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Localization of Sonic Hedgehog secreting and receiving cells in the developing and adult rat adrenal cortex

Leonardo Guasti^{a,*}, Alex Paul^b, Ed Laufer^b, and Peter King^a

^aCentre for Endocrinology, William Harvey Research Institute, Barts and the London, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK.

^bDepartment of Pathology and Cell Biology, Columbia University, New York, NY 10032, USA.

Abstract

Sonic hedgehog signaling was recently demonstrated to play an important role in murine adrenal cortex development. The organization of the rat adrenal differs from that of the mouse, with the zona glomerulosa and zona fasciculata separated by an undifferentiated zone in the rat, but not in the mouse. In the present study we aimed to determine the mRNA expression patterns of *Sonic hedgehog* and the hedgehog signaling pathway components *Patched-1* and *Gli1* in the developing and adult rat adrenal. *Sonic hedgehog* expression was detected at the periphery of the cortex in cells lacking CYP11B1 and CYP11B2 expression, while signal-receiving cells were localized in the overlying capsule mesenchyme. Using combined *in situ* hybridization and immunohistochemistry we found that the cells expressing *Sonic hedgehog* lie between the CYP11B2 and CYP11B1 layers, and thus *Sonic hedgehog* expression defines one cell population of the undifferentiated zone.

Keywords

Sonic hedgehog; adrenal cortex; development

1. Introduction

The adrenal cortex produces steroids, which mediate the mammalian stress response and control homeostasis, that are essential for life. The cortex is divided into three functionally distinct zones arranged around the medulla: underneath the capsule lies the zona glomerulosa (ZG, which secretes mineralocorticoids), next is the zona fasciculata (ZF, which secretes glucocorticoids) and then the zona reticularis (ZR, which secretes adrenal androgens in humans). Recent studies have shown the requirement for Sonic hedgehog (Shh) signalling for adrenal development (King et al., 2009; Ching et al., 2009; Huang et al., 2010), and lineage tracing studies have demonstrated that both Shh expressing cells in the subcapsular mouse cortex and Shh signal receiving capsule mesenchyme cells have properties of adrenocortical stem/progenitor cells, with their progeny populating the entire cortex (King et al., 2009). These data support previous hypotheses that the mesenchymal cells in the capsule represent a stem cell niche from which cells are recruited into the steroidogenic cortex (Salmon and Zwemer, 1941; Kim et al., 2009). However, studies in the rat identified a zone between the ZG and ZF which expresses neither CYP11B1 nor

*Corresponding author: Leonardo Guasti, Centre for Endocrinology, William Harvey Research Institute, Barts and the London, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK. Tel: +44 (0)20 78826243; Fax: +44 (0)20 78826197; l.guasti@qmul.ac.uk.

CYP11B2, the terminal enzymes required for corticosterone and aldosterone production, respectively, which was named the undifferentiated zone (ZU, or Zona Intermedia, Mitani et al., 1994). Cells bordering this zone display relatively high levels of proliferation, and the ZU has been proposed to represent a population of stem/progenitor cells able to differentiate into ZG or ZF cells. In this model, cells migrate from the ZU bidirectionally, either centrifugally into the ZG, or centripetally into the ZF (Mitani et al., 2003).

A zone equivalent to the ZU was not observed between the CYP11B1 and CYP11B2 positive cells in the mouse cortex (King et al., 2009). Rather, in the mouse the CYP11B2 positive cells are found in clusters underneath the capsule interspersed with clusters of Shh positive cells, both of which abut the CYP11B1 positive cells of the ZF (King et al., 2009). By comparing the gene expression profiles of mechanically separated capsule/ZG and inner zone (ZF/ZR/medulla) rat adrenal tissue fractions by microarray, we previously showed that the components of the Shh pathway were exclusively expressed in the capsule/ZG region in the adult rat cortex (King et al., 2009). Given these differences in zonal organization between the species, we sought to identify the specific location of the Shh signaling and responding cells in the developing and adult rat adrenal gland.

