REVIEW

The transient cortical zone in the adrenal gland: the mystery of the adrenal X-zone

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Abstract

The X-zone is a transient cortical region enriched in eosinophilic cells located in the cortical–medullary boundary of the mouse adrenal gland. Similar to the X-zone, the fetal zone in human adrenals is also a transient cortical compartment, comprising the majority of the human fetal adrenal gland. During adrenal development, fetal cortical cells are gradually replaced by newly formed adult cortical cells that develop into outer definitive zones. In mice, the regression of this fetal cell population is sexually dimorphic. Many mouse models with mutations associated with endocrine factors have been reported with X-zone phenotypes. Increasing findings indicate that the cell fate of this aged cell population of the adrenal cortex can be manipulated by many hormonal and nonhormonal factors. This review summarizes the current knowledge of this transient adrenocortical zone with an emphasis on genes and signaling pathways that affect X-zone cells.

Key Words

- adrenal cortex
- X-zone
- X-zone marker genes
- adrenal development and regression

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Introduction

Zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) are the three major cortical zones (definitive zones) in the mammalian adrenal gland. In human fetal adrenals, there is another cortical layer in the inner cortex next to the medulla named the 'fetal zone' (Ishimoto & Jaffe 2011). This transient cortical compartment grows rapidly from 8- to 30-week gestation leading to the fetal adrenal gland being 10- to 20-fold larger than the adult adrenal gland. Within the first two to three weeks after birth, the fetal zone undergoes rapid regression through apoptosis (Spencer *et al.* 1999). Although the origin and the lineage relationship between definitive zones and the fetal zone requires further investigation, lineage tracing experiments using mouse models have shown that definitive zones originate from the same group of cells (Zubair *et al.* 2008, King *et al.* 2009, Freedman *et al.* 2013). In mice, the adrenal capsule and the ZG contain stem and progenitor cells, respectively (King *et al.* 2009, Huang *et al.* 2010). Newly formed cortical cells from the stem/progenitor cells move inward to the corticomedullary boundary during development. Throughout this migration process, adrenocortical cells differentiate into concentric zones, which become definitive zones, whereas cortical cells in the fetal cortex are considered an aged cell population and accumulate in the corticomedullary boundary before they undergo regression. In mice, this transient cell population is called the X-zone. It is suggested that the fetal zone in human and the X-zone in mice are analogous structures (Yates *et al.* 2013). Although the timing of formation and regression of the fetal zone and the X-zone differ, these two populations share many common phenotypic, molecular and functional characteristics. Cells in both compartments produce steroid hormones that are critical for fetal development (Ishimoto & Jaffe 2011, Huang *et al.* 2012). A mutation in the same gene results in delayed regression of this transient population both in humans

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and mice (Muscatelli *et al.* 1994, Yu *et al.* 1998, Xing *et al.* 2017). Also, activin treatment induces the apoptosis of both fetal zone and X-zone cells (Spencer *et al.* 1999, Beuschlein *et al.* 2003). The regulatory mechanism of the growth and the regression of these aged cortical cells is not fully understood. However, mouse models have helped to identify many factors associated with the fate of this transient cell population in the adrenal cortex.

History of finding the X-zone: a unique response of the inner cortex during pregnancy

In the late 19th and early 20th centuries, increased adrenal gland weight during pregnancy was reported in many species, including guinea pigs (Guieysse 1899, Kolmer 1912), rabbits (Kolde 1913), rats (Herring 1920*a*) and mice (Masui & Tamura 1924). There were also reports showing controversial results, such as smaller rabbit adrenals during pregnancy (Gottschau 1883) and no enlargement in rat adrenals during pregnancy (Donaldson 1924). Although these disparate findings might be due to the varied genetic background and scale of the experimental design, the anatomic difference in adrenal cortex due to sex-specific physiological conditions has been noticed. At that time, many clinical reports also showed that female patients with adrenal cortical tumors tended to have male secondary sexual characteristics (Krabbe 1921). With other indications, such as feeding young animals with an adrenal substance that stimulates the growth of the testes, Vincent (1917) first linked the adrenal gland with the reproductive system and stated that the adrenal cortex is associated in its functions in some way with the development of the gonads.

