled homolog 2
Dax1	dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
CD24	heat stable antigen CD24 (HSA)
CD44	cell-surface glycoprotein
CD56	neural cell adhesion molecule (NCAM)
DHEA	dehydroepiandrosteron
GFP	green fluorescence protein
Gli1	Gli family zinc finger 1
LacZ	gene encoding β-galactosidase
MDR1/ABCB1	multidrug resistance 1, P-glycoprotein 1
Oct4	octamer binding transcription factor 4
Pbx1	pre-B-cell leukemia transcription factor 1
Ptch1	protein patched homolog 1
Sca-1	stem cell antigen-1
SCID	severe combined immunodeficiency
Sf1	steroidogenic factor 1
Shh	sonic hedgehog
tdRFP	tandem-dimer red fluorescent protein
Tpit	T-box factor, TPX19
Wnt	family of highly conserved secreted signaling molecules
X-zone	develops in the inner adrenal cortex of mice; similar to fetal zone in primates

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit- einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlicht vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Dr. Felix Beuschlein und Herrn Prof. Dr. Stefan Bornstein in München und Düsseldorf vor Ort und von Herrn Prof. Dr. Abken und Herrn Prof. Dr. Brüning in Köln betreut worden.

Köln, den 28. Januar 2014

Curriculum vitae

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Education

1981 - 1985	Mariengrundschule Appelhülsen, Germany
1985 - 1991	Johann-Conrad-Schlaun-Gymnasium, Germany
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1994 - 1995	Nursing Practical, Raphaelsklinik, Münster, Germany
1995 - 1998	Medical School, University of Münster, Germany
Summer 1997	'Physikum' (equivalent to final preclinical exam)
Summer 1998	'1. Staatsexamen'
1998 - 1999	Research fellowship: National Cancer Institute, NIH, USA
1999 - 2000	Medical School, University of Vienna, Austria
2000 - 2001	Medical School, University of Münster, Germany
Spring 2001	'2. Staatsexamen'
2001 - 2002	Senior year - First tertial: Internal Medicine Georgetown University, Washington DC,USA (supported by a DFA travel stipend) - Second tertial: Sugery, King's College of London, Gillingham, Kent, England - Third tertial: Anaesthesiology, University Hospital Münster, Germany
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2002 - 2003	Internship, German Diabetes Research Institute, Düsseldorf, Germany
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2004	Residency, German Diabetes Research Institute Düsseldorf, Germany

2004 - 2007	Residency, Department Internal Medicine II, Division of Endocrinology and Diabetology, University Hospital Freiburg, Germany
2008 - 2011	Residency, Division of Endocrinology & Diabetology, Department of Medicine IV, University Hospital Munich, Germany
Summer 2011	Board certification for Internal Medicine
2011 - 2013	Fellowship Endocrinology/Diabetology
Summer 2013	Board certification for Endocrinology/Diabetology

Doctorial Theses

1998 - 1999	National Cancer Institute, NIH, Bethesda, Maryland, USA & Heinrich Heine University of Düsseldorf, Germany Topic: Potential Role of p4.1 in Neuroblastomas
2003 - 2014	Interdisciplinary postgraduate program Molecular Medicine, University of Cologne, Germany

Awards

2004, Hannover: poster price of the German Diabetes Society; „Leptin reverses insulin resistance in patients suffering from lipodystrophy“

2005, Boston, USA: Merck Senior Fellows Award, Endocrine Society; “Pbx-1 haploinsufficiency impairs adrenocortical growth and steroidogenesis”

2005, Göteborg, Schweden: Travel Grant, European Congress of Endocrinology; “Pbx-1 haploinsufficiency results in impaired adrenocortical growth and steroidogenesis”

2009, Frauenchiemsee: Hugo von Ziemssen-Prize

2010, Munich: Outstanding tutor award (Tutorial)

Funding

2009, Foefole Starting Grant, LMU Munich

2010, Friedrich-Baur-Stiftung

Extramural Activities

Member of the organising committee of Young Active Research of the German Endocrine Society

Member of the Medical Commission of the German Fencing Association and team physician of the German fencing national team

Appendix: original publications contributing to this thesis work

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

Lichtenauer U, Shapiro I, Sackmann S, Drouin J, Scheele J, Maneck M, Klein C, Beuschlein F.
J Endocrinol. 2012 Dec;215(3):383-91. doi: 10.1530/JOE-12-0393. Epub 2012 Oct 5.

http://www.ncbi.nlm.nih.gov/pubmed/23042945

Pre-B-cell transcription factor 1 and steroidogenic factor 1 synergistically regulate adrenocortical growth and steroidogenesis.

Lichtenauer UD, Duchniewicz M, Kolanczyk M, Hoeflich A, Hahner S, Else T, Bicknell AB, Zemojtel T, Stallings NR, Schulte DM, Kamps MP, Hammer GD, Scheele JS, Beuschlein F.
Endocrinology. 2007 Feb;148(2):693-704. Epub 2006 Nov 2.

http://www.ncbi.nlm.nih.gov/pubmed/17082260

Side population does not define stem cell-like cancer cells in the adrenocortical carcinoma cell line NCI h295R.

Lichtenauer UD, Shapiro I, Geiger K, Quinkler M, Fassnacht M, Nitschke R, Rückauer KD, Beuschlein F.
Endocrinology. 2008 Mar;149(3):1314-22. Epub 2007 Dec 6.

http://www.ncbi.nlm.nih.gov/pubmed/18063677