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Sonic hedgehog signaling during adrenal development

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Abstract

It has been speculated for a number of years that Sonic hedgehog (Shh) signaling plays an important role in adrenal development. Over the past two years several reports have described the expression and function of *Shh* pathway genes in the adrenal cortex, using primarily mouse models. The key findings are that Shh signals produced by a population of partially differentiated cortical cells located in the outer cortex/zona glomerulosa are received by non-cortical mesenchymal cells located predominantly in the overlying capsule. This signal is required for growth of both the capsule and the cortex, but not for cortical zonation or steroidogenic cell differentiation. Using molecular genetic tools to define the adrenocortical cell lineages that are descended from both Shh signaling and receiving cells, both capsule and cortical cells were found to have properties of adrenocortical stem and/or progenitor cells. Here we place these observations within the context of prior studies on adrenal development, postnatal adrenal maintenance and adrenocortical stem/progenitor cell lineages.

Keywords

adrenal cortex; steroidogenic; Sonic hedgehog; Gli; development; signaling; Pallister-Hall syndrome

1. Introduction

Development of the adrenal gland, as for any other organ, is a complex process involving a highly choreographed interplay of cell behaviors. These involve both cell autonomous components, typically mediated by the output of cell-specific combinations of transcription factors, and non-autonomous components, mediated by multiple families of intercellular signaling molecules. In this review we focus on recent advances in defining the roles during adrenocortical development of the intercellular signal Sonic hedgehog, and the signaling pathway through which it acts, based primarily on data from mouse models. For more

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comprehensive reviews of the transcription factors and other signals involved in adrenal development see (Else and Hammer, 2005; Kim and Hammer, 2007; Val and Swain, 2010; Morohashi and Zubair, 2011; Wakil and Lalli, 2011).

2. Overview of adrenal development

Overt adrenal development begins at midgestation (Figure 1). The two adrenogonadal primordia (AGP) are initially visible as stripes of epithelial cells extending rostrocaudally just lateral to the dorsal midline of the coelomic epithelium at thoracic levels (Hatano et al., 1996). Each AGP contains a mixed population of adrenocortical and somatic gonad progenitor cells, all of which express the transcription factor Sf-1/Ad4BP (steroidogenic factor-1/Adrenal-4-binding protein, NR5A1; hereafter Sf-1), which is a key regulator of steroidogenic pathway gene expression and steroidogenic cell identity (Luo et al., 1994; Hatano et al., 1996; Morohashi, 1997). Shortly thereafter the proliferating Sf-1 cells delaminate from the epithelium and invade the underlying mesenchyme of the intermediate mesoderm. The vast majority of these cells migrate dorsolaterally to form the gonadal anlagen, while a subset from near the rostral end of the AGP that express the highest levels of Sf-1 migrate dorsomedially to form the adrenal anlagen, settling ventrolateral to the dorsal aorta (Ikeda et al., 1994; Hatano et al., 1996; Val et al., 2007). As the Sf-1 population expands it is invaded by neural crest cells that have migrated from the dorsal midline just lateral to the neural tube (Val et al., 2007; Kim et al., 2008). These neural crest cells will form the neuroendocrine cells of the adrenal medulla, and do not contribute to the adrenal cortex. Following the neural crest invasion, the adrenal primordium becomes encapsulated within a population of mesenchymal cells (Keegan and Hammer, 2002). The presumptive cortical and medulla populations proliferate rapidly, and sort into a central medulla and surrounding cortex, all encased in a stromal capsule that is several cell layers thick. The adrenal gland during these stages becomes heavily vascularized, and is innervated by projections from preganglionic motor neurons and sympathetic sensory neurons. Notably, while it is clear that the Sf-1 population derived from the coelomic epithelium contributes to the steroidogenic lineages of the cortex, potential contributions from the adjacent mesenchyme to these lineages have not been well defined. As the adrenal is growing it also erupts from the mesenchyme, and is contacted by the growing kidney, which develops from more caudal intermediate mesoderm. In the mouse the AGP forms at about 9.5–10.0 dpc, the adrenal anlagen segregates at about 10.5 dpc, neural crest invasion is complete by about 12.5 dpc and separation of the gland from surrounding mesenchyme is largely complete by about 14.5 dpc.

The adrenal Sf-1 cell population initially contributes to the fetal adrenal/X zone and subsequently to the definitive, or adult, adrenal gland (Zubair et al., 2006; 2008; 2009; Morohashi and Zubair, 2011). The fetal adrenal/X zone in mice is a transient population of unknown function that persists until sexual maturity in males, and first pregnancy in females (Miller, 1927; HersHKovitz et al., 2007). The cells of the definitive adrenal segregate into concentric steroid-producing zones, of which there are two in mice and rats. The zona fasciculata (ZF), which produces glucocorticoids in response to signals from the hypothalamus and pituitary (HPA axis), develops first and comprises the inner zone, while the zona glomerulosa (ZG), which produces the mineralocorticoid aldosterone under the control of the renin-angiotensin system (RAS), develops later and is the outer zone (Keegan and Hammer, 2002). In mice differentiation of the steroidogenic populations begins at about 11.5–12.5 dpc, when expression of basal steroidogenic enzymes such as the cholesterol side chain cleavage enzyme (scc, Cyp11a1), and accumulation of mitochondria and endogenous biotin become detectable (Schulte et al., 2007; Val et al., 2007; Paul and Laufer, 2011). Expression of the terminal enzymes begins later. Cyp11b1 (11-beta-hydroxylase), which labels the mature ZF, is detectable at about 13.5 dpc and is expressed at high levels one day

later. Cyp11b2 (aldosterone synthase), which labels cells with terminal steroidogenic capacity in the ZG, is not expressed until within one day or so of birth (Mitani et al., 1997; Wotus et al., 1998; King et al., 2009).

