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Fetal adrenal capsular cells serve as progenitor cells for steroidogenic and stromal adrenocortical cell lineages in *M. musculus*

Michelle A. Wood[1,*], Asha Acharya[2], Isabella Finco[1], Jessica M. Swonger[3], Marlee J. Elston[3], Michelle D. Tallquist[4] and Gary D. Hammer[1,5,‡]

ABSTRACT

The lineage relationships of fetal adrenal cells and adrenal capsular cells to the differentiated adrenal cortex are not fully understood. Existing data support a role for each cell type as a progenitor for cells of the adult cortex. This report reveals that subsets of capsular cells are descendants of fetal adrenocortical cells that once expressed *Nr5a1*. These fetal adrenocortical cell descendants within the adrenal capsule express *Gli1*, a known marker of progenitors of steroidogenic adrenal cells. The capsule is also populated by cells that express *Tcf21*, a known inhibitor of *Nr5a1* gene expression. We demonstrate that *Tcf21*-expressing cells give rise to *Nr5a1*-expressing cells but only before capsular formation. After the capsule has formed, capsular *Tcf21*-expressing cells give rise only to non-steroidogenic stromal adrenocortical cells, which also express collagen 1a1, desmin and platelet-derived growth factor (alpha polypeptide) but not Nr5a1. These observations integrate prior observations that define two separate origins of adult adrenocortical steroidogenic cells (fetal adrenal cortex and/or the adrenal capsule). Thus, these observations predict a unique temporal and/or spatial role of adult cortical cells that arise directly from either fetal cortical cells or from fetal cortex-derived capsular cells. Last, the data uncover the mechanism by which two populations of fetal cells (fetal cortex derived *Gli1*-expressing cells and mesenchymal *Tcf21*-expressing mesenchymal cells) participate in the establishment of the homeostatic capsular progenitor cell niche of the adult cortex.

KEY WORDS: Adrenal gland, Progenitor cells, Homeostasis, Mouse

INTRODUCTION

The adrenal capsule is histologically characterized as arising from cells of the intermediate mesoderm. After separation of the individual adrenal primordia (AP, fetal adrenal gland) and gonadal primordial (GP) from the shared adrenogonadal primordia (AGP), mesenchymal cells migrate and encapsulate the fetal adrenal gland between E11.5 and E12.5 in mice (Else and Hammer, 2005; Keegan and Hammer, 2002) or the 8th to 9th week of gestation in humans (França et al., 2013). The molecular mechanisms involved in this process are not well understood but the capsule has long been characterized as a simple structure that surrounds the gland. Studies support the hypothesis that homeostatic maintenance of the adrenal cortex occurs through an inward centripetal displacement of cortical cells from the periphery of the gland (capsule or subcapsular region) toward the cortico-medullary boundary where apoptosis occurs (Simon and Hammer, 2012). In the mouse, adrenal enucleation (removal of the medulla and much of the cortex, leaving only the capsule and peripheral cortex) is followed by formation of new cells that spread out beneath the capsule and proliferate until regeneration is complete (Simon and Hammer, 2012). The repopulating cells are proposed to arise from the capsule or undifferentiated subcapsular cells (Schaberg, 1955). In the normal homeostatic gland, adrenal cells turnover with time but the identity of the cells responsible for replenishment of the adult adrenal cortex remains to be determined.

Two potential sources of adrenocortical progenitor cells contribute to adrenal homeostasis: adrenocortical fetal precursors and the adrenal capsule (Wood and Hammer, 2011). Zubair et al. (Zubair et al., 2006; Zubair et al., 2008) showed that a fetal adrenocortical-specific enhancer (*FAdE*) activates and maintains nuclear receptor subfamily 5, group A, member 1 (*Nr5a1*, also known as Sf1 or Ad4bp) expression only in the fetal adrenal gland. *Nr5a1* encodes steroidogenic factor 1 (Sf1), an essential transcription factor for steroidogenesis, proliferation and differentiation of adrenocortical cells (Bland et al., 2004; Buaas et al., 2012; Fatchiyah et al., 2006; Katoh-Fukui et al., 2005; Lala et al., 1992; Luo et al., 1994; Morohashi et al., 1992; Rice et al., 1991; Sadovsky et al., 1995; Val et al., 2007). The adult cortex emerges between the fetal cortex and capsule, ultimately replacing the regressing fetal cortex. Cells using the *FAdE* can no longer contribute to the adult cortex after E14.5. Although adult adrenocortical cells do not use the *FAdE* to activate *Nr5a1* expression, virtually all adult adrenocortical cells are derived from fetal cells that once expressed *Nr5a1* under control of the *FAdE* (Zubair et al., 2008). A second series of studies examined the hypothesis that cells of the adrenal capsule serve as precursors for the underlying adult cortex. GLI-Kruppel family member GLI1 (*Gli1*)-expressing cells of the adrenal capsule give rise to *Nr5a1*-expressing cells in the adult cortex (Huang et al., 2010; King et al., 2009). Interestingly, a subset of peripheral adult adrenocortical cells express Sonic hedgehog (Shh), the morphogen that presumably induces *Gli1* expression and activation in cells of the adrenal capsule. Shh-expressing cells are known to serve as progenitor cells, embedded in the glomerulosa of the peripheral cortex, and are able to differentiate into the steroidogenic cells of the cortex throughout life (Ching and Vilain, 2009; Huang et al., 2010; King et al., 2009).

[1]Department of Internal Medicine, Division of Metabolism, Endocrinology and Diabetes, University of Michigan, Ann Arbor, MI 48109, USA. [2]Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA. [3]Molecular and Cell Biology Program, University of Hawaii, Honolulu, HI 96813, USA. [4]Center for Cardiovascular Research, John A. Burns School of Medicine, University of Hawaii, Honolulu, HI 96813, USA. [5]University of Michigan Cancer Center, University of Michigan, Ann Arbor, MI 48109, USA.
*Present address: Magee-Womens Research Institute, University of Pittsburgh, Pittsburgh, PA 15213, USA.

‡Author for correspondence (ghammer@umich.edu)

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Studies in this report examine whether these observations define two distinct lineages of the adult cortex or reflect a mechanism by which the homeostatic stem/progenitor niche of the adult cortex is established from the developing fetal cortex and capsule. A model emerged that integrates both observations by predicting that a subset of *FAdE*-using *Nr5a1*-expressing fetal adrenal cells, which abut the forming capsule, become embedded in the capsule as they extinguish *Nr5a1* expression. Such cells then express Gli1 and serve to populate the newly emerging *Nr5a1*-expressing cells of the adult cortex. Here, we report that fetal adrenocortical cells give rise to *Gli1*-expressing capsular cells that have been shown to serve as progenitor cells to maintain homeostatic replenishment of the adult cortex (Wood and Hammer, 2011). In addition, we show that transcription factor 21 (*Tcf21*)-expressing cells arising from the AGP contribute to the coalesced adrenal capsule and give rise to stromal cells of the adult adrenal cortex. Together, these studies reveal that the capsule as a complex niche for multiple cells types of separate fetal origins, which give rise to distinct lineages of adrenocortical cells during homeostatic maintenance.

RESULTS

Cells of the fetal adrenal cortex give rise to a subset of cells in the adrenal capsule

To determine whether fetal adrenal cells contribute to cells of the adrenal capsule, we used mice in which an IRES-Cre was inserted downstream of the *FAdE* and 5.8 kb of the *Nr5a1* proximal promoter [*FAdE-Ad4bp-Cre* (Zubair et al., 2008)]. *FAdE-Ad4bp-Cre* expression is restricted to the fetal adrenal cortex (not the adult adrenal cortex). The *FAdE-Ad4bp-Cre* mouse line is better suited to our studies than the *Nr5a1-Cre* lines (Bingham et al., 2006; Kim et al., 2008) where *Cre* is expressed in all steroidogenic cells and would preclude our ability to look specifically for fetal adrenal adrenocortical cell descendants. Thus, *FAde-Ad4bp-Cre* mice were crossed with mice that express a Tomato reporter ubiquitously until permanent recombination by Cre occurs, at which time cells and their descendants are indelibly tagged with EGFP [*FAdE-Ad4bp-Cre:R26R*$^{mTomato/mEGFP}$ (Muzumdar et al., 2007)]. This model permits identification of cells that have at some time actively expressed *Nr5a1* under control of the *FAdE*. *Cre* expression varied in penetrance, as indicated by expression of EGFP (Fig. 1A,B) and as was seen previously (Zubair et al., 2008). High-resolution, but not low-resolution, examination of the adrenal capsule revealed EGFP-expressing cells in the adrenal capsule that did not express Nr5a1 (Fig. 1C). On average, 5.78±0.84% of capsular cells per section were positive for EGFP in mice at E18.5 through P0.5 (*n*=5 animals). In adulthood, however, the number of capsular cells per section that were positive for EGFP (*n*=4 animals) dropped to 4.56±1.14%. Given the incomplete penetrance of the transgene in *FAdE-Ad4bp-Cre:R26R*$^{mTomato/mEGFP}$ mice and the sampling of sections evaluated, additional EGFP-expressing cells (*FAdE*-using)

