Molecular and Cellular Endocrinology 413 (2015) 168–177

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Fgfr2 is required for the expansion of the early adrenocortical primordium

Regine Häfner [a], Tobias Bohnenpoll [a], Carsten Rudat [a], Thomas M. Schultheiss [b], Andreas Kispert [a, *]

[a] Institut für Molekularbiologie, Medizinische Hochschule Hannover, Hannover, Germany
[b] Department of Genetics and Developmental Biology, Rappaport-Technion Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

ARTICLE INFO

Article history:
Received 17 May 2015
Received in revised form
21 June 2015
Accepted 22 June 2015
Available online 30 June 2015

Keywords:
Adrenocortical primordium
Proliferation
Coelomic epithelium
Conditional knockout
Tbx18
Fgfr2

ABSTRACT

The adrenal cortex is a critical steroidogenic endocrine tissue, generated at least in part from intermediate mesoderm of the anterior urogenital ridge. Previous work has pinpointed a minor role of the FGFR2IIIb isoform in expansion and differentiation of the fetal adrenal cortex in mice but did not address the complete role of FGFR2 and FGFR1 signaling in adrenocortical development. Here, we show that a *Tbx18*^{cre} line mediates specific recombination in the coelomic epithelium of the anterior urogenital ridge which gives rise by a delamination process to the adrenocortical primordium. Mice with conditional (*Tbx18*^{cre}-mediated) deletion of all isoforms of *Fgfr2* exhibited severely hypoplastic adrenal glands around birth. Cortical cells were dramatically reduced in number but showed steroidogenic differentiation and zonation. Neuroendocrine chromaffin cells were also reduced and formed a cell cluster adjacent to but not encapsulated by steroidogenic cells. Analysis of earlier time points revealed that the adrenocortical primordium was established in the intermediate mesoderm at E10.5 but that it failed to expand at subsequent stages. Our further experiments show that FGFR2 signaling acts as early as E11.5 to prevent apoptosis and enhance proliferation in adrenocortical progenitor cells. FGFR1 signaling does not contribute to early adrenocortical development. Our work suggests that FGFR2IIIb and IIIc isoforms largely act redundantly to promote expansion of the adrenocortical primordium.

1. Introduction

The adrenal cortex is a critical endocrine tissue that synthesizes and secretes different classes of steroid hormones in the control of body homeostasis and stress response (Walczak and Hammer, 2015). The adrenal cortex develops through a series of complex cellular processes from the intermediate mesoderm of the anterior urogenital ridge. At approximately embryonic day (E) 9.5 in the mouse, cells are supposed to delaminate from the coelomic epithelium covering the urogenital ridge and invade the underlying mesenchyme to form a contiguous adrenogonadal primordium. Starting at E10.5, a small anterior cell cluster separates from this primordium and moves dorso-medially to form the adrenal anlage adjacent to the dorsal aorta, while the remainder of the cells moves ventro-laterally to contribute to somatic cells of the gonads (Hatano et al., 1996; Luo et al., 1994; Uotila, 1940). From E11.5 to E12.5, neural crest cells invade the adrenal anlage. While the two progenitor populations proliferate rapidly and sort into a central medulla and surrounding cortex, mesenchymal cells probably of coelomic origin form a fibrous capsule around the composite adrenal anlage by E14.5. Neural crest derived medullary cells differentiate into neuroendocrine chromaffin cells that synthesize catecholamines and secrete them in response to sympathetic inputs (Anderson et al., 1991). The cortical region matures and forms a transient fetal zone (x-zone) and the definitive (adult) cortex. The fetal zone regresses after puberty in mice while the definitive cortex subdivides in a thin outer zona glomerulosa synthesizing mineralocorticoids and a thick inner zona fasciculata producing glucocorticoids. Homeostasis of the adrenal cortex is mediated by capsular and subcapsular progenitors that give rise to steroidogenic cells that move centripetally (for reviews see (Kim et al., 2009; Laufer et al., 2012; Walczak and Hammer, 2015).