Once the functional adult zones are established, they are maintained throughout life, although their size can be remodeled in response to physiological changes mediated via altered RAS or HPA axis activity (Mitani et al., 1994; 1996; Bland et al., 2003; Ulrich-Lai et al., 2006). In addition to this zonal remodeling, there is also a continuous low level turnover of cells in the steroidogenic zones. Most cortical proliferation occurs near the periphery of the adrenal (ZG through outer ZF), and cells slowly transit the gland towards the medulla, in a process known as centripetal displacement (Zajicek et al., 1986; Mitani et al., 2003; King et al., 2009). These and similar observations have led to the long-held idea that there exist adrenocortical stem or progenitor cells, located either in the outer steroidogenic cortex or in the non-steroidogenic capsule (Kim and Hammer, 2007; Kim et al., 2009; Wood and Hammer, 2011). However the location and molecular identity of these putative progenitor populations has been unclear.

3. The vertebrate hedgehog pathway

Sonic hedgehog (Shh) is a member of an evolutionarily conserved family of proteins that, along with Desert hedgehog (Dhh) and Indian hedgehog (Ihh) in mammals, plays important roles in embryonic development, adult stem cell maintenance and cancer (McMahon et al., 2003; King et al., 2008; Gupta et al., 2010; Ingham et al., 2011). Hedgehog (Hh) family proteins are secreted molecules that act upon target cells by binding to multiprotein receptor complexes. In vertebrates these complexes include Patched1 (Ptch1), the canonical Hh receptor that is a twelve pass transmembrane Hh binding protein, along with either of the single pass transmembrane Hh binding proteins Cdo (cell-adhesion-molecule-related/downregulated by oncogenes) or Boc (brother of Cdo/biregional Cdo-binding protein), or the GPI-anchored protein Gas1 (growth arrest specific) (Allen et al., 2011; Izzi et al., 2011). In the absence of Hh, Ptch1 is localized to the base of the primary cilium from which it, by an as yet not fully understood mechanism, excludes the seven pass transmembrane protein Smoothened (Smo). Inhibition of Ptch1 activity upon Hh binding leads to the derepression of Smo and its accumulation in the cilium. The transcription factors Gli2 and Gli3, which have both activator and repressor activities, are also found in the cilium and, in the absence of activated Smo, Gli2 is largely proteolytically degraded and Gli3 (and a proportion of Gli2) is cleaved to produce a transcriptional repressor, consequently inhibiting transcription of Hh responsive genes. Smo activation inhibits the proteolysis of these factors leading to loss of the repressive forms and accumulation of the full length activator forms, promoting transcriptional activation. Among the direct targets of Hh signaling is the transcriptional transactivator *Gli1*, which further augments Hh target gene transcription (Bai et al., 2002; Ahn and Joyner, 2004). Activation of the pathway thus shifts the ratio of Gli repressor and activator forms, allowing for a variable output proportionate to the magnitude of the Hh signal. The requirement for each Gli transcription factor in eliciting Hh signaling responses varies in a tissue-dependent manner, with differential requirements for transcriptional activator and repressor functions (Wang et al., 2000; Ingham and McMahon, 2001; Persson et al., 2002; Bai et al., 2004).

4. Shh is expressed by immature cells of adrenal steroidogenic lineages

Shh expression has been described in rodent adrenal glands, with the most extensive studies performed on murine embryonic and postnatal tissue using mRNA in situ hybridization analysis and transgenic reporters. In the embryonic mouse adrenal cortex, *Shh* mRNA expression is detected as early as 12.5 dpc, in clusters of cells at the periphery of the

adrenocortical anlagen (King et al., 2009). Similar patterns of mRNA expression were reported throughout embryogenesis (Bitgood and McMahon, 1995; Ching and Vilain, 2009; King et al., 2009; Huang et al., 2010) and into adulthood (Ching and Vilain, 2009). Two *Shh* reporter alleles were also used to monitor *Shh* expression. One contains a GFP:Cre fusion protein targeted to the *Shh* locus that is fused in frame with the endogenous *Shh* ATG, replacing the *Shh* coding sequence (Harfe et al., 2004), while the other contains a nuclear LacZ ORF downstream of an IRES sequence targeted to the 3' UTR of the *Shh* locus (Lewis et al., 2004). Both of these reporters also showed expression at the periphery of the cortex in clusters of cells from embryogenesis through adulthood (King et al., 2009).