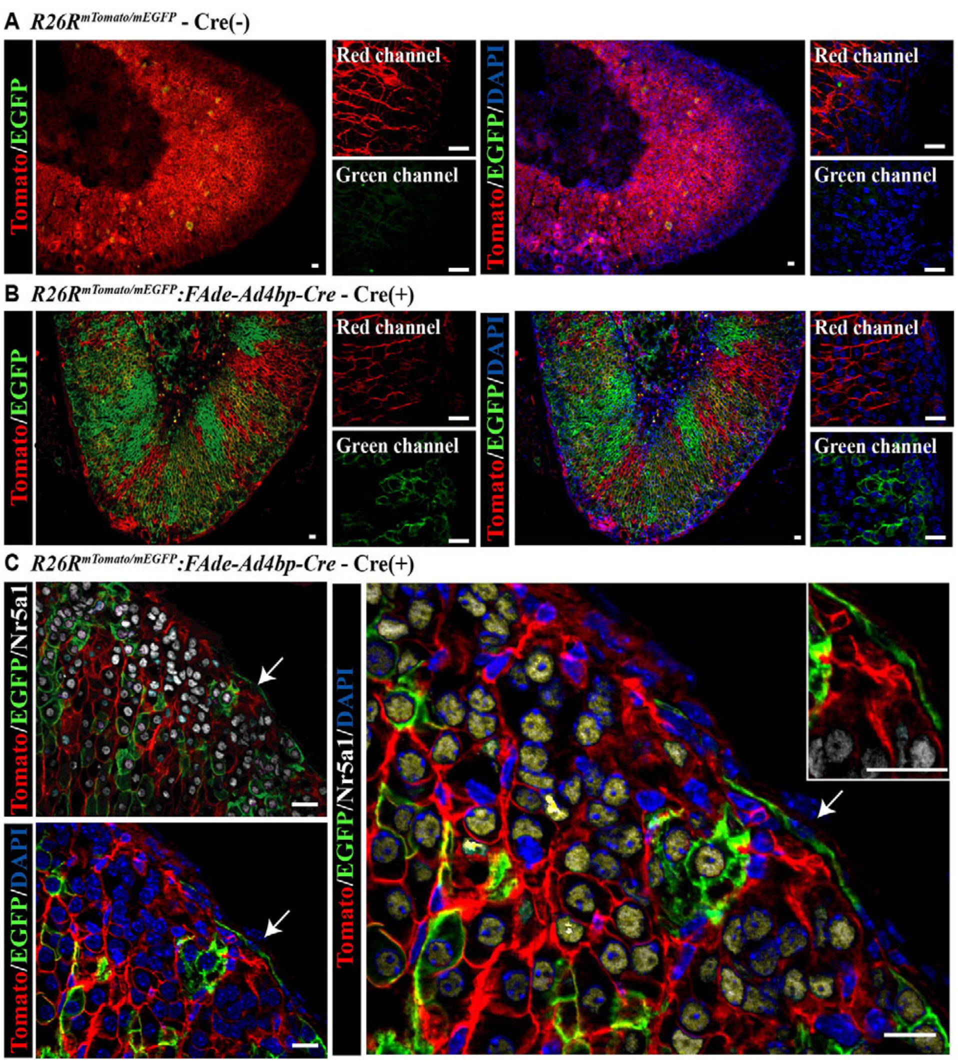

Fig. 1. Fetal adrenal cells give rise to capsular and steroidogenic cells of the adult adrenal gland. Using immunofluorescence (IF), cryosections from adult *FAdE-Ad4bp-Cre:R26R*$^{mTomato/mEGFP}$ mice reveal that in the absence of Cre (A), the ubiquitous Tomato reporter (red membrane *R26R*$^{mTomato/mEGFP}$ without Cre activation) is expressed throughout the gland but in Cre-expressing littermates (B), EGFP reporter expression (green membrane *R26R*$^{mTomato/mEGFP}$*:FAdE-Ad4bp-Cre*) is detected. Largest panels show red (549 nm excitation wavelength) and green (488 nm excitation wavelength) channels overlaid. High-power magnification insets on the right show each channel individually. (C) EGFP-expressing cells give rise to both *Nr5a1*-expressing cells in the cortex (white nuclei) and Nr5a1-negative cells in the adrenal capsule (arrows and inset). Scale bars: 20 µm.

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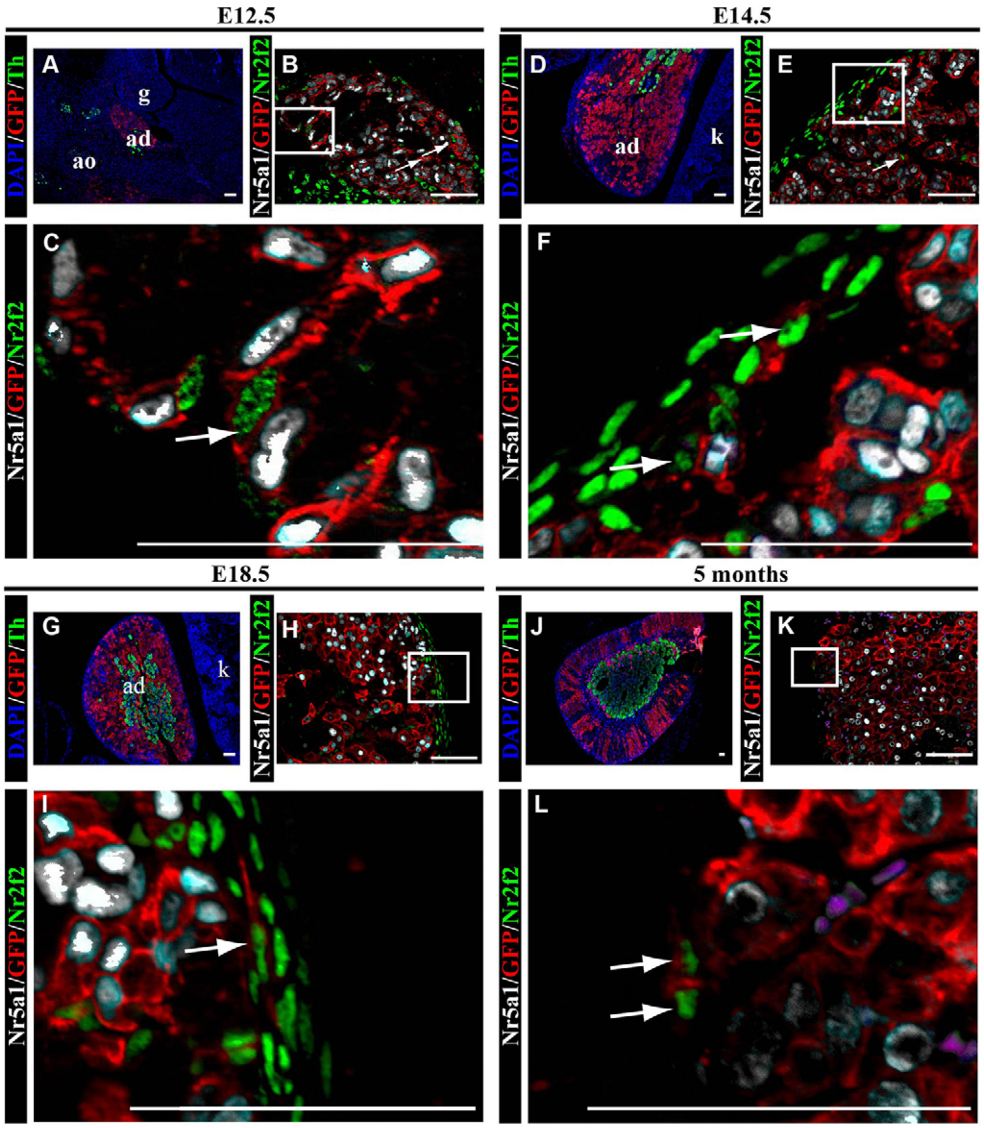

Fig. 2. Fetal adrenal cells give rise to cells of the adrenal capsule. Adrenal glands from *FAdE-Ad4bp-Cre:R26R*$^{mTomato/mEGFP}$ mice were evaluated by immunofluorescence in paraffin sections. In the fetal adrenal gland, at E12.5 (A), medullary cells expressing Th (green cytoplasm) and adrenocortical cells expressing EGFP (red membrane; *R26R*$^{mTomato/mEGFP}$*:FAdE-Ad4bp-Cre*) can be seen intermingling. EGFP-positive cells are indicative of fetal adrenocortical cell lineage (*FAdE-Ad4bp-Cre* expressing). (B) Low power and (C) high-power magnifications showing that *Nr2f2*-expressing capsular cells (green nuclei) and *Nr5a1*-expressing fetal adrenocortical cells (white nuclei) are intermingled. Both cell types co-express EGFP (red membrane, arrows), indicative of fetal adrenocortical lineage. (D) By E14.5, adrenal capsule formation and neural crest cell migration to the medulla, as reflected by Th expression (green cytoplasm) is complete. (E) Low-power and (F) high-power magnifications showing *Nr2f2*-expressing cells (green nuclei) are primarily in the adrenal capsule and some of these cells contain EGFP (red membrane), which is indicative of fetal adrenocortical lineage. These capsular cells expressing both Nr2f2 (green nuclei) and EGFP (red membrane) can be found in animals at all ages examined (white arrows) – E18.5 (G-I) and 5 months (J-L). (L) Magenta cells are red blood cells that are autofluorescent in every channel but do not stain with DAPI. C,F,I and L are enlargements of boxes in B,E,H and K, respectively. Blue in A,D,G and J indicates DAPI. Scale bars: 50 µm. Cyan results from overlay of white and red. ad, adrenal gland; g, gonad; ao, aorta; k, kidney.

are predicted to reside within the adrenal capsule. EGFP was not detected in tyrosine hydroxylase (Th)-expressing cells of the adrenal medulla (supplementary material Fig. S1C,D). Together, these results indicate that fetal adrenocortical cell descendants contribute to a population of adrenal capsular cells.

Capsular descendants of fetal adrenal cells express *Gli1* into adulthood

The adrenal capsule is composed of mesenchymal-like cells that envelop the gland. The mesenchymal cell marker nuclear receptor subfamily 2, group f, member 2 (Nr2f2, commonly known as CoupTFII), defines the majority of the coalescing capsular cells, stroma, vascular endothelium and smooth muscle cells of the adrenal gland and is maintained after birth and through adulthood where expression is less pronounced (supplementary material Fig. S2) (Suzuki et al., 2000; Tsai and Tsai, 1997). We use Nr2f2 throughout this paper as a marker of the Nr5a1-negative adrenal capsule as it is not known to be expressed in Nr5a1-expressing cells. Although the necessity of Nr2f2 in steroidogenic cell development has not been studied, Nr2f2 may negatively regulate the transcriptional activity of Nr5a1 (Shibata et al., 2003).

To determine whether descendants of fetal adrenal cells transition to Nr2f2-expressing capsular cells, we examined adrenal glands from *FAdE-Ad4bp-Cre:R26R*$^{mTomato/mEGFP}$ mice. At E12.5, prior to adrenal capsule formation, the fetal adrenal gland does not yet contain a distinct medulla, as detected by Th expression or a distinct capsule (Fig. 2A). However, EGFP-expressing cells (descended from fetal adrenal cells) co-expressing either Nr5a1 or Nr2f2 are mingled (Fig. 2B,C). By E14.5, the adrenal gland contains a distinct capsule and medulla (Fig. 2D). Nr2f2-expressing cells are now primarily found in the adrenal capsule and some of these capsular cells also express EGFP (descended from the fetal adrenal cortex; Fig. 2E-F). With a capsule fully encasing the gland, the medulla becomes more centrally located by E18.5 (Fig. 2G) and maintained in the adult (Fig. 2J). Fetal adrenocortical cell descendants (EGFP-expressing cells) in the capsule are evident through all ages evaluated and continue to colocalize with Nr2f2 (Fig. 2H,I,K). These results confirm that the adrenal capsule contains supporting mesenchymal cells and mesenchymal-like cells that descended from fetal adrenal cells and are maintained into adulthood.

Previous reports have shown that *Gli1*-expressing capsular cells give rise to adult adrenocortical cells (Huang et al., 2010; King et

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al., 2009). *Gli1* expression, as detected in *Gli1-LacZ* mice (Bai et al., 2002), is restricted to the adrenal capsule and appears in a subpopulation of Nr2f2-expressing cells (supplementary material Fig. S2). *Gli1*-expressing cells constitute 69.05±0.18% of the capsule at E18.5 and 63.82±0.06% of the capsule in adulthood. *FAdE-Ad4bp-Cre:R26R*$^{mTomato/mEGFP}$ mice were bred to *Gli1-LacZ* mice. Localization of EGFP-expressing cells (*FAdE* descendants) with Nr2f2 and β-gal would support the idea that these adrenal capsular cells give rise to the adult Nr5a1-expressing adrenocortical cells. Embryos from this cross were examined for colocalization of EGFP and β-gal in the adrenal capsule. *Gli1* promoter activity (β-gal expression) was not detected at E12.5, consistent with previous results (Ching and Vilain, 2009). Adrenal glands were evaluated starting at E14.5 (Fig. 3). Of the EGFP-expressing cells (descended from fetal adrenal cells) in the adrenal capsule, most stained for β-gal (indicative of *Gli1*-expressing cells) at all timepoints evaluated (E14.5 through 5 months; Fig. 3A-C). At E18.5 and in adulthood, 7.48±0.03% and 8.01±0.02% of the *Gli1*-expressing cells, respectively, co-express EGFP. The majority of the EGFP and β-gal co-expressing cells also expressed Nr2f2 (shown at E18.5 in Fig. 3D-F). Together, these data in conjunction with previous studies allow us to surmise that descendants of fetal adrenal cells that reside in the capsule are the *Gli1*-expressing cells that give rise to adult adrenocortical cells (Ching and Vilain, 2009; Huang et al., 2010; King et al., 2009). We also confirmed previous reports that the *Gli1*-expressing capsular cells give rise to the underlying adult adrenocortical cells (supplementary material Fig. S3).