Analyses of humans with congenital adrenal hypoplasia and of knockout mice have identified a number of molecular regulators of

* Corresponding author. Institut für Molekularbiologie, OE5250, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany.
E-mail address: kispert.andreas@mh-hannover.de (A. Kispert).

http://dx.doi.org/10.1016/j.mce.2015.06.022
0303-7207/

adrenocortical development. The steroidogenic factor 1 gene *(Sf1*, also known as *Nr5a1*), that encodes an orphan nuclear receptor expressed in the coelomic epithelium, the adrenogonadal primordium and the steroidogenic cells of the adrenal cortex (Ikeda et al., 1994), turned out as a key regulator of progenitor expansion, steroidogenic pathway gene expression and steroidogenic cell identity (Lala et al., 1992; Luo et al., 1994). *Sf1* transcription is positively regulated by Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (CITED2) (Bamforth et al., 2001), and negatively impacted on by Wilms tumor 1 homolog (WT1) (Bandiera et al., 2013; Moore et al., 1999), DAX1 (also known as nuclear receptor subfamily 0, group B, member 1, Nrob1) (Ito et al., 1997) and transcription factor 21 (TCF21) (Tamura et al., 2001). Sonic hedgehog (*Shh*) is expressed in the adrenocortex underneath the adrenal capsule. It is required for proliferation of capsular and adrenocortical cells but not for differentiation of the adrenocortex (Huang et al., 2010; King et al., 2009). Canonical WNT signaling maintains adrenocortical progenitors between E12.5 and E14.5 (Kim et al., 2008). WNT signaling within the outer adrenal cortex is subsequently involved in recruitment of progenitors, potentially through stimulation of *Shh* expression, and in differentiation of the zona glomerulosa (reviewed in (Drelon et al., 2015)). Insulin and insulin-like growth factor (IGF)1 receptor signaling have been implicated in induction of cell proliferation and maintenance of *Sf1* expression throughout the genital ridge and the adrenogonadal primordium beginning as early as E10.5 (Pitetti et al., 2013).

Fibroblast growth factors (FGF)s are a family of 23 secreted proteins that bind with high affinity to at least four members of a family of receptor tyrosine kinases, termed FGFR1-FGFR4. FGFRs occur in different splice variants that signal through different downstream modules to trigger in both a transcriptionally independent and dependent manner changes of cell behavior including migration, proliferation, apoptosis and differentiation in a variety of biological contexts (Laestander and Engstrom, 2014). FGFR2 signaling has been functionally implicated in adrenal development by a number of genetic loss-of-function experiments *in vivo*. Revest and colleagues showed that mice carrying an *Fgfr2* allele in which the IIIb variant is disrupted, exhibit numerous organ defects including hypoplastic adrenals at E16.5 (Revest et al., 2001). A couple of years later Kim and coworkers noted that mice with *Sf1cre*-mediated deletion of all splice variants of *Fgfr2* have a severe adrenal hypoplasia (Kim et al., 2007). More recently, it was found that mice with global deletion of the *Fgfr2IIIb* variant exhibit smaller adrenals with a thickened mesenchymal capsule and a slightly reduced expression of steroidogenic and zona fasciculata markers (Guasti et al., 2013). Although these studies suggest a (minor) role for the FGFR2IIIb isoform in development of the fetal adrenal cortex, the more severe adrenal hypoplasia noted in mice with conditional deletion of all *Fgfr2* splice variants in the adrenogonadal primordium argues for an additional or earlier requirement of other FGFR2 isoforms, and FGFR2 signaling, respectively, in adrenal development.

Here, we use a $Tbx18^{cre}$ line (Trowe et al., 2010) to completely delete *Fgfr2* in the coelomic epithelium of the anterior urogenital ridge. We show that *Fgfr2* (but not *Fgfr1*) has an early function in the expansion but not the differentiation of the adrenocortical primordium.

2. Methods

2.1. Mice

Mice were housed in rooms with controlled light and temperature. All mouse work was performed according to European and German legislation. The breeding of mutant mouse lines was approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (AZ 33.12-42502-04-13/1356).