However, evidence also suggested another way to link adrenal with gonads; that is, a factor released from the gonads may act on the adrenal gland. For example, a sex difference in adrenal gland size was recognized in rats (Hatai 1913, Jackson 1913). In addition to the increased adrenal gland weight, it was also noticed that there was an increased degeneration process in the reticular zone of the cortex during pregnancy in guinea pigs (Kolmer 1912). Masui and Tamura (Masui & Tamura 1924, Tamura 1926) then found that the ZR, the inner most cortical zone abutting the adrenal medulla, disappears completely due to fatty degeneration at the end of pregnancy in female mice. They also reported that castration results in an enlarged 'reticular zone' in male mice. Howard-Miller (1927) further studied how age and sex affect the inner cortex in mouse adrenals. She described degeneration due to pregnancy as well as spontaneous regression of the reticular zone in virgin female mice. She noticed that the degeneration could be fatty or nonfatty. She then named this 'highly developed' reticular zone in mice as the 'X-zone.'

Characterization of the X-zone: its origin and sexually dimorphic regression

X-zone cells are defined as a unique islet of eosinophilic cells in the inner cortex next to the medulla. X-zone cells can be found first at postnatal day (P) 8 and form a layer at the corticomedullary boundary at P10–P14. Depending on the mouse strain and age, the X-zone may contain cells with vacuolated (e.g., A/J mice and aged DDD mice) and/or nonvacuolated (e.g., SM/J mice and C57BL/6J mice) morphology or both (e.g., DDD mice at P35) (Tanaka & Matsuzawa 1995, Tanaka *et al.* 1995).

Based on the observation of lipid droplets in the cytoplasm, the X-zone was proposed to serve the same function as the outer cortical zones. For example, it exhibits an androgenic function as does the ZF in other species (Masui & Tamura 1924, Deanesly 1958, Nishida & Mochizuki 1963). The presence of lipid droplets and extensive agranular endoplasmic reticulum in the X-zone cells further supports the idea that these are steroid-producing cells (Sato 1968). However, the low expression of 3β-hydroxysteroid dehydrogenase (3βHSD) in the X-zone (Allen 1960, Hershkovitz *et al.* 2007) and the lack of androgen production postnatally in mouse adrenals (van Weerden *et al.* 1992) indicates that the X-zone is not the primary site of steroid hormone secretion. Although the steroidogenic activity of the fetal adrenal cortex is critical to the development of the fetal adrenal medulla and hypothalamic feedback suppression (Huang *et al.* 2012), the function of the X-zone remains unclear and requires further study.

In female mice, X-zone cells start to degenerate gradually as early as P32 (Howard-Miller 1927). The degeneration may occur between P32 and P200 subject to genetic variation (Daughaday 1941, Badr *et al.* 1968, Shire & Spickett 1968, Janat & Shire 1987, Di Curzio & Goldowitz 2011). The form of degeneration also varies in different strains in terms of the degree of vacuolization of the zone with or without the accumulation of lipid (Shire 1974). In pregnant females, the entire X-zone disappears at 5–15 days of the first pregnancy (Miller 1927, Jones 1952). Without pregnancy, the X-zone in female mice regresses gradually and disappears between 3 and 7 months of life

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(Miller 1927). In males, the X-zone disappears before P40 (Tamura 1926, Miller 1927) (Fig. 1).

Lineage tracing experiments using *lacZ* reporters driven by a specific steroidogenic factor 1 (SF1) promoter fragment (FAdE) show that X-zone cells in postnatal adrenal glands are descendants of the fetal adrenal cortex (Zubair *et al.* 2006, 2008). Lineage tracing using the *Shh-CreERT2* mouse line further showed that fetal cortical cells in the outer cortex labeled at embryonic day (E) 14.5 could differentiate into ZG and ZF cells (King *et al.* 2009). Although the outer cortex (*Shh*-positive cells) and capsule (*Gli1*-positive cells) are progenitor and stem cells that contribute to the inner cortical cell population (King *et al.* 2009, Huang *et al.* 2010), lineage tracing using the ZG-specific Cre (aldosterone synthase-Cre) demonstrates that the X-zone does not originate from the adult cortex (Freedman *et al.* 2013).