Tcf21 is expressed in the adrenal capsule and its expression decreases over time

Previous studies have revealed that Tcf21 can inhibit *Nr5a1* promoter activity and support a hypothesis that Tcf21 is a regulator of Nr5a1-expressing cell maintenance (Cui et al., 2004; Hidai et al., 1998; Tamura et al., 2001). We used *Tcf21*$^{+/LacZ}$ knock-in mice to characterize the temporal and spatial activity of the *Tcf21* promoter in the mouse adrenal gland (França et al., 2013; Quaggin et al., 1999; Shibata et al., 2003). Although the hormonal profile has not been examined, heterozygous *Tcf21*$^{+/LacZ}$ mice are expected to have normal adrenal function as they are viable, fertile and show no symptoms of adrenal hormone deficiencies. *Tcf21*$^{LacZ/LacZ}$ mice fail to show proper separation of the developing adrenal gland and gonad when compared with wild-type littermates (from P0.5 male mice; Fig. 4A-B). *Tcf21* promoter activity can be detected as early as E9.5 and is clearly present in a few cells of the urogenital ridge/AGP at E10.5 (supplementary material Fig. S4A,B).

By E12.5, β-gal activity is present in mesenchymal cells encapsulating the developing adrenal gland (supplementary material Fig. S4C,D). By E14.5, encapsulation is complete and *Tcf21* promoter activity can be detected throughout the capsule (data not shown). *LacZ* expression throughout the adrenal capsule is maintained through birth (Fig. 4C). In the postnatal adrenal gland,

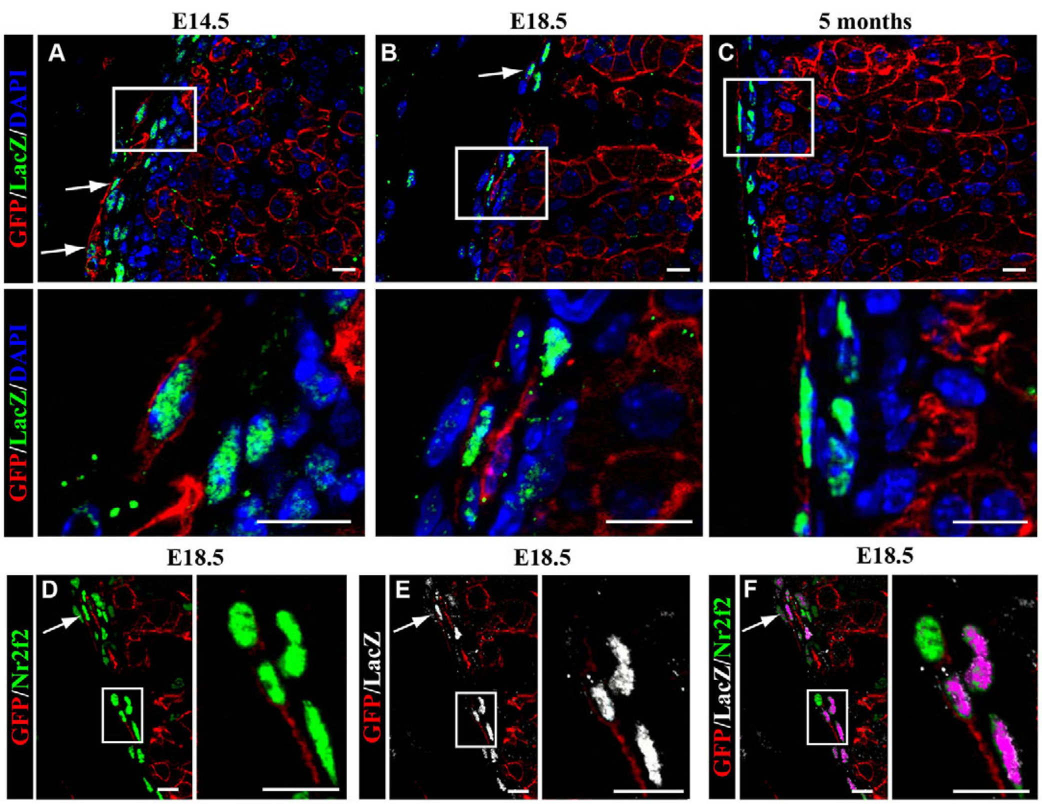

Fig. 3. Capsular descendants of the fetal adrenal cortex express *Gli1*. Adrenal glands from *FAdE-Ad4bp-Cre:R26R*$^{mTomato/mEGFP}$*:Gli1-LacZ* mice were evaluated by immunofluorescence in paraffin sections. At all ages examined, E14.5 (A), E18.5 (B,D,F) and at 5 months (C), some cells expressing β-gal (*LacZ*, green nuclei; indicative of *Gli1* promoter activity) also express EGFP (red membrane; indicative of fetal adrenocortical lineage, *R26R*$^{mTomato/mEGFP}$*:FAdE-Ad4bp-Cre* expressing). Bottom panels in A-C are enlargements of boxed areas in the top panels. White arrows indicate cells expressing both β-gal (*Gli1-LacZ*, green nuclei) and EGFP (red membrane). (D-F) In the capsule of an E18.5 adrenal gland, EGFP-expressing cells (red membrane) co-express both Nr2f2 (green nuclei; D) and β-gal (*Gli1-LacZ*, white nuclei; E) with triple overlay in F. Magenta indicates overlap of green (Nr2f2) and white (Gli1-*LacZ*). Boxed areas in D-F are shown enlarged in the right panels. White arrows in D and E indicate cells expressing both markers. In F, white arrow indicates cells expressing EGFP, *LacZ* and Nr2f2. Scale bars: 10 µm.

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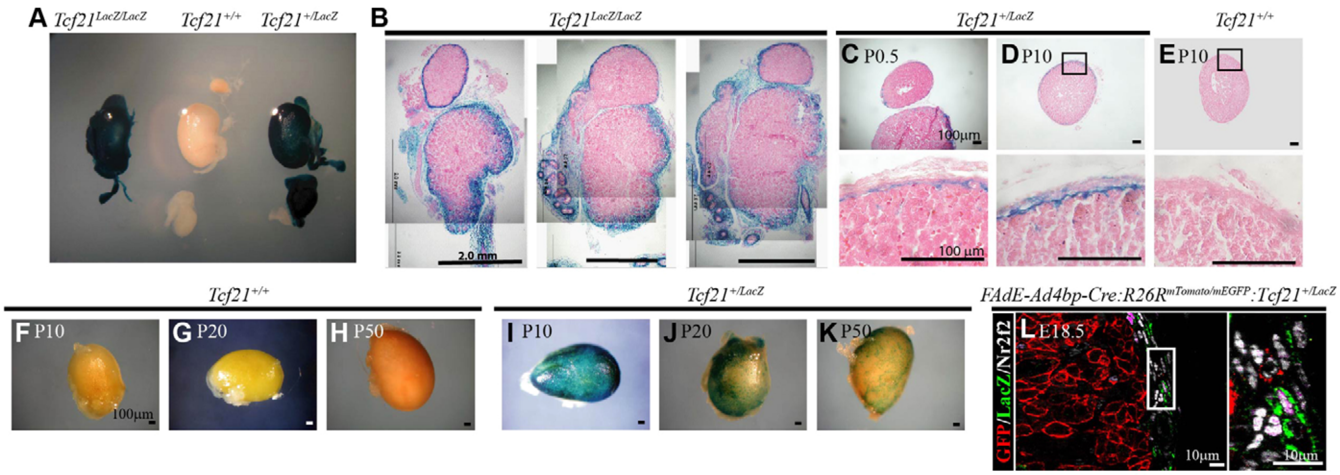

Fig. 4. *Tcf21* is expressed in the adrenal capsule but does not colocalize with descendants of fetal adrenocortical cells. Adrenal glands harvested from *Tcf21*$^{+/+}$, *Tcf21*$^{+/LacZ}$ or *Tcf21*$^{LacZ/LacZ}$ mice were evaluated by whole-mount β-gal staining to characterize *Tcf21* promoter activity during adrenal gland development. (A) Whole-mount staining of adrenal gland, kidney and gonad from male littermates of each genotype at P0.5 show β-gal activity in +/*LacZ* and *LacZ*/*LacZ* animals. (B) Improper separation of the adrenal gland and gonad can be seen in organs from *Tcf21*$^{LacZ/LacZ}$ mice. All panels are from the same adrenal, gonad and kidney unit. Scale bars: 2 mm. Cross-sections of P0.5 (C) and P10 (D) adrenal glands reveal staining predominantly in the capsule with a few positive cells in the adrenal cortex when compared with wild-type controls (*Tcf21*$^{+/+}$; E). Postnatal *Tcf21*$^{+/+}$ (wild type) adrenal glands lack β-gal activity at P10 (F), P20 (G) and P50 (H). Postnatal *Tcf21*$^{+/LacZ}$ adrenal glands show decreasing capsular β-gal activity from P10 (I) through P20 (J) and P50 (K). All cross-sections were counterstained with Eosin (pink). Scale bars: 100 μm. (L) Adrenal glands from *FAdE-Ad4bp-Cre:R26R*$^{mTomato/mEGFP}$*:Tcf21*$^{+/LacZ}$ mice were used for lineage tracing and evaluation by immunofluorescence in paraffin sections. EGFP expression (red membrane; *R26R*$^{mTomato/mEGFP}$*:FAdE-Ad4bp-Cre*) in descendants of fetal adrenal cells (*FAdE-Ad4bp-Cre* expressing) did not overlap with β-gal expression (*LacZ*, green cytoplasm; *Tcf21*-expressing cells; *Tcf21*$^{+/LacZ}$), despite both EGFP and β-gal co-localizing individually with Nr2f2 (white nuclei). Panel on the right is an enlargement of the box in the left panel. Scale bars: 10 μm. Abbreviations: ad, adrenal gland; g, gonad; k, kidney.

Tcf21 promoter activity is present in the majority of capsular cells until around 10 days after birth (Fig. 4D-F,I). At this time, the number of cells with *Tcf21* promoter activity gradually decreases until around the time of puberty (Fig. 4G-J). Small populations of cells maintain β-gal activity through adulthood (Fig. 4H,K). These results suggest that the *Tcf21* promoter is expressed in a subpopulation of cells of the adrenal capsule throughout life.