$Tbx18^{GFP}$ ($Tbx18^{tm2Akis}$) and $Tbx18^{cre}$ ($Tbx18^{tm4(cre)Akis}$) mice were previously generated in the laboratory at the Medizinische Hochschule Hannover (Christoffels et al., 2006; Trowe et al., 2010). Mice expressing the double fluorescent cre reporter line *(Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato-EGFP)Luo}$, synonym: $R26^{mTmG}$) (Muzumdar et al., 2007), mice with *loxP* sites flanking exon 4 of the *Fgfr1* locus ($Fgfr1^{tm5.1Sor}$; synonym: $Fgfr1^{fl}$) (Hoch and Soriano, 2006), and mice with *loxP* sites flanking exons 7 to 10 of the *Fgfr2* locus ($Fgfr2^{tm1Dor}$, synonym: $Fgfr2^{fl}$) (Yu et al., 2003) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained on an NMRI outbred background. Embryos for mutant analysis were derived from matings of $Tbx18^{cre/+}$;$R26^{mTmG}$;$Fgfr1^{fl/+}$;$Fgfr2^{fl/+}$ male and $Fgfr1^{fl/fl}$;$Fgfr2^{fl/fl}$ female mice. Embryos for *Fgfr1/Fgfr2* expression analysis were obtained from NMRI matings. Matings were set up in the evening and vaginal plugs checked in the morning afterward. Noon was taken as E0.5. Urogenital systems and embryos were dissected in PBS, fixed in 4% paraformaldehyde (PFA) in PBS and stored in methanol at −20 °C. For genotyping by PCR genomic DNA prepared from yolk sacs or tail biopsies was used.

2.2. Organ cultures

Urogenital ridges from E11.5 embryos of $Tbx18^{cre/+}$;$R26^{mTmG}$;$Fgfr1^{fl/+}$;$Fgfr2^{fl/+}$ x $Fgfr1^{fl/fl}$;$Fgfr2^{fl/fl}$ matings were dissected, explanted on Transwell permeable supports (Corning Inc.) and cultured with DMEM/F12 medium (Gibco) supplemented with 10% fetal calf serum (Lonza) and 1% penicillin/streptomycin solution (Hyclone Laboratories) at the atmosphere-medium interface at 37 °C and 5% CO_2. Replacement of culture medium and documentation took place every 24 h.

2.3. Histological and immunofluorescent analysis

For histological analysis, embryos were fixed as stated before, paraffin embedded and sectioned to 5 μm. Sections were stained with hematoxylin and eosin, following standard procedures.

Immunofluorescence analysis was done on 5 μm sections with the following antibodies: rabbit anti-SF1 (TransGenic Inc., preparation of antibodies by Dr. Ken-Ichirou Morohashi, 1:200), rabbit anti-TH (ABIN723635, antikoerper-online.de, 1:800), mouse anti-GFP (11814460001, Roche, 1:200), rabbit anti-GFP (sc-8334, Santa Cruz, 1:200). Fluorescent staining was performed using Alexa-488/555-conjugated secondary antibodies (A11034; A11008; 711-487-003; A21202; A21422; A21428, Invitrogen/Dianova; 1:400) or biotin-conjugated secondary antibodies (Dianova; 1:400) and the TSA Tetramethylrhodamine Amplification Kit (Perkin–Elmer).

Tagging with primary antibodies was performed at 4 °C overnight after antigen retrieval (Antigen unmasking solution, Vector Laboratories; 10 min, 100 °C), blocking of endogenous peroxidases (3% H_2O_2/ddH_2O, 15 min), and incubation in blocking solutions provided with the kits. Sections were mounted with Mowiol (Roth). All sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei.

2.4. Proliferation and apoptosis assays

To analyze apoptosis with the TUNEL assay, stained sections were treated according to the protocol provided with the ApopTag Fluorescence Apoptosis detection kit (S7111, Millipore) before DAPI-staining and mounting. For cell proliferation analysis the incorporated 5-bromo-2′-deoxyuridine (BrdU) on 5 μm sections was detected fluorescently (1170376, Roche, 1:100) as described