X-zone markers

Three unique makers have been identified in the inner most adrenal cortex including the X-zone. In the adrenal gland, these marker genes are specifically expressed in this particular region. However, no X-zone phenotype has been reported in knockout (KO) mouse models of these marker genes.

20-α-Hydroxysteroid dehydrogenase (20αHSD)

20αHSD was first identified in the ovary as a progesterone-metabolizing enzyme (Wiest 1959). Its expression in the mouse adrenal gland then has been associated with sex hormones in which estrogen induces its expression and testosterone causes a loss of expression (Ertel & Ungar 1968, Stabler & Ungar 1970). In adrenals, expression of 20αHSD mRNA (*Akr1c18*) (Pelletier *et al.* 2003) and its enzyme activity (Ungar & Stabler 1980) is dependent on the presence of the X-zone, with the cellular expression pattern of *Akr1c18* and 20αHSD in the adrenal gland confirming its specific expression in the X-zone (Pelletier *et al.* 2003, Hershkovitz *et al.* 2007). Although this enzyme is highly expressed in the X-zone, targeted disruption of *Akr1c18* does not affect adrenal morphology or X-zone growth/regression (Hershkovitz *et al.* 2007).

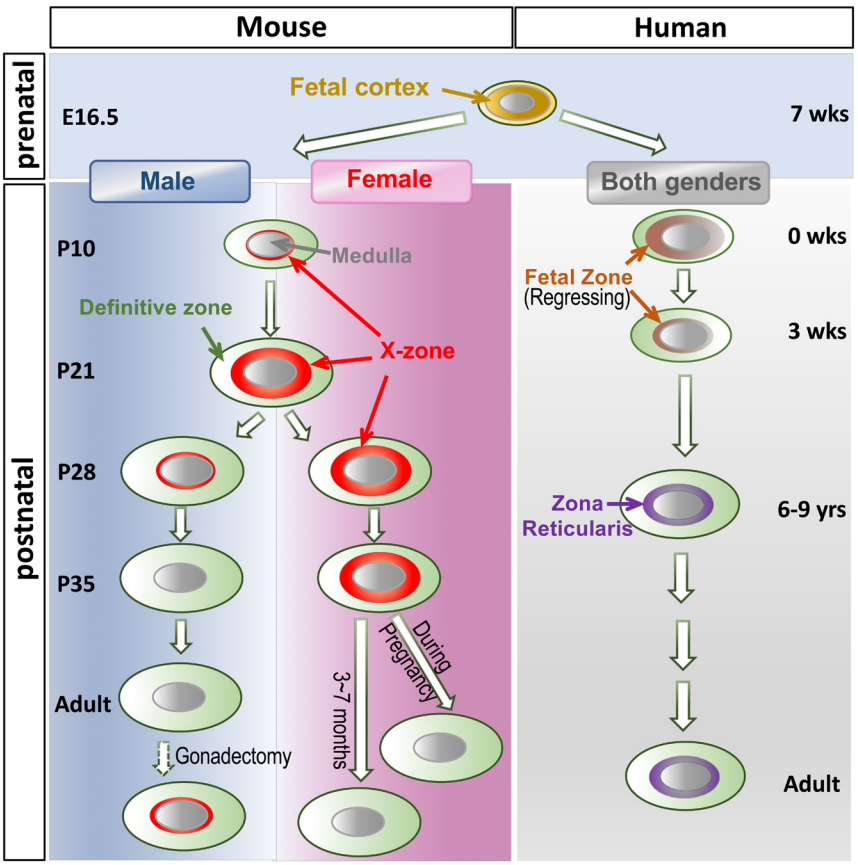

Figure 1
Timeline of the formation and regression of the inner cortex in mouse and human adrenals.