Tcf21-expressing capsular cells are not descendants of the *Nr5a1*-expressing fetal adrenocortical cells

To investigate the hypothesis that *Tcf21*-expressing cells arise from Nr5a1-expressing fetal adrenocortical cells, we crossed *Tcf21*$^{+/LacZ}$ mice with *FAdE-Ad4bp-Cre:R26R*$^{mTomato/mEGFP}$ to evaluate colocalization of EGFP (descendants of fetal adrenal cells) with β-gal (*Tcf21* promoter activity) in the adrenal capsule. Capsular EGFP-expressing cells also expressed Nr2f2; however, we were unable to find EGFP-expressing cells that also expressed β-gal (Fig. 4L). These data, along with the evidence presented above that (1) *Tcf21* is expressed in the presumptive AGP and (2) *Tcf21*-null mice exhibit incomplete separation of the AP and GP (from the AGP), suggest that *Tcf21*-expressing cells of the capsule arise from a population of cells of the AGP. As *Tcf21*-expressing capsular cells are not descendants of Nr5a1-expressing, fetal adrenal cells, we wanted to investigate the population(s) of cells that the *Tcf21*-expressing cells might contribute to or whether they remained capsular throughout life.

Prior to adrenal capsule establishment, *Tcf21*-expressing cells give rise to *Nr5a1*-expressing cells

To determine the lineage of *Tcf21*-expressing cells, we used mice in which a tamoxifen-inducible Cre recombinase was knocked into the *Tcf21* locus [*Tcf21*$^{+/iCre}$ (Acharya et al., 2011)] crossed to *R26R*^{tdTomato} mice, where *Tcf21*-expressing cells and their descendants are indelibly tagged with Tomato. Because *Tcf21* promoter activity has been detected as early as E9.5 (Hidai et al., 1998; Lu et al., 1998; Quaggin et al., 1998), we conducted initial studies where Cre recombinase activation was induced prior to fetal adrenal primordia coalescence and prior to adrenal capsule formation. Evaluation of tissues at E18.5 revealed Tomato expression (*Tcf21* lineage) in two populations of cells (Fig. 5A,B). We also found that Nr5a1-expressing cells that arose from Tcf21-expressing cells at E8.5 (induced at E8.5) persisted until P21 (Fig. 5C). First, Tomato-expressing cells were present in the vicinity of the peripheral cortex and expressed Nr5a1. Second, Tomato-expressing cells were present in the adrenal capsule and most did not express Nr5a1. It remains unclear whether the expression of Nr5a1 in the Tomato-expressing cortical cells (Tcf21 lineage) reflects (1) residual Nr5a1 expression in the cells of the AGP prior to the transition to *FAdE*-using fetal adrenal steroidogenic cells and prior to capsule formation, or (2) adult cortical cells that are descendants of the *Tcf21*-expressing capsular cells.

After adrenal capsule formation, *Tcf21*-expressing cells give rise to cortical stromal cells but not to *Nr5a1*-expressing cells

Given the ambiguity of Tcf21 lineage prior to adrenal capsule formation, we analyzed the lineage of *Tcf21*-expressing cells after capsule formation (~E14.5) to determine their contribution to homeostatic maintenance of the adrenal cortex. We stimulated Cre recombination at E14.5 and examined adrenal glands through E18.5 (Fig. 5D,E) and postnatally (6 weeks, Fig. 5F). A subset of adrenocortical cells was derived from *Tcf21*-expressing cells but these descendants did not express Nr5a1. Descendants were closely associated with the capsule and throughout the adrenal cortex. In contrast to the round Nr5a1-expressing cells, descendants of *Tcf21*-

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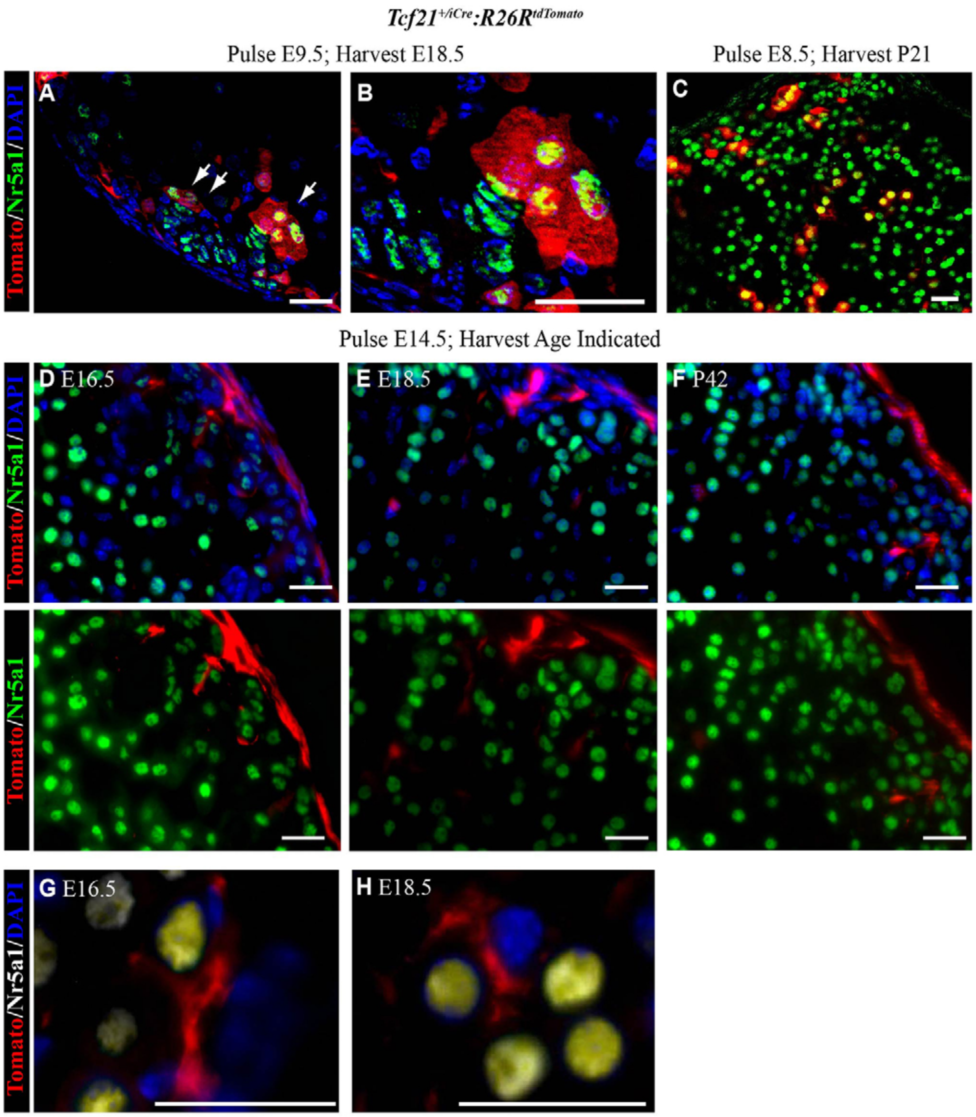

Fig. 5. *Tcf21*-expressing cells give rise to steroidogenic adrenocortical cells prior to adrenal capsule formation and non-steroidogenic adrenocortical cells after adrenal capsule formation. Adrenal glands from *Tcf21*$^{+/iCre}$:*R26R*^{tdTomato} mice were harvested at E18.5 after tamoxifen induction at E9.5 (prior to adrenal capsule formation) and were evaluated by immunofluorescence in cryosections (A,B). Tomato-expressing (red cytoplasm; *Tcf21*$^{+/iCre}$:*R26R*^{tdTomato}) descendants of *Tcf21*-expressing cells were found both in the capsule and in a few adrenocortical cells that expressed Nr5a1 (green nucleus, white arrows). (B) An enlargement of A. Similar to A and B, in C the adrenal glands were harvested at P21 after tamoxifen induction at E8.5. (A-C) Yellow indicates overlap of red and green. Arrows in A indicate cells expressing both Nr5a1 and tomato, and thus descendants of Tcf21-expressing cells. (D-H) Adrenal glands from *Tcf21*$^{+/iCre}$:*R26R*^{tdTomato} mice harvested at various times after administration of tamoxifen to pregnant females at E14.5 (after adrenal capsule formation). Adrenocortical cells failed to show co-expression of *Nr5a1* (green nuclei) and Tomato (red cytoplasm) at E16.5 (D), E18.5 (E) and P42 (F). In G (E16.5) and H (E18.5), Tomato expression (red cytoplasm) belongs to cells lacking *Nr5a1* expression (white nuclei). (G,H) Yellow tint indicates overlap of white and blue staining. DAPI was used to visualize nuclei in all panels (blue). Scale bars: 20 μm.

expressing cells were more elongated, spindle shaped and often had extensions of cytoplasm that appeared to wrap around the Nr5a1-expressing cells (Fig. 5G,H). Together, these results indicate that after adrenal capsule formation, *Tcf21*-expressing cells do not give rise to Nr5a1-expressing cells but rather to stromal cells of the adult adrenal cortex. Stromal cells of the adrenal cortex and their functions have not been well delineated in prior studies; therefore, to better define the Tcf21 stromal lineage in the adrenal gland, we examined these cells for stromal proteins.

Stromal cells in other organs are commonly fibroblastic and produce collagen 1a1. Therefore, we bred *Tcf21*$^{+/iCre}$:*R26R*^{tdTomato} mice to mice expressing GFP under control of the collagen 1a1 promoter [*ColGFP* (Lin et al., 2008)]. Descendants of *Tcf21*-expressing cells had a high degree of co-localization between Tomato and GFP predominantly in the adrenal cortex (Fig. 6A-C). Desmin (Des) and α smooth muscle actin (Acta1) are markers of smooth muscle cells (SMC) but are not always expressed in the same population of cells as actin typically identifies vascular SMCs. Desmin was expressed by a high percentage of cells of the Tcf21 lineage, when examined in adrenal glands from *Tcf21*$^{+/iCre}$:*R26R*^{tdTomato} mice at P21 after a single induction of tamoxifen at P7 (Fig. 6D,E). By contrast, we did not observe a high degree of Acta1 expression by descendants of *Tcf21*-expressing cells (Fig. 6F,G). We did find that similar to the lineage in the heart, the *Tcf21* lineage in the adrenal gland is also located in vessel adventitia near Acta1-expressing cells. Fibroblasts and vSMC can be distinguished by the expression of platelet-derived growth factors receptors [α and β polypeptide, Pdgfra and Pdgfrb (Acharya et al., 2012; Smith et al., 2011)]. To determine whether Pdgfra- and *Tcf21*-expressing cells contribute to the fibroblast lineage, we stained for Pdgfra and β-gal expression in *Tcf21*$^{+/LacZ}$ mice. Pdgfra was expressed predominantly in the adrenal capsule (Fig. 6H,I) and colocalized with *Tcf21* promoter activity (Fig. 6J,K). Moreover, Pdgfra expression in *Tcf21*$^{LacZ/LacZ}$ is reduced compared with *Tcf21*$^{+/LacZ}$ littermates (Fig. 6L,M). Evaluation of adrenal glands from knock-in mice that express the fusion protein histone 2B-GFP under control of the *Pdgfra* promoter (*Pdgfrα*$^{+/GFP}$) confirmed that nuclear GFP was expressed in the adrenal capsule, in adrenocortical stromal cells, and in cells of the adrenal medulla (Fig. 6N). However, GFP-expressing (*Pdgfra*-expressing) cells were not Nr5a1-expressing adult adrenocortical cells (Fig. 6N,O). We evaluated whether descendants of *Tcf21*-expressing cells give rise to *Pdgfra*-expressing cells by crossing our *Tcf21*$^{+/iCre}$:*R26R*^{tdTomato} mice with *Pdgfrα*$^{+/GFP}$ mice. When Cre recombination was induced after