Defining the onset of *Shh* expression is important for interpreting *Shh* mutant phenotypes. *Shh* mRNA expression was not detected in the adrenogonadal primordium (AGP) prior to the separation of the adrenal and gonadal anlagen, nor was it detected in the intermediate mesoderm or developing gonad (King et al., 2009). Genetic Cre-Lox lineage analyses using the *Shh*-GFP:Cre allele as a Cre driver in combination with conditional R26-LacZ or R26-YFP reporters also revealed that descendants of cells that had expressed *Shh* were not detectable in the AGP, the intermediate mesoderm or the gonad at least through 13.5 dpc (King et al., 2009). Labeled cells were first detected in the adrenal anlage at 11.5 dpc, although only a very small fraction of cells were marked, and increasing numbers were present thereafter (King et al., 2009). Previous reports suggest there is a lag of up to 12 hours before reporter expression is detectable from the onset of Cre expression (Danielian et al., 1998), placing the timing of initial *Shh*-GFP:Cre expression around 11.0 dpc. These results, in conjunction with the *Shh* reporter data, reveal that *Shh* is not expressed in the lineages that give rise to the adrenal gland, and suggest that expression begins in a subset of the adrenal founder cell population almost immediately after they have segregated from the AGP.

Shh mRNA or transgenic reporter expression were compared to expression on adjacent sections or by multichannel coimmunofluorescence with either pan-adrenocortical markers such as Sf-1 or endogenous biotin, or zonal markers such as Cyp11b2 (ZG) or Cyp11b1 (ZF) to define the population of cells expressing *Shh*. These studies consistently revealed that all cells expressing *Shh* also express pan-adrenocortical markers (King et al., 2009; Paul and Laufer, 2011). Furthermore the vast majority do not express either Cyp11b2 or Cyp11b1, although the *Shh* cells are unambiguously located within the ZG, with cell clusters that express *Shh* interspersed with clusters that express Cyp11b2 (King et al., 2009). The few *Shh* cells that do overlap with Cyp11b2 or Cyp11b1 cells were marked by weak LacZ expression, and it is likely that this represents perdurance of the LacZ protein rather than *bona fide* *Shh* expression, as GFP protein expressed from the *Shh*-GFP:Cre allele does not extend into the ZF (King et al., 2009). Taken together these data indicate that *Shh* expression marks a population of adrenocortical cells that are within the steroidogenic lineages, but that do not express the terminal enzymes required for mineralocorticoid or glucocorticoid biosynthesis.

A study of *Shh* expression in the rat adrenal gland (Guasti et al., 2011) also reported *Shh* mRNA near the cortical periphery. However unlike in the mouse, the rat adrenocortical cells that express *Shh* are not found in clusters immediately underneath the capsule, but rather are present in a continuous layer immediately underneath the ZG cells that express Cyp11b2. Interestingly there is also a very thin layer of cells that do not express *Shh* between the *Shh* cells and the ZF cells that express Cyp11b1. Thus *Shh* is expressed within the outer undifferentiated zone previously described in the rat, a region that also expresses Pref-1 (Halder et al., 1998) and Dab-2 (Romero et al., 2007).

SHH expression in human adrenal glands has not yet been described. However the *CYP11B2* domain, as defined by mRNA or protein expression is discontinuous in the human ZG (Boulkroun et al., 2010; Nishimoto et al., 2010), and the DAB2 expression domain encompasses the *CYP11B2* domain, while also extending laterally to mark regions that lack *CYP11B2* expression (Boulkroun et al., 2010). By analogy with the rodent expression patterns, this suggests human *SHH* is likely to be expressed in patches of the ZG, more similar to mouse than rat.

5. Shh signals to non-steroidogenic cells of the capsule and interstitial mesenchyme

Since Shh is a secreted signaling protein, it is important to define which cells in the adrenal gland respond to this signal during normal embryonic development as well as postnatally. Genes encoding two components of the *Shh* signaling pathway are targets of *Shh* signaling and their expression is upregulated in cells actively transducing an Shh signal. One of these is the Shh receptor, *Ptch1*, which is expressed at low levels in the absence of Shh, and this expression is increased when cells transduce a Hh signal (Goodrich et al., 1996). The other is the transcription factor *Gli1*, which is not expressed in the absence of a signal, but is induced upon signal transduction (Ahn and Joyner, 2004). Both of these genes were used to identify putative Shh target cells, and provided similar results.

Both mRNA expression and transgenic LacZ reporter alleles (Goodrich et al., 1997; Bai et al., 2002) were used to assess *Ptch1* and *Gli1* expression in the mouse adrenal (Ching and Vilain, 2009; King et al., 2009; Huang et al., 2010). In the embryonic adrenal, *Ptch1* and *Gli1* expression are detectable from about e12.5 at the cortical periphery, consistent with the approximate onset of *Shh* expression. While initial reports suggested that *Gli1* expression is undetectable postnatally (Ching and Vilain, 2009), subsequently it was found to be present throughout adulthood (King et al., 2009). Comparison with morphological and molecular markers revealed that *Ptch1* and *Gli1* are expressed by cells that are in the adrenal capsule, with higher expression in cells closer to the cortex. In addition, during embryonic development and at early postnatal ages *Gli1* cells are scattered throughout the forming cortex. However in all cases the *Gli1* cells do not also express Sf-1 (King et al., 2009; Huang et al., 2010), and *Gli1* is thus not expressed within cells of the steroidogenic lineages. Taken together these studies suggest that the cells that actively respond to Shh are present throughout life in the inner capsule, overlying the cells that produce the Shh signal. During adrenal development Shh-responsive cells also appear to consist of interstitial mesenchyme that was perhaps incorporated into the coalescing adrenal anlagen, as well as surrounding mesenchyme of the intermediate mesoderm, neither of which express Sf-1.