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Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing gamma polypeptide (*Pik3c2g*)

Pik3c2g was identified as an X-zone marker by comparing the adrenal microarray data from the wild-type and *Gata6* steroidogenic tissue-specific conditional knockout (cKO) mice, a mouse model that lacks the X-zone and exhibits gonadal-like differentiation of the adrenal cortex following gonadectomy (Pihlajoki *et al.* 2013), as discussed subsequently. The specific expression of *Pik3c2g* also was confirmed by quantitative polymerase chain reaction (qPCR) using whole adrenals from different sexes and developmental stages as well as cells from different cortical zones collected by laser microdissection (Pihlajoki *et al.* 2013). The protein encoded by *Pik3c2g* belongs to the phosphoinositide 3-kinase (PI3K) family, which is mainly expressed in liver but also can be found in prostate, testis, small intestine, kidney and pancreas at a lower level (Misawa *et al.* 1998, Rozycka *et al.* 1998). Although PI3-kinase has roles in signaling pathways involved in cell proliferation, survival and metabolism, no adrenal gland phenotype has been reported in mice carrying mutations associated with the PI3K family (Harada *et al.* 2005, Harris *et al.* 2011, Yoshioka *et al.* 2012, Braccini *et al.* 2015).

Thyroid hormone receptor β1 (TRβ1)

TRβ1 was recently reported as a novel marker of the inner cortex, including the X-zone (Huang *et al.* 2015). The cellular expression pattern of TRβ1 partially overlaps that of 20αHSD; however, in the adrenal inner cortex, there is also a TRβ1$^+$/20αHSD$^-$ zone surrounding the 20αHSD$^+$ X-zone. In male mice, the TRβ1$^+$ zone regresses with the 20αHSD$^+$ X-zone, whereas in female mice, the TRβ1$^+$ zone remains in parous mice. The unique expression of TRβ1 in the inner cortex indicates that cells in definitive zones share a similarity with X-zone cells; that is, both are TRβ1$^+$ when they become aged cells.

Major factors that affect the X-zone

The following hormones and genes have been reported by several studies as being linked to the development and/or regression of the X-zone.

Sex steroid hormones

The sexual dimorphism of X-zone regression suggests a possible role of sex steroid hormones on inner cortical cells. In male mice, castration increases the size of the inner cortex (Masui & Tamura 1924) and prevents its regression (Miller 1927). A secondary X-zone also reappears in adult males after castration (Callow & Deanesly 1935, Howard 1939, Hirokawa & Ishikawa 1975). Although ovariectomy in 3-month-old females results in a thicker X-zone 1 week postoperatively, the X-zone still regresses gradually with age in ovariectomized mice similar to that in control mice (Matsuura & Suzuki 1986). Also, estrone or estradiol injection has no or limited effect on X-zone degeneration (Warning 1942, Dickson 1969, Holmes & Dickson 1971*b*), suggesting that factors from the ovary do not affect X-zone regression. Findings from gonadectomy studies suggest that a molecule(s) from the testis might be the main factor responsible for X-zone regression. Indeed, testosterone treatment leads to rapid X-zone regression in all conditions, including in adolescent males and females, adult females, castrated adult males, adolescent ovariectomized females and adult ovariectomized females (Starkey & Schmidt 1938, Hershkovitz *et al.* 2007). Moreover, testosterone treatment before the X-zone prevents X-zone formation in both sexes (Starkey & Schmidt 1938). A recent study on tissue-specific androgen receptor (AR) KO mice in which the Cre expression is driven by the *Cyp11a1* promoter confirms that AR is essential for regression of the X-zone (Gannon *et al.* 2017). However, another genetic mouse model with spontaneously mutated AR (the Tfm mice) that leads to low androgen-binding activity, small testes and a lack of male accessory glands (Lyon & Hawkes 1970, He *et al.* 1994) still shows normal regression of the X-zone (Shire 1976). The disparate results from AR cKO and Tfm mice suggest that the testosterone effect on X-zone regression may not solely rely on androgen binding to its receptor. The loss of androgens through castration or androgen action via AR cKO impact adrenal cortex structure, gene expression and protein localization differently (Gannon *et al.* 2017). In male mice, the X-zone regresses before testosterone increases, supporting the idea that testosterone is not the sole mediator of X-zone regression under normal conditions. For instance, androgen could be a precursor and its metabolites responsible for X-zone regression.