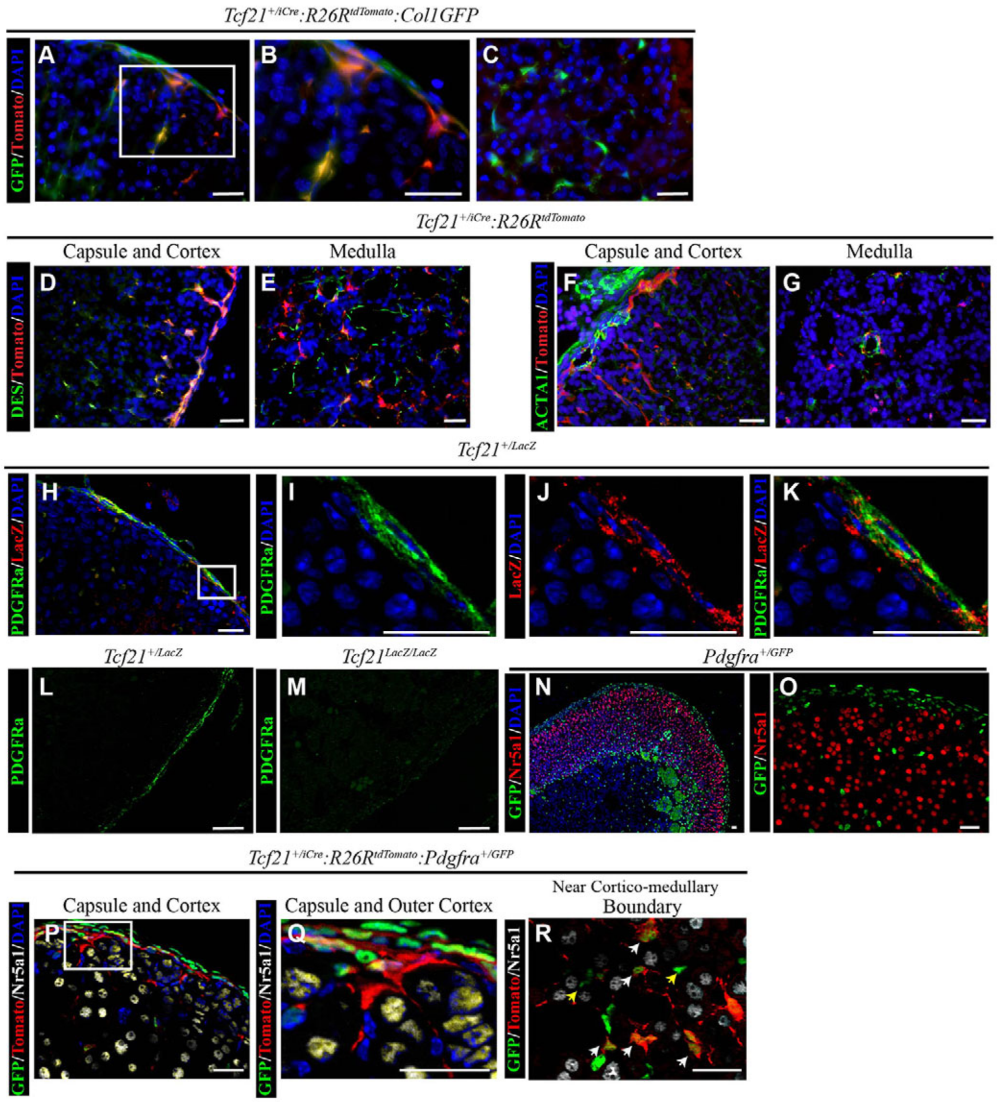

Fig. 6. *Tcf21*-expressing cells give rise to stromal cells after adrenal capsule formation. (A-C) Adrenal glands from 8-week-old *Tcf21$^{+/iCre}$:R26R^{tdTomato}:ColGFP* mice were harvested after a 2-week induction with tamoxifen starting at 4 weeks of age. Adrenal glands analyzed by immunofluorescence of cryosections. GFP (green, stromal cells; *Col1GFP*) and Tomato (red, Tcf21 lineage; *Tcf21$^{+/iCre}$:R26R^{tdTomato}*) were found to colocalize (yellow) in both the adrenal capsule and in spindle-shaped cells of the cortex (A,B) and medulla (C). (B) An enlargement of the boxed area in A. (D-G) Adrenal glands from *Tcf21$^{+/iCre}$:R26R^{tdTomato}* were harvested at P21 after a single tamoxifen induction at P7 and analyzed by immunofluorescence in cryosections. Desmin (DES, green) and Tomato (red, Tcf21-lineage) were found to colocalize in the adrenal capsule, cortex (D) and medulla (E). Smooth muscle actin (ACTA, green) and Tomato (red, Tcf21-lineage) were found in close proximity but do not appear to colocalize (F,G). (H-M) Adrenal glands from P0.5 *Tcf21$^{+/LacZ}$* mice were harvested, embedded in paraffin and analyzed by immunofluorescence to characterize Pdgfra expression. Pdgfra (green membrane) is predominantly present in the adrenal capsule with *Tcf21* (as detected by anti-β-gal antibody; red cytoplasm; H,I; *Tcf21$^{+/LacZ}$*). Both Pdgfra (I, green membrane) and β-gal (J, *LacZ*, red cytoplasm) appear to be co-expressed in the adrenal capsule (K). (I-K) Enlargements of boxed area in H. Pdgrfa expression (green) is diminished in adrenal glands from *Tcf21$^{LacZ/LacZ}$* mice (M) when compared with wild type (L). (N,O) Immunofluorescence on paraffin sections of adrenal glands from *Pdgfra$^{+/GFP}$* reporter mice reveal GFP protein (green nuclei; *Pdgfra$^{+/GFP}$*) in the adrenal capsule and some cells of the adrenal cortex but not in steroidogenic *Nr5a1*-expressing cells (red nuclei). Large cytomegalic cells at the corticomedullary boundary are common in adult adrenal glands and are autofluorescent. (P-R) Adrenal glands were harvested from *Tcf21$^{+/iCre}$:R26R^{tdTomato}:Pdgfra$^{+/GFP}$* mice at E18.5 after tamoxifen administration at E14.5 and analyzed by immunofluorescence in cryosections. Tomato expression (indicative of Tcf21 lineage; red cytoplasm) was colocalized with GFP (green nuclei) in *Pdgfra*-expressing cells of the adrenal capsule and cortex but did not colocalize with Nr5a1-expressing adrenocortical cells (white nuclei). (Q) Enlargement of the boxed area in P. Tomato- and GFP-expressing cells could be found throughout the cortex, including close to the corticomedullary boundary (R). White arrows indicate cells co-expressing Tomato and GFP; yellow arrows indicate GFP-expressing cells without Tomato. Nuclei in all panels except L, M and O are visualized with DAPI. Scale bars: 20 μm.

adrenal capsule formation at E14.5 and adrenal glands were harvested at E18.5, the Tomato-expressing cells (*Tcf21*-descendants) were found to colocalize with GFP-expressing cells (*Pdgfra* expressing; Fig. 6P-R). Not all Tomato-expressing cells also expressed GFP, and not all GFP-expressing cells expressed Tomato. Thus, our studies show that Tcf21-expressing cells of the capsule give rise to stromal SMCs, which express desmin, and to Pdgfra-expressing fibroblastic cells but not to vascular SMCs. Further studies are required to fully characterize the stroma of the adrenal gland.

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DISCUSSION

The adrenal capsule and outer regions of the adrenal cortex have long been hypothesized to contain progenitor cells that mediate adrenocortical maintenance. Studies presented here represent the first to explore the origin of the capsule and to report two mutually exclusive capsular progenitor populations of the adult adrenal cortex. The formation of the adrenal capsule has been described histologically as the coalescence of mesenchymal cells (from the intermediate mesoderm) around the AP (fetal adrenal cells only), ultimately encasing the gland (Keegan and Hammer, 2002; Kim et al., 2009). Previous studies have shown that *Gli1*-expressing cells of the adrenal capsule give rise to steroidogenic cells of the adrenal cortex (Ching and Vilain, 2009; Huang et al., 2010; King et al., 2009). King et al. (King et al., 2009) have specifically shown that *Gli1*-expressing cells give rise to Nr5a1-expressing cells up through 120 days of life and *Gli1*-expressing cells tagged as late as P23 could contribute to Nr5a1-expressing cells 21 days later. Additionally, *Gli1*-expressing cells give rise to Shh-expressing cells of the peripheral adrenal cortex that centripetally contribute to the steroidogenic zones of the differentiated adrenal cortex. Quantification revealed that descendants were expressing cytochrome P450, family 11, subfamily B, polypeptide 1 and 2 (Cyp11b1, ~26% of reporter expressing cells; Cyp11b2, ~37% of reporter expressing cells), markers of the zona fasiculata and glomerulosa, respectively. Other studies by Zubair et al. (Zubair et al., 2008) showed that cells from the Nr5a1-expressing fetal adrenal cortex also contribute to the population of adult adrenocortical cells. It has remained unknown whether these data define dual lineages of the adult adrenal cortex or perhaps two temporally distinct components of a singular lineage cascade.

The data presented here demonstrate that fetal adrenal cells give rise to *Gli1*-expressing cells of the adrenal capsule that are retained at all ages examined, despite incomplete penetrance of the *FAdE-Ad4bp-Cre* activity. Based on the previous studies outlined above, we infer that the *Gli1*-expressing descendants of the fetal adrenal cells are indeed these progenitors. As such, *FAdE*-using *Nr5a1*-expressing fetal adrenal cells do give rise to the Nr5a1-expressing adult adrenal cortex, albeit after becoming Nr5a1-negative, *Gli1*-expressing capsular cells. If the *FAdE-Cre* transgene were completely penetrant, a higher number of *Gli1*-expressing cells would be expected to display EGFP, indicating they are descendants of *Nr5a1*-expressing fetal adrenal cells and further supporting the conclusion that the *Gli1*-expressing capsular cell population is derived from *FAdE-Cre*-expressing fetal adrenal cells and is the same progenitor population that gives rise to *Nr5a1*-expressing adult adrenocortical cells. Our studies do not rule out the possibility that fetal adrenal cells are able to give rise directly to adult adrenal cells. It is also possible that there is more than one origin of *Gli1*-expressing cells. Because weak expression from the *FAdE* is observed in the anterior part of the gonad and thoracic region, it could be argued that the promoter construct used for lineage tracing of *FAdE-Cre*-expressing cells is leaky (Zubair et al., 2008). This would induce 'ectopic' expression of Cre and, hence, EGFP expression that results in descendant cells that express EGFP that are not derived from bona fide fetal adrenal cells (i.e. false positive). However, experiments have shown that endogenous *Nr5a1* mRNA is expressed in the region anterior to the adrenal primordium at E10.5. The expression of Dax1 during fetal adrenal gland development might suggest a role in the suppression of *Nr5a1* activation through the *FAdE*. Therefore, we feel that this model accurately reflects *Nr5a1* expression as driven by the *FAdE*. Most importantly, the *FAdE* promoter has allowed us to examine the fate of fetal adrenal cells distinct from those of the adult adrenal gland.