These data imply that even though *Shh* is expressed by peripheral cortical cells with steroidogenic potential, the steroidogenic lineages themselves are insensitive to Shh, and do not directly require this signal for their development, maturation or maintenance. Consistent with this idea, genetic deletion of the obligate Shh pathway signal transducer *Smo* from the steroidogenic lineages, using a conditional floxed *Smo* allele (Long et al., 2001) in combination with an Sf-1-cre driver allele (Bingham et al., 2006), resulted in normal adrenocortical growth, zonal organization, and steroidogenic cell differentiation (King et al., 2009).

6. Shh is required for the development and maintenance of the adrenal capsule and cortex

6.1 Adrenal development in Pallister-Hall syndrome in humans and mouse models

Several lines of evidence implicate Shh signaling as critical for normal adrenocortical development. The first derives from studies of humans with Pallister-Hall syndrome (PHS; OMIM 146510), for which the diagnostic criteria include hypothalamic hamartoma and central digit polydactyly (Biesecker et al., 1996). PHS is a dominant genetic disorder that displays variable expressivity. It is caused by truncations in the open reading frame encoding the GLI3 protein, which result in the production of GLI3 with constitutive transcriptional repressor activity (Kang et al., 1997). Five of the six original PHS cases presented with adrenal hypoplasia or aplasia (Hall et al., 1980), which might be caused by impaired Hh signaling during adrenal development. However as these patients also suffer from hypopituitarism, the adrenal phenotypes were proposed to be secondary to the pituitary defects (Hall et al., 1980). Subsequent descriptions of PHS patients have not described adrenal phenotypes, so it is unclear how frequently morphological adrenal defects are present, although approximately half of PHS patients do have pituitary hypoplasia.

Mice homozygous for a *Gli3* mutation (*Gli3*^{Δ699/Δ699}) that encodes a protein truncated in a similar fashion to PHS mutations are not viable, but were reported to have embryonic adrenal aplasia (Böse et al., 2002). Murine *Pomc* (proopiomelanocortin), which encodes the precursor polypeptide to the pituitary to adrenal peptide signal ACTH (adrenocorticotrophic hormone), is not required for adrenal development (Karpac et al., 2005; 2007). Thus the *Gli3* mutant phenotype is unlikely to be secondary to a pituitary defect, which provides support for the idea that the *Gli3*^{Δ699/Δ699} mutation is directly affecting adrenal development.

However during the course of studies unrelated to adrenal development, we recently observed adrenal glands in *Gli3*^{Δ699/Δ699} mutants (Figure 2). To further investigate whether these adrenal glands have gross abnormalities, we examined sectioned mutant and control adrenal tissue isolated from 18.5 dpc embryos. We found that the mutant adrenals had a cortex and medulla encased in a mesenchymal capsule. We stained the adrenal tissue with streptavidin to detect endogenous biotin, which is exclusively elevated in all cells with steroidogenic potential in the cortex (Paul and Laufer, 2011), and for the expression of tyrosine hydroxylase, which marks medullary neuroendocrine cells. We did not observe any gross differences between mutant and control tissue in the staining patterns detected by either of these markers. Whether there are more subtle defects in adrenal growth or differentiation will require additional analysis. Why the adrenal glands initially were not detected in this mutant is unclear. It might be due to differences in the genetic backgrounds between the two colonies, or perhaps because the adrenals are displaced relative to the aplastic or hypoplastic kidneys found in the mutant animals (Böse et al., 2002). Nonetheless homozygous truncating mutations in *Gli3* are clearly compatible with substantially normal adrenal development. This also provides support for the idea that the severe adrenal defects in some PHS patients, who are heterozygotes for *GLI3* mutations, are secondary to pituitary dysfunction.

6.2 Murine Shh null phenotypes

Three recent studies (Ching and Vilain, 2009; King et al., 2009; Huang et al., 2010) have examined the phenotypic consequences resulting from conditional deletion of a floxed *Shh* allele using the Sf-1-cre driver. All three reported that the mutant mice were viable, and that the mutant adrenals were significantly smaller than in controls. Furthermore one group found that mutant mice had only one adrenal (Ching and Vilain, 2009), although both

adrenals were present in the mice studied by the other two groups (King et al., 2009; Huang et al., 2010). The cause of this discrepancy is unclear, but conditional deletion of *Shh* is clearly compatible with adrenal formation.