Studies have shown a variety of different results on the effects of pseudopregnancy on X-zone regression (Jones 1952, Holmes & Dickson 1971*b*, Hershkovitz *et al.* 2007). Blastocysts or trophoblast transplantation under the kidney capsule and progesterone injection were both found to cause X-zone regression (Holmes & Dickson 1971*a*,*b*), suggesting that progesterone can trigger this process.

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Promoter analysis of 20αHSD showed binding sites for the glucocorticoid receptor (GR), progesterone receptor (PR) and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), but not for AR (Hirabayashi *et al.* 2004). This finding suggests that the reduced 20αHSD levels observed in pregnant female mice (Hershkovitz *et al.* 2007) may be primarily due to increased progesterone levels rather than the regression of the X-zone. Therefore, it is possible that several factors in the hypothalamic–pituitary–gonadal axis are involved in X-zone regression.

Luteinizing hormone (LH)

Equine LH can rescue X-zone regression in hypophysectomized virgin female mice, suggesting LH as an X-zone maintenance factor (Jones 1949, Deacon *et al.* 1986, Beuschlein *et al.* 2003). If LH can act as a growth/survival factor for the X-zone, elevated LH and LH receptor (LHR) levels (Kero *et al.* 2000) then likely explain why castrated mice show no X-zone regression and/or can develop a secondary X-zone. However, under normal conditions without castration, *Lhr* is not expressed in male and female mouse adrenals regardless of whether the X-zone is present (Kero *et al.* 2000). Moreover, in bLHβ-CTP transgenic mice, a model with chronic LH hypersecretion and strong adrenal LHR expression, the overall adrenal weight is only slightly increased at age 1 month or 5 weeks without any difference in X-zone size (Kero *et al.* 2000, Beuschlein *et al.* 2003). Instead, bLHβ-CTP virgin female mice show complete X-zone regression at age 5 months, which is sooner than in control virgin females (Kero *et al.* 2000). These findings suggest that LH might elicit its growth-promoting effects on the X-zone only under hypophysectomized or gonadectomized conditions through a secondary mediator(s). Thus, the negative feedback suppression of LH secretion is unlikely to be the primary reason for X-zone regression with testosterone treatment.

Thyroid hormone

The effect of thyroid hormone on adrenals has been known since the 1920s, when it was determined that administration of thyroid extract results in the hypertrophy of the inner cortical layer in many species, including mouse, rat, rabbit, cat and guinea pig (Herring 1920*b*). In the mouse, thyroid extract or thyroxine enlarges the adrenal X-zone of both sexes, increases the lipoid quantity in and cell size of X-zone cells and prolongs the survival of the X-zone (Preston 1928, Gersh & Grollman 1939). Consistent with a growth-promoter role for thyroid hormone in the X-zone, the X-zone is poorly developed in mice with hypothyroidism (Shire & Beamer 1984). KO mouse models further show that thyroid hormone treatment elicits its response in the X-zone mainly through TRβ1 (Huang *et al.* 2015). However, the X-zone in TRβ1 and TRβ1/TRβ2 global KO mice still develops and regresses normally (Huang *et al.* 2015), suggesting that thyroid hormone is not the primary regulator controlling X-zone formation/regression during normal development.