In vivo, *Tcf21* knockout mice have defects in multiple tissues and die at birth (Lu et al., 2000; Quaggin et al., 1999). In the knockout, separation of the GP from the AP is incomplete and the anterior end of the testis remains continuous with the AP (Cui et al., 2004). These previous studies support a crucial role for Tcf21 in the development of both the gonad and the adrenal gland. During testis development, Tcf21 is normally expressed in Nr5a1-negative interstitial stromal cells. However, in *Tcf21* null GFP knock-in mice, GFP expression is detected in Nr5a1-expressing cells and the number of Nr5a1-expressing Leydig cells is increased, thus supporting the hypothesis that Tcf21 normally represses *Nr5a1* expression in these potential Leydig cell progenitors (Cui et al., 2004). As Leydig cells and adrenocortical steroidogenic cells both arise from the AGP, we set out to characterize *Tcf21* expression and evaluate the role of Tcf21 in the maintenance of adrenocortical cells. We characterized *Tcf21* expression in the adrenal gland from E9.5 through adulthood, where it is expressed predominantly in the adrenal capsule at all ages examined after E14.5. We found that *Tcf21*-expressing cells give rise to cells in the adrenal cortex. Prior to fetal adrenal primordia coalescence and prior to adrenal capsule formation, *Tcf21*-expressing cells and/or cells derived from *Tcf21*-expressing cells at E9.5 give rise to steroidogenic cortical cells and non-steroidogenic capsular cells. It remains unclear whether the expression of Nr5a1 in the Tomato-expressing cortical cells (Tcf21 lineage) reflects (1) Tcf21 expression in cells that gave rise to Nr5a1 cells prior to capsule formation in the fetal adrenal gland or (2) adult adrenal steroidogenic cells that are direct descendants of the fetal adrenal steroidogenic cells (without first becoming a capsular cell).

The stroma of the adrenal gland has not been studied extensively, nor have the relationships between adrenal stromal cells and the steroidogenic adrenocortical cells been systematically examined. Prior studies in *Pdgfra* and *Pdgfrb* double knockout mice predict a potential role of Pdgf signaling in adrenal stromal cell biology (Schmahl et al., 2008). These mice display reduced adrenocortical thickness and a decreased number of Cyp11b1-expressing cells (Schmahl et al., 2008). Global loss of Pdgfrb led to a 50% decrease of pericytes in the adrenal gland but loss of Pdgfra alone does not appear to affect steroid-producing cells of the adrenal (Brennan et al., 2003; Hellström et al., 1999). Tcf21 and Pdgfra are both crucial for development of the epicardial-derived cardiac fibroblasts, with Tcf21-expressing cells giving rise to Pdgfra-expressing cells in the epicardium and myocardium (Acharya et al., 2012; Smith et al., 2011). Our studies revealed that stromal cells of the cortex arise from capsular *Tcf21*-expressing cells and express collagen, desmin and Pdgfra but not smooth muscle actin. Further studies are required to understand fully the stromal-steroidogenic cell interactions in the adrenal cortex, but these data are the first to describe that a capsular cell is capable of giving rise to stromal cells of the adrenal cortex.

An understanding of the origin of cells in the adrenal gland is beginning to come into focus (Fig. 7A). Neural crest cells give rise to cells of the adrenal medulla. Multiple subpopulations contribute to the adrenal capsule, including mesenchymal *Tcf21*-expressing cells and *Gli1*-expressing cells derived from the fetal gland. *Gli1*-expressing capsular cells in turn give rise to adult adrenocortical cells. In addition, *Tcf21*- and *Pdgfr*-expressing cells are also present in the adrenal capsule and give rise to stromal cells in the adrenal cortex. Together, the fetal adrenal cortex, the medulla, the capsule and the adult cortex contribute to the ultimate development of a mature organ (Fig. 7B). These data contribute to an updated model of adrenal organogenesis and maintenance (Fig. 7C). Briefly, after separation of the AP from the AGP, the fetal adrenal primordia is

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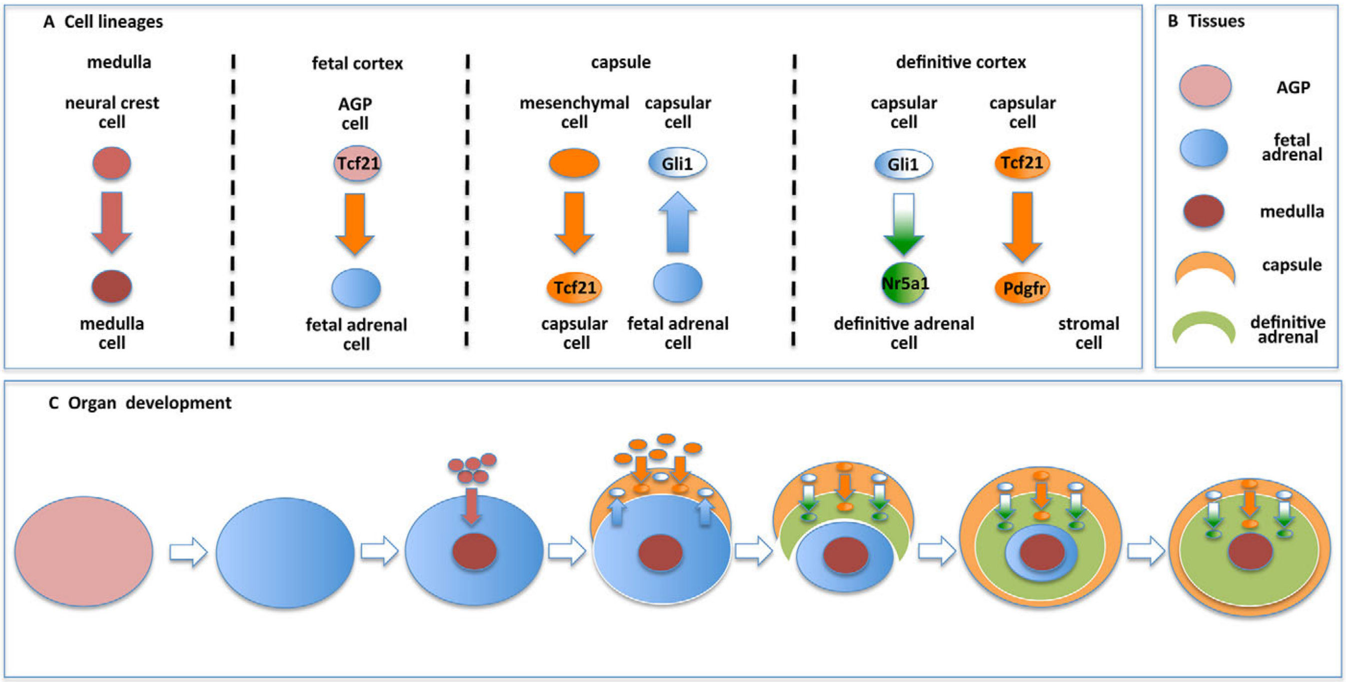

Fig. 7. Model of adrenal lineage and homeostasis. (A) Multiple cell lineages contribute to cells of the differentiated adrenal gland. Neural crest cells (red) give rise to catecholamine-secreting cells of the adrenal medulla. *Tcf21*-expressing cells contribute to the pool of fetal adrenal cells and arise from a mesenchymal cell lineage prior to contributing to the adrenal capsule (orange). Fetal adrenal cells (blue) contribute to a subpopulation of capsular cells that express *Gli1* (white). *Gli1*-expressing capsular cells give rise to steroidogenic adrenocortical cells (green) of the adult gland, whereas *Tcf21*-expressing capsular cells give rise to Pdgfr-expressing stromal adrenocortical cells (orange). (B) The four tissue lineages represented in C. (C) During adrenal organogenesis starting with adrenogonadal primordia (AGP), cells from the neural crest invade the fetal adrenal primordia to form the medulla, and mesenchymal cells contribute to the adrenal capsule. Fetal adrenal cells contribute to the adrenal capsule. As the adult cortex replaces the fetal cortex, *Tcf21*-expressing and *Gli1*-expressing cells serve as progenitors in the adrenal capsule that give rise to stromal and steroidogenic adrenocortical cells, respectively. Upon completion of organogenesis, the capsular progenitor cells are retained throughout adulthood and contribute to homeostatic maintenance of the adrenal cortex.

invaded by migrating neural crest cells to form the adrenal medulla, whereas mesenchymal cells serve to encapsulate the fetal gland. Once established, cells from the adrenal capsule contribute to the expanding steroidogenic and stromal cells of the adult cortex, replacing the fetal adrenal. Once organogenesis is complete, the *Gli1*-expressing cells and the *Tcf21*-expressing cells of the adrenal capsule continue to contribute to adrenal gland homeostasis. Although it remains uncertain under what circumstances these capsular cells are engaged to repopulate the underlying adult cortex, it is becoming increasingly clear that extracellular factors [i.e. wingless-related MMTV integration site 4 (Wnt4), insulin-like growth factor 2 (Igf2), delta-like 1 homolog (Dlk1, also known as Pref1)] and intracellular nuclear factors [i.e. nuclear receptor subfamily 0, group B, member 1 (Nr0b1, also known as Dax1); catenin (cadherin-associated protein), β 1 (Ctnnb1); nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)] participate in the homeostatic maintenance of the adult adrenal cortex (Simon and Hammer, 2012). Future studies are predicted to expand our knowledge of the complexity of the adrenal capsule and its role in the homeostatic maintenance of multiple cell types in the adrenal cortex.

MATERIALS AND METHODS

Experimental models in *M. musculus*

Experiments involving mice were performed in accordance with institutionally approved protocols under the auspice of the University Committee on Use and Care of Animals at the University of Michigan or the Institutional Animal Care and Use Committees of UT Southwestern Medical Center. Veterinary care was provided according to standards in the *Guide for Care and Use of Laboratory Animals*, the Animal Welfare Act Regulations, and the *Public Health Service Policy on Humane Care and Use of Laboratory Animals*. Mice used have been previously described: $Tcf21^{+/LacZ}$ [kindly provided by S. Quaggin (Quaggin et al., 1999)], $Tcf21^{+/iCre}$ (Acharya et al., 2011), $Pdgfr\alpha^{+/GFP}$ (Hamilton et al., 2003), *FAdE-Ad4bp-Cre* [kindly provided by K. Morohashi (Zubair et al., 2008)], $Gli1Cre\text{-}ER^{T2}$ [kindly provided by A. Dlugosz, University of Michigan Medical School, Ann Arbor, USA (Ahn and Joyner, 2004)], *Gli1-LacZ* [kindly provided by A. Dlugosz (Bai et al., 2002)] and $Col1\text{-}GFP^{Tg/0}$ [kindly provided by J. Duffield (Lin et al., 2008)]. Reporter strains used in this study include: $R26R^{tdTomato}$ (Madisen et al., 2010), $R26R^{mTomato/mEGFP}$ (Muzumdar et al., 2007) and $R26R^{LacZ}$ (Soriano, 1999). For each experiment, 4-10 animals were evaluated at each timepoint.