There is also evidence suggesting that the conditional deletion of *Shh* does not completely eliminate all adrenocortical Hh signaling activity. When a *Gli1-LacZ* reporter allele was crossed onto the conditional *Shh* mutant background, some LacZ expression was still present in the mutant adrenal capsule. By contrast, when the same reporter was present in *Shh* null homozygotes, no LacZ expression was detectable (King et al., 2009). These results suggest that in the conditional mutants either recombination at the *Shh* locus was incomplete, or that another source of Hh activity was present in the conditional mutant adrenals. However there are no convincing reports of *Ihh* or *Dhh* expression in the normal adrenal cortex. Furthermore the total loss of Gli1-LacZ expression in the *Shh* null mutants suggests that *Shh* is the only source of Hh activity associated with the adrenal, at least during embryogenesis. Thus the conditional mutants might be hypomorphs, rather than complete nulls for adrenal *Shh*.

Further scrutiny of the conditional *Shh* mutant adrenals revealed that there are defects in both the capsule and cortex. The mutant capsule was thinner, with only about one-third the cell density of that in controls (King et al., 2009; Huang et al., 2010). Examination of cell cycle and apoptotic markers revealed that mutant capsule cells proliferate less than in controls, but do not undergo excess programmed cell death (Huang et al., 2010). As the capsule is a direct target of the Shh signal, these data indicate a primary function of cortical Shh is apparently to support the growth of a complete capsule, as well as to maintain it postnatally. More direct experiments will be required to determine if Shh is a capsule cell mitogen, or if the proliferative effects are due to other, perhaps secondary, signals acting on the capsule mesenchyme.

Despite the fact that Shh does not signal directly to cortical cells, the effects of *Shh* deletion on the cortex are dramatic. Adult mutant adrenal glands are 5–10× smaller than in controls, but the medulla is roughly of normal size. Thus the cortex is disproportionately reduced in *Shh* mutants, and the growth defects are visible during embryogenesis, as early as 13.5 dpc (Ching and Vilain, 2009; King et al., 2009; Huang et al., 2010). The medulla can be acentric and abut the overlying thin capsule, and sometimes ectopic islands of medulla cells are present, although the medullary cells differentiate apparently normally and express neuroendocrine markers (Ching and Vilain, 2009; King et al., 2009; Huang et al., 2010). Despite the reduction in cortical size, zonation appears normal, with an outer ZG and inner ZF, displaying appropriate expression of Sf-1, Cyp11a1, 3betaHSD, Cyp21, Cyp11b2 and Cyp11b1 (Ching and Vilain, 2009; King et al., 2009; Huang et al., 2010). However the individual cells in both the ZG and ZF of mutants are hypertrophic in adults (Ching and Vilain, 2009; King et al., 2009; Huang et al., 2010). Thus the predominant cortical defect is a failure to grow, but not to differentiate or undergo appropriate zonation.

In mutant animals circulating corticosterone levels are approximately normal perinatally, but are several fold reduced compared to controls at one year, when plasma ACTH levels are elevated (Huang et al., 2010). This suggests that the ZF cells in mutant adrenals have sufficient steroidogenic capacity when body mass is small, but fail to compensate fully for the adrenal hypoplasia as the animals grow.

The morphological defects in the *Shh* mutants present a conundrum: if the Shh signal is received only by cells that are predominantly located in the capsule and are outside the steroidogenic lineages, why is the mutant cortex so severely affected? One possibility is that the capsule is required for the survival or growth of the cortical population because it

produces secondary signals that act on the cortical cells. No increase in cell death was observed in the mutants, strongly suggesting there is no Shh-dependent trophic signal produced by the capsule required to maintain cortical viability (Huang et al., 2010). By contrast, fewer PCNA positive cells were detected in the cortex of *Shh* mutants (Ching and Vilain, 2009), which is consistent with the idea of a secondary signal required for cortical growth. The identity of a putative Shh-dependent secondary signal that is produced by the capsule but acts on the cortex is currently unknown, although there are some candidates.

The concept of reciprocal signaling pathways involving *Shh* is well established (Laufer et al., 1994; Niswander et al., 1994; McMahon et al., 2003), and there are at least two families of signal that might play such a role in the adrenal gland, based on previously described phenotypes. One possibility is that a Shh-dependent canonical Wnt signal is produced by the capsule. Canonical Wnt signals are transduced via intracellular signaling pathways that utilize the stabilization of cytoplasmic beta catenin and its subsequent translocation to the nucleus to regulate target gene expression (Wakil and Lalli, 2011). Beta catenin levels are elevated in the ZG, and beta catenin is required for the growth and differentiation of the embryonic adrenal cortex (Kim et al., 2008). Furthermore transgenic reporters of canonical Wnt pathway activity are elevated in the ZG (Kim et al., 2008; Ferrer-Vaquer et al., 2010). In addition Shh/Wnt feedback signaling between epithelial and stromal compartments has been documented in other tissues, such as during regeneration of the bladder epithelium (Shin et al., 2011). Thus it is plausible that expression of one or more Wnt ligands in the capsule is dependent on Shh activity.

The fibroblast growth factor (FGF) pathway is another candidate system that might interact with Shh signaling. *Fgfr2* and *Fgfr4* are expressed in the developing adrenal cortex (Stark et al., 1991). Germline deletion of *Fgfr2-IIIb* leads to adrenal dysgenesis (Revest et al., 2001), as does conditional deletion of *Fgfr2* from the *Sf-1* lineage (Kim et al., 2007). Thus response to an FGF signal is required in cortical cells, although the requisite ligands and their possible expression in the capsule are not known.