Activin and inhibin

Activin and inhibin belong to the transforming growth factor-β (TGFβ) family and are secreted mainly by the gonads and adrenals. The roles of inhibin and activin in adrenal gland physiology and tumorigenesis have focused along several lines of research (Hofland & de Jong 2012). In human adrenals, all three activin and inhibin subunit proteins are expressed in definitive zones and the fetal zone (Spencer *et al.* 1992). Despite the fact that the inhibin α subunit is expressed throughout the fetal adrenal, it is expressed most intensively in the innermost area of the fetal zone in human adrenals (Voutilainen *et al.* 1991, Billiar *et al.* 1999). In mice, inhibin-deficient nulliparous females show gradual regression of the X-zone more rapidly than control mice, exhibiting no remaining X-zone by the age of 18 weeks (Beuschlein *et al.* 2003). However, this X-zone regression requires the presence of ovarian tumors. Gonadectomy in these mice instead leads to X-zone growth and the development of an adrenal tumor that expresses a high level of LHR and other gonadal cell markers (Matzuk *et al.* 1994, Beuschlein *et al.* 2003, Looyenga & Hammer 2006). Since inhibin-deficient mice with ovarian tumors do not have elevated estradiol or testosterone levels, the regression of the X-zone in inhibin-deficient mice must be due to gonadal factors other than sex steroids. Indeed, in 5-week-old double transgenic mice that have inhibin deficiency and the bLHβ-CTP transgene, the adrenals have no X-zone and are significantly smaller than those in inhibin single mutant mice or wild-type mice. Other than the elevated LH level, the double transgenic mice have larger ovarian tumors and higher activin levels at age 5 weeks. All these data suggest the idea that the regression of the X-zone in inhibin-deficient mice might be due to increased plasma activin levels as a result of the developing ovarian tumor (Beuschlein *et al.* 2003). Since activin types I and II

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receptors are present in mouse adrenals, with a strong expression of activin receptor subtypes IA and IIB in the X-zone, activin may have a direct effect on X-zone cells (Beuschlein *et al.* 2003). This idea is supported by a series of *in vitro* and *in vivo* studies: human primary adrenal cell culture experiments show that activin A not only inhibits the proliferation of specific fetal adrenocortical cells in a dose- and time-dependent manner (Spencer *et al.* 1990), but also induces their apoptosis (Spencer *et al.* 1999). In addition, activin A enhances adrenocorticotropic hormone (ACTH)-stimulated cortisol secretion in cultured fetal zone cells, but not in adult cortical cells (Spencer *et al.* 1992). In mouse primary adrenal cell culture, activin treatment leads to a significant reduction in cell number only in cultures obtained from female mice containing the X-zone. Activin treatment shows no effect in cultures from adult male mice (Beuschlein *et al.* 2003). In a mouse study *in vivo*, activin specifically induces apoptosis in the X-zone 3 and 6 h after injection (Beuschlein *et al.* 2003). However, no phenotype of the X-zone or the adrenal gland has been reported in activin-deficient mice (Matzuk *et al.* 1995).

While the effect of activin on X-zone regression in inhibin-deficient mice has been characterized, the function of inhibin on the X-zone remains to be determined. The gonadectomy-induced adrenal tumors in inhibin-deficient mice suggest a critical role for inhibin in adrenocortical cell differentiation. A transgenic mouse model carrying the simian virus 40 T-antigen-coding region driven by the mouse inhibin α subunit promoter presents with adrenal tumors originating from the X-zone (Rilianawati *et al.* 2000). Since inhibin shows no mitogenic effect on adrenocortical cell cultures (Spencer *et al.* 1992), whether inhibin can directly exert its function on the fetal cortex or the X-zone, or works indirectly to affect these tissues, requires further investigation.

GATA-binding protein-6 (GATA6)

In human fetal adrenals, GATA6 is expressed in the fetal zone and is more prominent in the definitive zone (Kiiveri *et al.* 2002). In adult human adrenals, GATA6 is expressed abundantly in the ZF and ZR with a clear predominance in the ZR (Kiiveri *et al.* 2002). GATA6 is a member of the GATA family of transcription factors and it regulates the expression of many steroidogenic enzymes, including P450 side-chain cleavage (Jimenez *et al.* 2003) and 3βHSD (Martin *et al.* 2005). Consistent with its expression in the inner cortex, GATA6 has a role in androgen synthesis (Flück & Miller 2004). Reporter assays in human adrenocortical cell lines have showed that GATA6 works with SF1 and/or specificity protein 1 (SP1) to activate 17α-hydroxylase (P450c17) (Jimenez *et al.* 2003, Flück & Miller 2004). GATA6 expression also positively correlates with P450c17 in human adrenal tumors (Kiiveri *et al.* 2005).