Analysis of mouse adrenal gland histology and immunohistochemistry

Adrenal glands were collected at the indicated ages, fixed, processed and sectioned as previously described (Kim et al., 2008). Tissue sections (6 μm) from paraffin blocks were treated with boiling 10 mM citric acid (pH 2 or pH 6) for 20 minutes followed by 20-minutes cooling if antigen retrieval was required. Slides were washed twice for 5 minutes in phosphate-buffered saline (PBS) and incubated with 2% non-fat dry milk in PBS for 1 hour followed by primary antibody at 4°C overnight. Slides were washed and incubated with secondary antibodies. Tissue sections from frozen samples were allowed to dry at least 3 hours at room temperature. Dried sections were rehydrated in PBS for 15 minutes and permeabilized with PBS+0.1% Triton-X 100 for 10 minutes. Sections were treated with antigen retrieval solution (0.1 mg/ml Proteinase K; 50 mM Tris, pH 8; 5 mM EDTA, pH 8 in PBS) for 5 minutes at 37°C and washed with PBS three times for 5 minutes. Slides were incubated with 5% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA) in PBS + 0.1% Triton-X 100 for at least 1 hour at room temperature followed by incubation with primary and secondary antibodies as above. Antibody dilutions were made in PBS containing 5% FBS. Antibody details are provided in supplementary material Table S1. Fluorescence of the *R26R*^{tdTomato} reporter was detected without an antibody in frozen sections and did not fluoresce in paraffin sections without an antibody (supplementary material Fig. S1A,B). Fluorescence microscopy was conducted on a Zeiss Axiovert 200 with a Hamamatsu ORCA-ER camera, a Zeiss LSM 5 Pascal confocal system or a Zeiss ApoTome using its structured illumination to provide high-resolution images for each ample and images were captured with an AxioCam MRm. Scale bars are indicated with each image.

Whole-mount staining for β-galactosidase (β-gal) in mouse adrenal glands

Dissected adrenal glands were washed in PBS with 2 mM $MgCl_2$ and fixed for 1 hour in 1% formaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P-40 and 1 mM $MgCl_2$ in 1×PBS. Adrenal glands were washed with 1×PBS three times for 5 minutes and stained in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.04% X-gal and 1 mM $MgCl_2$ in PBS at room temperature overnight. Samples were then rinsed three times in PBS and fixed for 2-4 hours with 4% paraformaldehyde (PFA) in PBS at 4°C and embedded in paraffin for sectioning and analysis. Images were taken with an Olympus DP21 camera attached to a Nikon SMZ800 stereomicroscope. X-gal-stained adrenal glands were sectioned and samples were subjected to rehydration and eosin staining for 3 seconds followed by dehydration to allow visualization of histology compared with β-gal activity. Light microscope images were obtained using an Olympus DP70 camera attached to a Nikon Optiphot-2.

Tamoxifen induction and tissue fixation for immunohistochemistry of adrenal gland sections

Tissue lineage analyses were conducted by evaluating *Tcf21*$^{+/iCre}$ knock-in mice carrying the *R26R*^{tdTomato} reporter on a mixed 129/C57Bl6 background crossed to wild-type females or through analyzing *Gli1-CreER*^{T2} mice (Ahn and Joyner, 2004) crossed with the reporter strain *R26R*^{LacZ} (Soriano, 1999). Noon on the day of a vaginal plug was designated as E0.5; pregnant females were administered tamoxifen (100 mg/kg body weight) via gavage or intraperitoneal (IP) injection at indicated times. Tamoxifen (156708, MP Biomedicals, Solon, OH) was dissolved in 10% ethanol and 90% sunflower oil (S5007, Sigma) to a final concentration of 20 mg/ml. Postnatal inductions of *Gli1-CreER*^{T2} mice occurred via once daily IP injections for 2 weeks starting at P21 and harvested at 5 and 25 weeks of age. All other adult tracings were conducted by providing tamoxifen containing chow. Assuming a body weight of 25 g/mouse and an intake of 4 g/day, the resulting dosage is 40 mg per kg body weight per day. Samples for Tomato detection were fixed with 4% PFA for 4 hours, incubated in 10%, 20% and 30% sucrose in series for 3 hours to overnight each, and embedded in Tissue Tek OCT Compound (Sakura Finetek USA, Torrance, CA) for frozen sections. No Cre activity (as determined by R26R reporter activity) was detected following inductions when only oil was administered to Cre-expressing mice or when tamoxifen was administered to mice not expressing Cre. Samples for β-gal detection were processed as above. Inductions at the indicated time points were performed a minimum of three times with similar results and confirmed using more than one reporter strain.

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Competing interests
The authors declare no competing financial interests.

Author contributions
M.A.W. contributed to the conceptualization, writing and overall experimentation of the paper as a whole; A.A., J.M.S. and M.J.E. contributed through conducting the Tcf21 lineage-tracing experiments and characterization of the lineage; I.F. contributed through conducting Gli1 lineage-tracing experiments; M.D.T. contributed to the conceptualization of studies and Tcf21 lineage-tracing experiments; G.D.H. contributed to the conceptualization of studies and writing of the manuscript.

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Supplementary material
Supplementary material available online at
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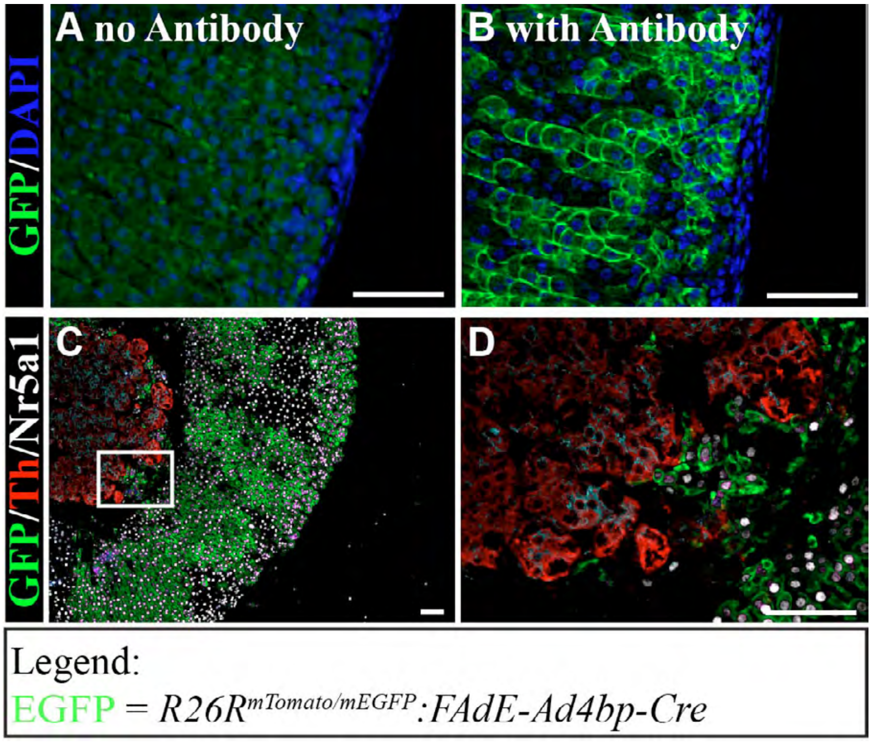

Figure S1: Specificity of GFP Antibody in Paraffin Sections and Cre Expression is Restricted to Fetal Adrenal Descendant Cells. Tomato and EGFP can be detected endogenously in cryosections, however for the majority of our studies, paraffin sections were used. In paraffin sections, a GFP antibody can be utilized to detect EGFP expression by immunofluorescence (IF) and thereby identify cells with active recombination by Cre. IF was carried out on paraffin sections of adrenal glands from adult mice. A. No primary antibody. B. Anti-GFP shows green cytoplasm in cortical cells indicative of Cre-recombination. Cre recombination is specific to cells derived from fetal adrenocortical cells (expressing *FAdE-Ad4bp-Cre*), including Nr5a1-expressing cells (white nuclei). C. Co-staining with anti-GFP (green), anti-Nr5a1 (white), and anti-Th (red) shows cre recombination in Nr5a1-expressing cells but not in the Th expressing medulla. Panel D is magnified from the box in Panel C. Scale bars = 50 μm.

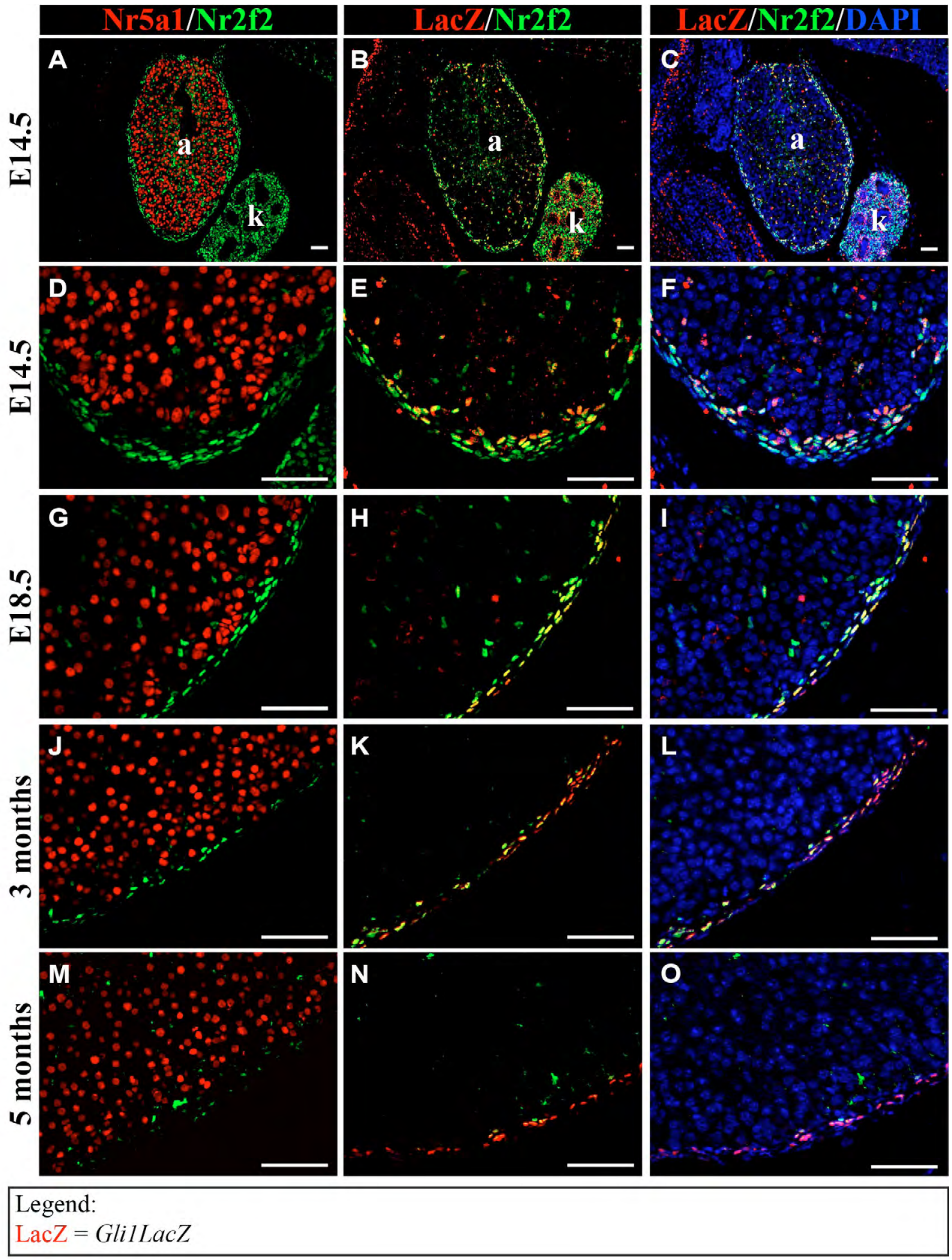

Figure S2: Adrenal Capsule Contains Nr2f2- and Gli1-Expressing Cells. *Gli1-LacZ* mice were used to characterize the cellular milieu of the adrenal capsule by immunofluorescence in paraffin sections. After formation of the adrenal capsule, Nr2f2 (green nuclei) is expressed from E14.5 (panels A-F), through E18.5 (panels G-I), and into adulthood (3 months; panels J-L) where upon cessation of organogenesis, Nr2f2 levels decrease (5 months; panels M-O). Panels B, E, H, K, and N show that β-galactosidase expression (LacZ, indicative of *Gli1* expression; red nuclei) can also be detected throughout the adrenal capsule at all ages evaluated. As shown in panels A, D, G, J, and M, Nr5a1 expression (red nucleus) is restricted to the adrenal cortex. Panels D-E are high power images from A-C, respectively. Yellow indicates overlap of green and red nuclei. Nuclei are shown by DAPI in panels C, F, I, L, and O. a = adrenal, k = kidney. Scale bars = 50 μm.