The possibility that Shh-dependent signals might be produced by the capsule raises the question of which cells might be their relevant targets. One cell population that was proposed to contain the affected cells is a putative cortical progenitor population located in the outer cortex, possibly within the ZG (Huang et al., 2010), although their molecular identity was not defined. Intriguingly the *Shh* expression pattern itself provides some insight into this issue. As *Shh* is expressed by partially differentiated ZG cells, it raised the issue of whether the *Shh* cells are progenitors of other cortical cell types in the murine adrenal gland (King et al., 2009). Genetic lineage analyses were performed using either constitutive or tamoxifen-inducible Cre expressed from the *Shh* locus in combination with conditional floxed reporters. When the constitutive Cre was used, it was found that *Shh* expressing cells give rise to cells in all cortical zones, including Cyp11b2 cells in the ZG, but not in the capsule or medulla. This result reveals that *Shh* expression marks cortical progenitors, at least during embryonic development, when *Shh* expression begins.

When the inducible Cre was used and activated at 14.5 dpc, *Shh* cell descendants were initially present at the periphery of the cortex, and over time grew deep into the cortex. Interestingly all the labeled cells did not simply move centripetally, but rather the labeled population expanded into columns spanning the cortex, indicating some progenitor cells persisted at the cortical periphery. Qualitatively similar results were obtained when the tamoxifen treatment was given to adults, although the cells were only followed for two weeks and transited from the ZG into the ZF. Thus *Shh* expression marks a cortical progenitor population that is itself a candidate target of a Shh-dependent signal from the capsule.

An alternative explanation for the *Shh* mutant phenotype is that a cortical stem or progenitor cell population is located in the capsule, and that these cells are dependent on Shh signaling. In this scenario, capsule cells would invade the cortex, and convert from a mesenchymal, Sf-1 negative phenotype to a cortical Sf-1 positive identity in a Shh-dependent manner. Shh might maintain these cells in the capsule by promoting their proliferation, or might facilitate their steroidogenic conversion or invasion into the cortex. The idea that cortical stem cells are present in the capsule has been around for decades, but there has been scant experimental evidence in its support.

Using inducible genetic lineage tracing similar to that performed for *Shh*, *Gli1* cells were indeed found to contribute to steroidogenic lineages (King et al., 2009; Huang et al., 2010). When the *Gli1* lineage was labeled during embryogenesis, labeled cells were detected in the adrenal glands for up to four months, the longest period described. These cells were present in the capsule, as well as the cortex. Adrenal glands examined a few days after labeling contained cortical *Gli1* descendants adjacent to the capsule (King et al., 2009; Huang et al., 2010), while those examined after periods of weeks to months contained occasional columns of labeled cells extending from the capsule deep into the cortex, sometimes reaching the medulla border (King et al., 2009). The descendants of *Gli1* cells located in the cortex also expressed Sf-1 and *scc*, indicating they had steroidogenic potential, while those in the capsule did not (King et al., 2009; Huang et al., 2010). As Sf-1 expression was not detected in the *Gli1* cells themselves (King et al., 2009; Huang et al., 2010), this provides support for the idea that these cells converted from a *Gli1* positive, Sf-1 negative identity to a *Gli1* negative, Sf-1 positive identity.

These *Gli1* lineage studies raise an additional point about adrenocortical development, which is that they imply that not all Sf-1 cells in the cortex are descended from the initial Sf-1 population that delaminated from the coelomic epithelium. Instead, they suggest that the cortex is a mixture of coelomic epithelial Sf-1 cells that invade the intermediate mesoderm mesenchyme and Sf-1 cells recruited from this mesenchyme directly. Interestingly it has recently been found that the steroidogenic Leydig cells of the testes are derived from both coelomic epithelium and mesonephric mesenchyme (DeFalco et al., 2011).

Could depletion of the *Gli1* lineage account for the *Shh* mutant phenotype? At this point it is too early to tell. First, the *Gli1* lineage analyses were not quantitative so it is unclear what proportion of the cortex is actually derived from the capsule lineage. In the absence of this information, we cannot easily assess the impact of depleting these cells. Second, we do not know whether establishment or maintenance of this lineage or its transformation into Sf-1 positive cells is actually dependent on Shh activity. We only know that the cells are marked by the *Gli1* driver. It would be useful in this regard to have a *Shh*-independent Cre driver that labels all capsule/mesenchyme cells, but is not expressed in the coelomic epithelium or Sf-1 cells.