In mice, SF1-Cre-mediated GATA6 ablation leads to a lack of the X-zone in virgin females and castrated males. These *Gata6* cKO mice also have gonadal-like cells in the adrenal cortex when gonadectomized (Pihlajoki *et al.* 2013). Since mouse adrenals only express P450c17 at embryonic stages (Keeney *et al.* 1995), the ectopic expression of P450c17 in gonadectomized *Gata6* cKO adrenals suggests a possible role of GATA6 in cortical cell differentiation. However, it also is possible that the effect of GATA6 depletion on X-zone development is indirect, since *Gata6* is expressed mainly in the capsular and subcapsular cells, but not in the X-zone (Pihlajoki *et al.* 2013). Considering *Inhba* and *Inhbb* are elevated in *Gata6* cKO adrenals (Pihlajoki *et al.* 2013) and the expression of *Inhba* positively correlates with *Gata6* expression in mouse adrenocortical cells (Looyenga & Hammer 2006), the effect of GATA6 on X-zone development might be secondary to effects on inhibin and/or activin.

Protein kinase cAMP-dependent type I regulatory subunit alpha (*Prkar1a*)

Prkar1a is a tumor suppressor gene that encodes a regulatory subunit in the cAMP-dependent protein kinase (PKA) pathway. A lack of *Prkar1a* leads to constitutive PKA activation. A tetracycline-controlled (Tet-Off) antisense RNA mouse model shows that depletion of *Prkar1a* leads to PKA activation in the adrenal with X-zone persistence in parous female and adult male mice (Griffin *et al.* 2004). Knockdown of PRKAR1A in the human adrenocortical cancer cell line H295R decreases the expression of SMAD3, a downstream effector of the TGFβ pathway, and blocks TGFβ-induced apoptosis (Ragazzon *et al.* 2009). This anti-apoptotic effect also is observed in the adrenals of *Akr1b7-Cre*-mediated *Prkar1a* KO (AdKO) mice, in which *Prkar1a* is conditionally deleted in ZF cells and X-zone cells (Lambert-Langlais *et al.* 2009) resulting in inhibition of the dexamethasone-induced apoptosis (de Joussineau *et al.* 2014). Since PKA signaling activates the mammalian target of rapamycin (mTOR) in human and mouse adrenocortical cells and leads to increased cell survival, it has been proposed that activation of the mTOR pathway could be responsible for the resistance to dexamethasone-induced apoptosis in AdKO mice

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(de Joussineau *et al.* 2014). Adrenals in AdKO mice also show increased proliferation, a persistent X-zone, and an enlarged, mislocated X-like zone (20αHSD-positive) in aged parous female mice (Sahut-Barnola *et al.* 2010). Furthermore, these 20αHSD-positive cells are positive for AKR1B7, GATA4 and P450c17, suggesting *Prkar1a* could affect their differentiation and/or cell fate. The enlarged X-zone in AdKO mice could be due to conversion of the inner ZF into 20αHSD-positive cells and/or a direct effect of *Prkar1a* on X-zone cells. A recent report using two different Cre mouse lines to remove *Prkar1a* either in fetal cortex (FAdE-CreERT2) or definitive cortex (aldosterone synthase-Cre) shows that a lack of *Prkar1a* in fetal cortical cells (X-zone) does not lead to adrenal abnormalities (Dumontet *et al.* 2018), supporting the idea that *Prkar1a* has a unique function in the ZF to differentiate these cells into 20αHSD-positive cells.