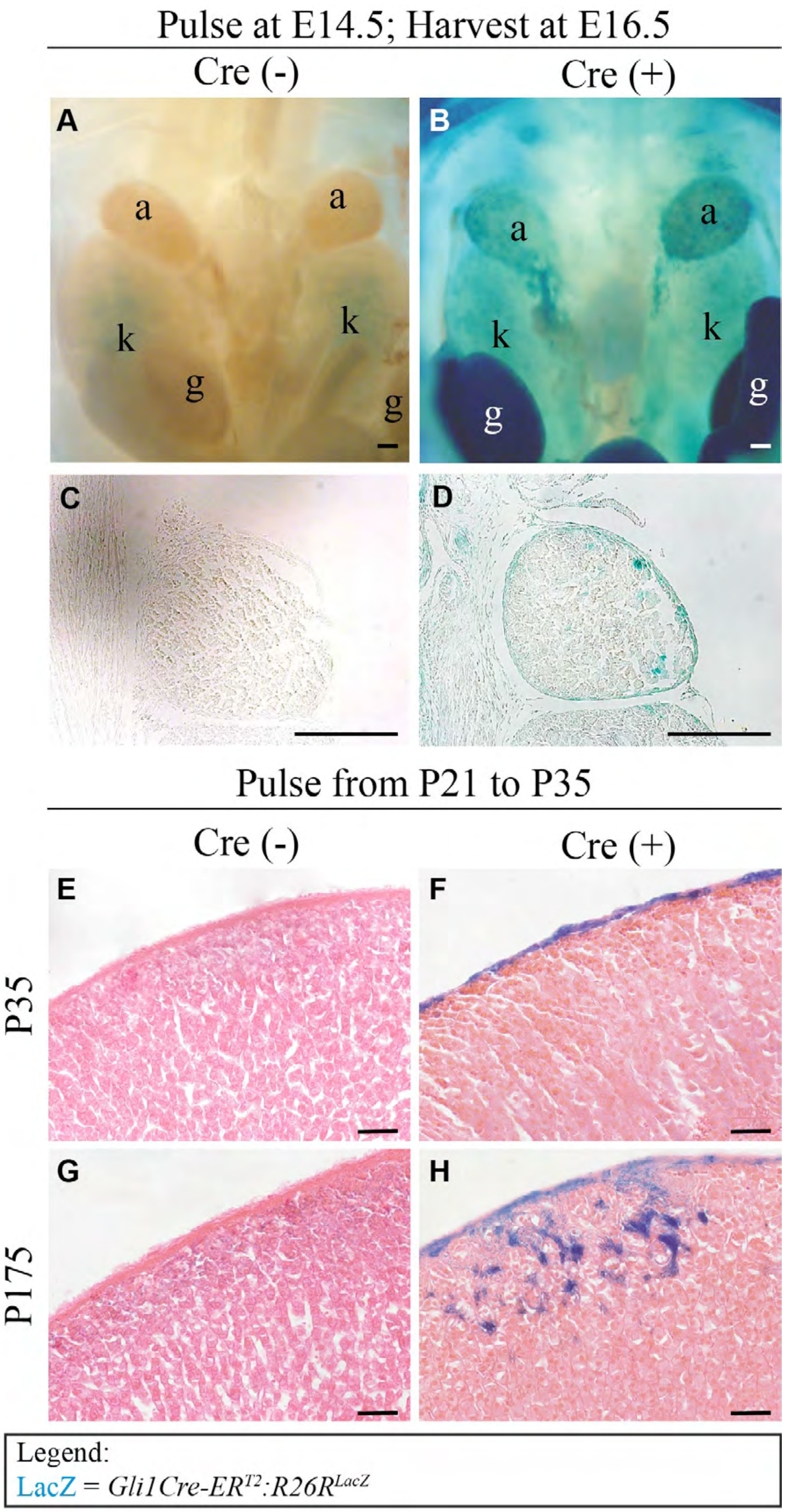

Figure S3: *Gli1*-Expressing Cells of the Adrenal Capsule Give Rise to Adrenocortical Cells. In panels A-D, embryos were harvested at E16.5 from pregnant *Gli1Cre-ER^{T2}:R26R^{LacZ}* (Ahn and Joyner, 2004) mice that were administered tamoxifen at E14.5. In Panels A and C, adrenals from animals lacking Cre expression have no β-galactosiadase (βgal) activity (blue cytoplasm) as detected by whole mount LacZ staining. In contrast, panels B and D show that animals with Cre expression reveal βgal activity throughout the adrenal capsule and some cells with βgal activity in the adrenal cortex. Panels C and D are sagittal sections from embryos. In panels E-H, postnatal *Gli1Cre-ER^{T2}:R26R^{LacZ}* mice administered tamoxifen from P21 through P35, display βgal activity predominantly in the adrenal capsule of Cre expressing mice at P35 (F) and in cells of the adrenal cortex at P175 (H) when compared to animals lacking Cre expression (E, G). Scale bars = 100 μm.

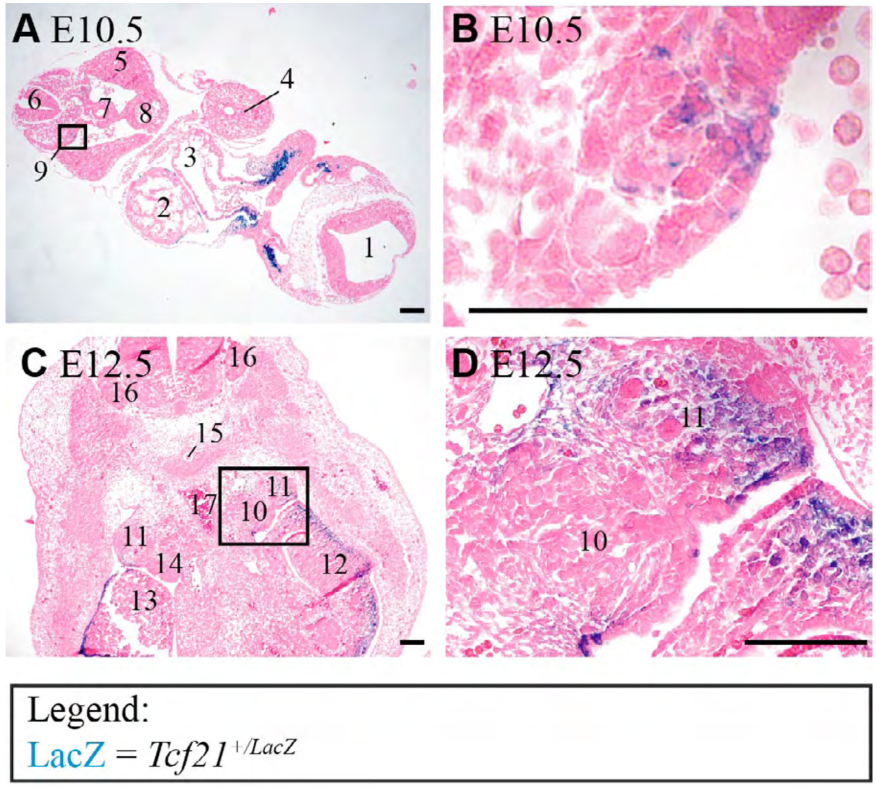

Figure S4: Characterization of *Tcf21* Promoter Activity in the Embyronic Adrenal Gland. Embyros harvested from *Tcf21*$^{LacZ/+}$ mice were evaluated by whole mount LacZ staining. Transverse sections of E10.5 (panels A and B) and E12.5 (Panels C and D) embryos reveal βgal activity (blue cytoplasm) in the developing adrenogonadal primordia and coalescing adrenal primordia, respectively. Panels B and D are enlargements of boxes in Panels A and C. Key: (1) caudal region of fourth ventricle; (2) common ventricular chamber of heart (3) bulbus cordis region of heart; (4) caudal extremity of notochord; (5) hindlimb bud; (6) neural tube and lumen; (7) hindgut; (8) umbilical artery; (9) urogenital ridge; (10) adrenal primordial; (11) mesonephric tubules; (12) stomach; (13) liver; (14) rostral extremity of gonadal ridge; (15) descending aorta; (16) dorsal root ganglion; (17) aorta. Scale bars = 100 μm.

Table S1. Antibodies used for immunohistochemistry

A. Primary antibodies

Antibody	Species	Company	Catalog code	Working Dilution
Nr2f2 (CoupTFII)	Mouse	R & D Systems (Minneapolis, MN)	pp-H7147-00	1:200
GFP	Chicken	Abcam (Cambridge, MA)	ab13970	1:2000
GFP	Rabbit	Invitrogen (Carlsbad, CA)	A11122	1:500
LacZ	Chicken	Abcam	ab9361	1:1000
LacZ	Rabbit	Millipore (Billerica, MA)	AB1211	1:1000
Pdgfra	Rabbit	Cell Signaling Technology (Danvers, MA)	3164S	1:500
Nr5a1	Rabbit	Proteintech Group (Chicago, IL)	Custom	1:1000
Th	Mouse	Millipore (Billerica, MA)	MAB318	1:200
Acta1	Mouse	Sigma (St Louis, MO)	A2547 (Clone 1A4)	1:200
Desmin	Rabbit	ThermoScientific/Pierce (Rockford, IL)	PA5-16705	1:200

B. Secondary antibodies from Jackson ImmunoResearch (West Grove, PA, USA) used at 1:500 dilution

Host	Reactivity	Fluorophore	Catalog code
Donkey	Chicken	AlexaFluor 647	703-605-155
Donkey	Chicken	Dylight 488	703-485-155
Donkey	Goat	AffiniPure 488	705-545-147
Donkey	Goat	AffiniPure Cy3	705-165-147
Donkey	Goat	AlexaFluor 647	705-605-147
Donkey	Mouse	Dylight 649	715-495-150
Donkey	Rabbit	Dylight 649	711-495-152
Goat	Chicken	Dylight 549	103-505-155
Goat	Mouse	Dylight 488	115-485-146
Goat	Mouse	Dylight 549	115-505-146
Goat	Rabbit	Dylight 549	111-505-144
Rabbit	Goat	Dylight 488	305-485-045
Rabbit	Goat	Dylight 549	305-505-045