2. Materials and methods

2.1 Animals

Rats and mice were housed in rooms with controlled light and temperature and treated under the Home Office Animals (Scientific Procedures) Act 1986 or according to Columbia University Institutional Animal Care and Use Committee guidelines. Animals were sacrificed by CO₂ asphyxiation, and tissue collected in ice-cold PBS. Wistar rat embryos and adult mouse adrenals were fixed in 4% paraformaldehyde (PFA, Sigma) for varying times: mouse adrenals, 2 hrs; rat embryos e13.5 and e14.5, 8 hrs; e15.5, 12 hrs; e17.5 and e19.5, 16 hrs; cryoprotected in 30% sucrose and embedded in OTC compound (Fisher). Sagittal slices were obtained on a freezing-microtome (Leica GM 1510 S or Leica 3050 S) at 12 µm (embryos) or 5 µm (adult mouse adrenal) thickness, and mounted on Superfrost Plus slides (VWR). Adult male (Wistar and Sprague Dawley) and female (Lewis) rat adrenals were snap frozen or embedded in paraffin. Paraffin embedded sections were obtained using a microtome (Leitz 1512) at 6 µm thickness. Shh-LacZ mice were maintained on a mixed 129.B6.SW background, and genotyped by PCR as described (Jeong et al., 2004; King et al., 2009).

2.2 RNA probe design, labeling and non-radioactive in situ hybridization (NR-ISH)

RNA was extracted from adult adrenal tissues using RNeasy Mini kit (QIAGEN), and cDNA was prepared from random-primed total RNA using Maloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT, Promega). Shh, Ptch1, Gli1, tyrosine hydroxylase, collagen type I (Col1a1) and SF-1 cDNA fragments were PCR amplified using the following primers: Shh F: 5'-AAGCTTCGAGTGACTGAGGG-3', R: 5'-CTGGACTTGACTGCCATTCC-3' (957 bp); Ptch1 F: 5'-ACCAAGTGGACAGTTGGGAG-3', R: 5'-GAAGGCAGTGACATTGCTGA-3' (860 bp); Gli1 F: 5'-ACTAGAGGGCTACAGGGGGA-3', R: 5'-TCCAGCTGAGTGTTGTCCAG-3' (1046 bp); Col1a1 F: 5'-ACGTCCTGGTGAAGTTGGTC-3', R: 5'-CGATCCAGTACTCTCCGCTC-3' (1079 bp); tyrosine hydroxylase F: 5'-GCTACCGAGAGGACAGCATC-3', R: 5'-GGGCTGTCCAGTACGTCCAAT-3' (628 bp); SF-1 F: 5'-TGAAGCAGCAGAAGAAAGCA-3', R: 5'-ACCTCCACCAGGCACAATAG-3'. Amplified cDNAs were cloned into the dual promoter vector pGEM-T easy (Promega) and linearized with the appropriate restriction enzymes. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were synthesized by *in vitro*

transcription in the presence of DIG-labeling mix (Roche) using ~1 µg of linearized template and T7 or SP6 RNA polymerase (New England Biolabs). The concentration and integrity of each RNA probe was analyzed by gel electrophoresis and spectrophotometrically; for each probe, the transcription reaction was diluted with diethylpyrocarbonate (DEPC)-treated H₂O to a concentration of 100 ng/µl transcript, aliquoted, and stored at -80°C. All probes were used at a concentration of 400–800 ng/ml of hybridization buffer. Each probe was designed to include regions of low nucleotide identity with other related family members or other sequences located in the NCBI nucleotide database.

Sections were either dehydrated through ascending alcohol series (paraffin) or fixed in PFA (fresh frozen or cryoprotected). NR-ISH was performed as described in Guasti et al., (2005). When co-labeling was desired, slides, after *in situ* hybridization staining, were blocked with 5% normal goat serum (Sigma) and incubated with mouse monoclonal antibodies (anti-CYP11B1 and CYP11B2) diluted 1:20 in PBS/Triton X-100 0.05% (T-PBS)/Na Azide 0.1% overnight at room temperature. After three washes with T-PBS, slides were incubated for 2 hr with goat anti-rabbit Alexa Fluor 488 secondary (Invitrogen) diluted 1:1000 in PBS and, after further washes, reacted with 4', 6-diamidino-2-phenylindole (DAPI, Sigma) for 1 min. Images were acquired using a Leica DMR microscope (Leica), and digital images were captured using a Leica DC200 camera (Leica) and DCViewer software (Leica). For co-labeling, images were imported into Photoshop (Adobe Systems, San Jose, CA), and bright field *in situ* hybridization images were converted into black and white, inverted and pseudo-colored to red before merging them with the immunofluorescence images in RGB mode.