An alternative hypothesis to account for the origin of the Sf-1 cells has been proposed (Wood and Hammer, 2011). In this model, all Sf-1 positive cells throughout the cortex are derived from the same initial population that expresses Sf-1. Some of the founder cells maintain Sf-1 expression and form the fetal adrenal, while the remaining cells downregulate Sf-1 expression and enter the nascent capsule, before being recruited into the definitive cortex and reactivating Sf-1 expression. If this latter model is correct, then one would expect the capsule to be descended from Sf-1 cells. At a gross level this does not appear to be the case, as the Sf-1-Cre BAC transgene driver (Bingham et al., 2006) does not label the capsule, at least when analyzed postnatally (King et al., 2009). However it is possible that a population of Sf-1 cells undergoes this dual transformation during embryogenesis, and is

depleted from the capsule by early postnatal ages. Lineage analyses using inducible Cre recombinase expressed under the control of a fetal adrenal enhancer element (FAdE) derived from sequences located within the murine Sf-1 fourth intron provide some support for this idea (Zubair et al., 2008; Morohashi and Zubair, 2011). They reveal that if recombination is induced shortly after formation of the adrenal anlagen, then both the fetal adrenal/X-zone and definitive adrenal are labeled. This result suggests that both the fetal and definitive adrenal cortex are derived from the same founder population, rather than being of mixed coelomic epithelial and mesenchymal origin. Resolving this issue will require more precise lineage analyses than have been reported to date.

7. Perspectives

The sophisticated mutational and lineage analyses described here have provided significant new insights into adrenocortical development, and the key signaling and lineage relationships are highlighted in Figure 3. However substantial issues remain. With respect to the function of the *Shh* signal, a key question is how signaling to the overlying capsule impacts the underlying cortex. *Shh* function might modulate reciprocal signaling back to the cortex, or influence the behavior of the mesenchymal cell population that undergoes a transformation into steroidogenic cortical lineages, or it might function through a combination of both modalities. More detailed analyses at cellular resolution should help address these issues. Interestingly these studies emphasize the importance of the adrenal capsule, a critical component of the gland that has received scant experimental attention, particularly in studies of adrenal development. Indeed the correlation between capsule and cortical defects in *Shh* mutants indicates that mutant analyses of genes that are expressed solely in cortical cell populations should also include an examination of capsule morphology and gene expression patterns.

It is also striking that the *Shh* pathway gene expression patterns that are present in the developing adrenal gland persist into adulthood. This implies that *Shh* function is likely to be maintained in adults as well. In particular one can envision that *Shh* signaling is important to maintain the capsule, and that this might in turn help maintain the size of the cortex. The experimental models examined to date all reduced *Shh* signaling starting during embryogenesis. However it is an intriguing question whether these defects would arise *de novo* if *Shh* signaling were abrogated postnatally. In this context, it is also noteworthy that while the conditional *Shh* mutant mice had normal glucocorticoid levels when the animals were young and small, they became deficient as the animals aged and grew. This also raises the possibility that human patients with defects in *Shh* signaling might suffer from previously undetected adrenal dysfunction, either under normal conditions, or perhaps when elevated adrenal steroid production is required. The requirement for *Shh* signaling for normal capsule and cortical growth also raises the possibility that the *Shh* pathway might support the hyperproliferative characteristics of adrenocortical adenoma or carcinoma. Each of these issues is clearly worthy of further investigation.

Finally, *Shh* and *Gli1* expression as markers of candidate cortical stem or progenitor cells has the potential to provide novel insights into our understanding of how adrenal lineages are regulated. However we first need to better define these cell populations, and the lineages to which they can contribute. It is important to recognize that we do not yet know whether either of these genes marks an equipotential cell population, as there might be additional heterogeneity within the capsule or ZG. Furthermore while *Shh* cells can become Cyp11b2 or Cyp11b1 cells, whether this transition is strictly unidirectional, and whether there is a strict progression from one cell type to another is as yet unknown. In addition whether there are temporal variations in these lineage relationships, for instance between development and postnatal maintenance of the gland, is also important to define. Despite these uncertainties,

how these lineages participate in the normal and diseased adrenal cortex remain fascinating questions for the future.

Highlights

- Shh signal produced by partially differentiated outer cortical/zona glomerulosa cells
- Signal received only by non-cortical mesenchyme cells, predominantly in capsule
- Capsule and cortex growth need Shh; zonation and steroidogenic differentiation do not
- Pallister-Hall syndrome mice (*Gli3*^{Δ699/Δ699}) have adrenal glands
- Lineage analyses show *Shh* and *Gli1* expression mark cortical stem/progenitor cells

Abbreviations

HPA	hypothalamic-pituitary-adrenal axis
RAS	renin angiotenin system
PHS	Pallister-Hall syndrome
AGP	adrenogonadal primordium
Shh/Ihh/Dhh	Sonic/Indian/Desert hedgehog
Ptch1	patched 1
Smo	smoothened
ZG	zona glomerulosa
ZF	zona fasciculata
POMC	proopiomelanocortin
ACTH	adrenocorticotrophic hormone
scc	cholesterol side chain cleavage enzyme, Cyp11a1
Sf-1/Ad4BP	steroidogenic factor-1/adrenal-4-binding protein; NR5A1
TH	tyrosine hydroxylase
GFP/YFP	green/yellow fluorescent protein
LacZ	beta galactosidase
ORF	open reading frame
IRES	internal ribosome entry site
UTR	mRNA untranslated region
dpc	days post coitus

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Figure 1. Summary of the major stages of murine adrenal development

(A) Cells expressing Sf-1 delaminate from the dorsal coelomic epithelium (ce) and invade the overlying mesonephric mesenchyme (m). The adrenal anlagen (a) forms from cells that migrate dorsomedially, while the gonadal anlagen precursors (g) settle dorsolaterally. The adrenal primordium is invaded by migratory sympathoadrenal neural crest cells (nc) that will form the neuroendocrine medulla. Subsequently the gland becomes encapsulated by mesenchymal cells.