2.3 Immunostaining

Sections were either air dried (cryosections) or deparaffinized (paraffin embedded), treated with 3% hydrogen peroxide in PBS for 30 mins, and then boiled with Antigen Unmasking solution (Vector Laboratories). They were then blocked with 5% normal goat serum (Sigma) and incubated overnight with anti-CYP11B1, anti-CYP11B2 (both 1:20) or anti-Inner Zone Antigen (IZA, 1:20). Sections were washed in T-PBS and incubated with a biotinylated goat anti-mouse IgG secondary (Vector Laboratories) diluted 1:500 in T-PBS for 2 h. They were washed, treated with avidin-biotin complex (ABC Elite kit, Vector Laboratories) according to the manufacturer's instructions, rinsed again and incubated with diaminobenzidine with or without nickel (Vector Laboratories). The reaction was stopped with H₂O and slides were dehydrated and coverslipped using DPX mounting medium (Fisher). Images were acquired as described above.

Immunofluorescent detection of Shh-LacZ and CYP11B2 on mouse sections was performed according to King et al., 2009.

3. Results and discussion

3.1 Shh is expressed in the subcapsular region of the developing rat adrenal cortex

The mRNA expression pattern of genes encoding *Shh*, its receptor *Ptch1* and a transcription factor induced by Hh signaling, *Gli1*, was determined using non-radioactive *in situ* hybridization (NR-ISH). This approach was chosen as we failed to obtain reproducible results on tissue sections with a set of commercially available antibodies raised against Hedgehog pathway components (not shown). We therefore designed and tested antisense RNA probes for these genes, and confirmed they specifically detect *Shh*, *Ptch1* and *Gli1* expression in previously documented embryonic locations (Supplementary Figure 1).

We next examined gene expression patterns in the region of the developing adrenal gland. Adrenal capsule cells are not easy to distinguish from the cortex and surrounding tissues

based solely on their morphology prior to e16.5. Therefore we used a probe specific for the capsule marker collagen type I (Col1A1; Otis et al., 2007), to identify capsule cells. *SF-1* expression was used to identify cells of the adrenal cortex, while *tyrosine hydroxylase* (TH) expression was used to identify sympathoadrenal cells of the forming medulla. Unambiguous *Shh* expression was first detected at e14.5, in cells located underneath the capsule (Fig 1). Later in development (e17.5 and e19.5) *Shh* expression is clearly localised directly underneath the capsule in a region that does not express the ZG marker CYP11B2, or the ZF markers CYP11B1 and inner zone antigen (IZA) (Min et al., 2004). However, cells in this location do express *SF-1*. Mitani et al. also did not detect CYP11B2 expression at e19.5 in the rat, indicating that a mature ZG has not formed by this age. They did observe a region underneath the capsule of e18 rat adrenal glands that appeared not to express CYP11B1 (Mitani et al., 1999) and suggested that this might represent the undifferentiated zone (ZU) in the embryonic adrenal gland. Our results are consistent with this observation and suggest that *Shh* is a marker of the embryonic ZU.

3.2 *Shh* receiving cells are localized primarily in the capsule of the developing adrenal gland

The localization of *Shh* receiving cells was determined by means of *Ptch1* and *Gli1* mRNA expression. Cells expressing *Ptch1* and *Gli1* were present almost exclusively in the adrenal capsule from early stages of adrenal development (Fig 2), which is similar to the murine expression patterns (King et al., 2009, Huang et al., 2010). At all embryonic stages some *Ptch1* and *Gli1* positive cells can also be observed in the cortex just beneath the capsule. This is particularly noticeable at e17.5 and e19.5 (arrows in Fig 2J, K, M and N). Non-steroidogenic *Gli1*-positive cells were also observed in the subcapsular murine adrenal cortex (King et al., 2009) and these may be an equivalent population in the rat.

3.3 *Shh*, *Ptch1* and *Gli1* expressing cells in the adult adrenal

In the adult adrenal, *Shh* mRNA was detected exclusively in the subcapsular region of the cortex (Fig 3A), and all cells expressing *Shh* were *SF-1* positive (Fig 3B). When adjacent sections were stained with antibodies to CYP11B1 and CYP11B2 (Fig 3B), it appeared that the majority of *Shh* producing cells express neither CYP11B1 nor CYP11B2 and are located between the CYP11B2 and CYP11B1 positive layers, indicating that *Shh* also marks the ZU in the adult. To determine the location of the *Shh* positive cells more precisely, we combined non-radioactive *in situ* hybridization for *Shh* mRNA with immunofluorescence staining for CYP11B1 and CYP11B2 on the same section: in agreement with the analyses of adjacent sections, all cells that express *Shh* mRNA are CYP11B1 negative, while in the ZG some *Shh* positive cells nearest the capsule are also CYP11B2 positive (Fig 3C). Interestingly, not all the cells of the ZU are *Shh* mRNA positive, with a one to three cell wide layer of *Shh*/CYP11B1 negative cells clearly present between the *Shh* positive layer and the ZF (white brackets). This was observed in three different rat strains (Wistar, Lewis and Sprague Dawley; upper panels of Fig 3D). Based on these observations, we propose that the adult ZU can therefore be further divided into an Outer-ZU (CYP11B1/CYP11B2 negative, *Shh* positive) and a smaller Inner-ZU (CYP11B1/CYP11B2/*Shh* negative). *Ptch1* and *Gli1* expression was found to be almost exclusively in the capsule (Fig 3E).

These data indicate that the developmental expression and localization of the *Shh* signal pathway are largely similar in the rat and mouse adrenal but with some interesting differences. The general timing of expression and the subcapsular *Shh* expression and capsular location of signal receiving cells are similar in the two species. However, the subcapsular arrangement of CYP11B2 and *Shh* positive cells clearly differs between mouse and rat adrenals. In the mouse, *Shh* and CYP11B2 positive cells form discrete clusters which abut each other and both the capsule and ZF (lower panel of Fig 3D, King et al., 2009).

However, in the rat (Fig 3B and C) the Shh expressing cells form a continuous layer between the CYP11B2 positive cells of the ZG and the CYP11B1 positive cells of the ZF.

Separation of the Shh cell layer from the capsule by the CYP11B2 positive cells under the non-remodelling conditions employed here, with adrenals taken from unstressed animals on standard chow, raises an interesting question about adrenocortical cell origins in the rat. If, as in the mouse, capsular cells delaminate and enter the cortex to become steroidogenic, then either they migrate through the ZG as undifferentiated CYP11B2 negative cells, or they transiently acquire a CYP11B2 positive differentiated status before downregulating CYP11B2 and upregulating Shh upon entering the ZU. In the embryo, where we have detected relatively frequent transformation of capsule cells in the mouse, this is not an issue, as the differentiated ZG has not yet formed. Postnatally, our murine genetic lineage tracing data indicate this is a relatively rare event (King et al., 2009), and thus we favour the idea of small numbers of migrating CYP11B2 negative cells, which we anticipate would be difficult to detect, in the ZG layer. We have also found that the thickness of the ZG varies between animals (Fig 3D), and under normal conditions some Shh cells may be in close proximity with the capsule. Thus it is also possible that migration from the capsule occurs preferentially in such regions.

If the ZU does indeed represent a stem/progenitor pool then bidirectional differentiation of the Shh positive cells would presumably be required to produce the ZG and ZF. Indeed, we do observe some Shh/CYP11B2 positive cells (Fig 3D), and they might represent cells migrating outwards from the Shh layer and converting to a ZG identity. This would therefore be equivalent to those with the similar phenotype observed in the mouse (Fig 3D lower panel; King et al., 2009). Centrifugal migration of BrdU labeled cell populations from the ZU into the ZG was described for rats transferred onto a low sodium diet (Mitani et al., 2003). Our data are consistent with the idea that the cells marked by BrdU in those experiments are descendants of the Shh positive cells of the ZU. Whether some or all of these ZG cells subsequently migrate through the ZU to become ZF cells under normal or extreme physiological conditions is an interesting question.

A recent study of human adrenal zonation shows CYP11B1/CYP11B2 negative cells both between CYP11B2 positive cell clusters and between the CYP11B1 and B2 positive layers (Nishimoto et al., 2010), suggesting that the zonation of the human cortex has features of both the mouse and rat. We consider the ZU to be a region of relatively undifferentiated, SCC and SF-1 positive, CYP11B1 and B2 negative, cells, present in all species examined so far, albeit with variable distribution.

The demonstration that the ZU in the rat can be separated into a Shh positive, outer zone and a Shh negative, inner zone is very interesting. The Shh positive region appears to be similar to that of preadipocyte factor-1 (Pref-1, DLK-1), which is also expressed in the outer region of the ZU. Pref-1 is downregulated following adrenal enucleation, and re-expressed following re-establishment of the ZF (Halder et al., 1998). It is well documented as an inhibitor of adipogenesis (Sul, 2009), and in light of this has been suggested to be a negative regulator of adrenocortical differentiation. Its apparent co-expression with Shh suggests that interplay may exist between these two secreted factors in modulating the growth and development of the adrenal gland and this clearly warrants further investigation. The function of the Shh-negative inner ZU is not known. It is unclear whether a similar population exists in the mouse, where steroidogenic cells that lack Shh, CYP11B1 and CYP11B2 expression have not been observed. However, given the non-laminar distribution of the Shh and CYP11B2 positive cell clusters in the murine ZG, such cells might be more difficult to detect than in rat. The inner ZU might represent a transition zone where cells have lost Shh expression but have not yet upregulated CYP11B1 expression as they migrate

centripetally, or it might represent a more basal stem cell population (Mitani et al., 2003), that could upregulate Shh expression depending on the requirement for de novo production of ZG or ZF cells, such as during remodelling. These possibilities are currently under investigation, along with the search for markers of the inner ZU.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