(B) As the gland grows the cortical cells sort into a central medulla and outer cortex. The cortex is composed of fetal adrenal cells that are established before the outer definitive adrenal population.

(C) The cortical cells segregate into functional zones. Some ZG cells do not completely differentiate, although they do express Sf-1 and some steroidogenic biosynthetic pathway enzymes. Fully differentiated steroidogenic ZG cells express Cyp11b2, while differentiated ZF cells express Cyp11b1.

(D) Transverse section of 11.5 dpc mouse embryo immunostained for expression of Sf-1 (green) and PECAM, which marks endothelial cells (red), and counterstained for DNA (blue). By this age the adrenal (a) and gonadal (g) primordia are clearly segregated, and the adrenal is embedded in the mesonephric mesenchyme (m).

(E) Whole mount ventral view of an *Sf-1-Cre^{Tg}; R26^{mT/mG}* 12.5 dpc embryo in which Cre-mediated recombination has induced GFP reporter expression (green) in the Sf-1 lineage in the adrenal (a) and gonadal (g) anlagen. All non-recombined cells express tdTomato, and are labeled in red.

(F) Whole mount ventral view of an *Sf-1-Cre^{Tg}; R26^{mT/mG}* 14.5 dpc embryo. By this age the metanephric kidneys (k) have grown adjacent to the adrenal glands.

(G) Section of adult adrenal gland coimmunostained for expression of Cyp11b2 (green) and Cyp11b1 (red). Note that some immature ZG cells do not express Cyp11b2. Scale bar: 10 microns.

Abbreviations: D, dorsal; V, ventral; M, medial; L, lateral; n, notochord; nt, neural tube; nc, neural crest; d, dorsal aorta; im, intermediate mesoderm; m, mesonephric mesenchyme; ce, coelomic epithelium; agp, adrenogonadal primordium; a, adrenal; g, gonad; k, kidney; c, capsule; zg, zona glomerulosa; zf, zona fasciculata; x, X-zone; m, medulla.

Methods: All experiments with live animals were performed according to Columbia University Institutional Animal Care and Use Committee guidelines. Mice were maintained on a mixed Sw.B6.129 background and fed standard laboratory chow. *Sf-1-Cre; R26-mT/mG*

mice were generated from *Sfl-cre*^{Tg(Hi)} (Kim et al., 2008) and *R26*^{mT/mG} (Muzumdar et al., 2007) parents. Noon of the day of vaginal plug detection was considered 0.5 dpc. Tissue was collected and immunostained as described (King et al., 2009).

Figure 2. PHS mice have adrenal glands. Tissue was collected from embryonic wild type (+/+) or *Gli3*^{Δ699/Δ699} mutant (Böse et al., 2002) (Δ699/Δ699) mice and the adrenal glands examined (A,B) Representative whole mount images of tissue dissected from 16.5 dpc embryos. Adrenal glands of similar size and shape (yellow arrowheads) are visible in both control (A) and mutant (B) embryos, although the mutant kidneys (K) are dysmorphic or absent. (C,D) Representative multichannel fluorescence images of control (C,C') and mutant (D,D') 18.5 dpc adrenal tissue. Similar staining patterns of endogenous biotin, which labels all cortical cells with steroidogenic potential, and tyrosine hydroxylase (TH), which labels neuroendocrine cells of the medulla, are present in adrenals of both genotypes. Boxed regions enlarged in lower panels (C',D'). Scale bars: 100 microns.

Methods: All live animal procedures were approved and performed according to University of Duisberg-Essen institutional guidelines. Mice were maintained on a mixed 129.B6 background and fed standard laboratory chow. *Gli3*^{Δ699/+} mice (Böse et al., 2002) were intercrossed to generate mutant and control sibling embryos. Noon on the day of vaginal plug detection was designated 0.5 dpc. Cryosectioned tissue was stained as described (Paul and Laufer, 2011).

Figure 3. Gene expression, signaling and lineage relationships between the adrenal capsule and cortex

(A) *Shh* is expressed by clusters of incompletely differentiated Sf-1 positive cells in the ZG (pale green), and signals (red arrow) to *Gli1* positive cells in the overlying inner capsule (yellow). Both Wnt and FGF signaling are required for adrenal development and maintenance, and potentially expression of these ligands in the capsule depends on *Shh*. These ligands might signal (orange arrows) to the *Shh* expressing progenitor cells and/or the Cyp11b2 expressing cells (light green) in the ZG, all of which potentially express beta catenin, *Fgfr2* and *Fgfr4*.

(B) Lineage tracing studies indicate that *Gli1* positive but Sf-1 negative cells in the capsule or non-capsule mesenchyme can convert to Sf-1 positive steroidogenic lineages during development of the gland (black arrows) (King et al., 2009; Huang et al., 2010), at least in

part via a *Shh* expressing intermediate (King et al., 2009). *Shh* expressing cells differentiate to become both Cyp11b1 and Cyp11b2 positive, but not Gli1 positive cells (white arrows).