Shh	Sonic hedgehog
ZU	Undifferentiated Zone
ZF	Zona Fasciculata
ZG	Zona Glomerulosa
ZR	Zona Reticularis

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Figure 1. Shh is localized at the periphery of the developing adrenal cortex

Shh (A and E) mRNA at e14.5 and e15.5, respectively, is localized mainly in the subcapsular compartment of the adrenal cortex. The capsule (“C”) is unstained. At these stages, cortical cells are already strongly positive for SF-1 transcript (C and D for e14.5 and G for e15.5) and medullary cells, some of which appear to be still migrating (arrows in H), are expressing tyrosine hydroxylase (TH). At later stages, when CYP11B1 (I and J for e17.5, Q and R for e19.5), but not CYP11B2 (K for e17.5, S for e19.5, arrow shows adrenal position. L is an adult section used as a positive control) is detected, the subcapsular expression of Shh (M and N (higher magnification view of boxed section in M) for e17.5, U and V (higher magnification view of boxed section in U) for e19.5) is very clear. Note that Shh mRNA expressing cells are not CYP11B1 or IZA positive, but are SF-1 mRNA positive (compare panel N with J and P (higher magnification of boxed section in O) or panel V with R and T), suggesting that Shh expression marks cells in the ZU. Sections incubated with Shh sense probe (B, F and n) did not show any specific staining. K: kidney. Scale bars: 200 μm.

Figure 2. Shh receiving cells are localized in the capsule of the developing adrenal cortex
Ptch1 mRNA expression at the indicated ages is shown on left panels (A, D, G, J, M), *Gli1* expression in the middle panels (B, E, H, K, N). *SF-1* in situ for e14.5 is shown in C, while the other panels on the right indicate *Col1A1* mRNA expression (F, I, L, O). Arrows in J, K, M and N indicate cluster of positive cells in the cortex. Scale bar: 200 μ m

Figure 3. Localization of *Shh* expressing cells in the adult rat adrenal

A) Low magnification image of adrenal sections incubated with *Shh* antisense (AS) and sense (S) riboprobes. Staining using antisense probe is present only at the periphery of the cortex and absent in the medulla (Med). The sense probe resulted in no specific staining. **B)** Adjacent adrenal sections were processed for *Shh* and SF-1 NR-ISH, and CYP11B1 (B1) and CYP11B2 (B2) immunohistochemistry. Note that the majority of *Shh* expressing cells are located in the Undifferentiated Zone, between the ZG and ZF. Cells expressing *Shh* mRNA (at a lower level) are located directly underneath the capsule therefore potentially CYP11B2 positive. **C)** Combined *Shh* NR-ISH and CYP11B1/CYP11B2 immunofluorescence. Nuclei are stained with DAPI. A minority of CYP11B2 positive cells in the ZG are also *Shh* positive (yellow arrows). White brackets indicate a layer of cells above the ZF that are not *Shh* positive (Inner ZU). **D)** The Outer- and Inner-ZU can be identified in the adrenal cortex from rats of different strains (upper panels). Sections derived from rats of the indicated strains were processed for *Shh* NR-ISH and then incubated with antibodies to CYP11B1/CYP11B2. Cells in between white brackets are *Shh*/CYP11B1/CYP11B2 negative and represent the Inner-ZU, while the majority of the *Shh* positive cells are in the Outer-ZU (some CYP11B2/*Shh* double positive cells indicated with yellow arrows). Expression of *Shh* and CYP11B2 in the adult mouse adrenal cortex is shown in the lower panel, where sections derived from adult *Shh*-lacZ mouse adrenal tissue were processed for lacZ and CYP11B2 expression by immunofluorescent staining: clusters of lacZ positive cells (green arrows), rather than a continuous layer, are juxtaposed with clusters of CYP11B2 positive cells (red arrows). Occasionally, double positive cells can be detected (yellow arrow). **E)** *Ptch1* and *Gli1* mRNA expression in the adult adrenal. Vertical bars indicate the capsule. Scale bars = 50 μ m