Dose-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome (DAX1)

DAX1 is a nuclear receptor identified as a downregulator of other transcription factors, such as retinoic acid receptor alpha (RARα) and retinoid X receptor alpha (RXRα) (Muscatelli *et al.* 1994). It is expressed mainly in adrenals and gonads (Zanaria *et al.* 1994, Swain *et al.* 1996) and acts as a repressor of steroidogenic gene expression (Lalli *et al.* 1998) and SRY (sex-determining region Y gene) function (Swain *et al.* 1998). A lack-of-function mutation of DAX1 results in delayed regression of the X-zone in male mice (Yu *et al.* 1998, Beuschlein *et al.* 2002, Xing *et al.* 2017), and no regression of the fetal zone in humans (Muscatelli *et al.* 1994). Although *Dax1*-null male mice are sterile due to impaired spermatogenesis, their serum LH- and follicle-stimulating hormone (FSH) levels are normal (Yu *et al.* 1998). Delayed regression of the X-zone in *Dax1*-mutant mice again serves as evidence that a factor(s) other than sex hormones likely regulates X-zone regression. The similarity in the delayed X-zone regression phenotype found in the SUMOylation-deficient-$Sf1^{2KR/2KR}$ mice (Lee *et al.* 2011, Xing *et al.* 2017) links DAX1 and its negative regulation of *Sf1* to X-zone regression. *In vitro* studies show that SF1 SUMOylation is required to increase the binding of DAX1 to SF1 to repress FAdE activity in the *Sf1* gene. *In vivo* mouse models demonstrate that a lack of DAX1 or SF1 SUMOylation results in FAdE activation in the X-zone. Since FAdE is specifically expressed in the fetal cortex, delayed regression of the X-zone in these two models could be due to loss of inhibition of FAdE activity by the SUMOylated-SF1/DAX1 complex (Xing *et al.* 2017). However, the loss of SF1 SUMOylation or of DAX1 does not completely block regression of the X-zone. The X-zone in the mutant male mice still regresses gradually and disappears completely at the age of 15 weeks (Xing *et al.* 2017), indicating that DAX1 and SF1 only determine the proper timing of X-zone regression.

Other genes and factors

Ablation or deficiency of the following genes and factors also has been reported to result in X-zone phenotypes. However, the mechanisms underlying these effects on the X-zone are unclear. Further studies using tissue-specific knockout models are required because the reported phenotypes could be due to secondary effects from the global knockout of these genes.

Pre-B-cell transcription factor 1 (*Pbx1*)

In *Pbx1*-haploinsufficient mice, the size of the X-zone is reduced (Lichtenauer *et al.* 2007). *Pbx1* has been associated with the expression of P450c17 (Kagawa *et al.* 1994), which is expressed in mouse adrenals only at embryonic stages: *Pbx1* is expressed in the adrenogonadal primordium and is an upstream regulator of *Sf1* (Schnabel *et al.* 2003, Zubair *et al.* 2006). In *Pbx1* KO mouse embryos, early expression of *Sf1* (at E10.0) in the coelomic epithelium is reduced dramatically (Schnabel *et al.* 2003). Adrenals fail to develop in the *Pbx1* KO mouse, but remain rudimentary, despite initiation of sexual differentiation. Since SF1 also activates *Pbx1* promoter activity, it is possible that the reduced X-zone in *Pbx1* haploinsufficiency is due to synergistic actions of these two transcription factors in the regulation of downstream effectors of adrenocortical differentiation (Lichtenauer *et al.* 2007).

Adrenal X zone degeneration 1 and 2 (Exz1 and Exz2)

There appears to be a strain variation of the X-zone regression in virgin female mice. Regression of the X-zone starts at 60–70 days of age in adult BALB/cBy mice, whereas the X-zone persists in C57BL/6 animals at a similar age. Exz1 and Exz2, two quantitative trait loci (QTLs), were identified as genetic factors that may cause this early X-zone regression in BALB/cBy mice (Shire & Spickett 1968, Janat & Shire 1987). The genetic position of Exz1 is on chromosome 16 and may be linked to *Ly7*. Exz2 resides on chromosome 12 and its location is identical to

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that of the *Pre-1* (*Serpina1*) locus. How *Ly7* and *Pre-1* link to other X-zone-related factors is unclear and their roles in the adrenal cortex require further investigation.

PROP paired-like homeobox 1 (*Prop1*)

Female *Prop1* KO mice have an underdeveloped X-zone at 5 weeks, which undergoes early regression and becomes undetectable at 8 weeks (Nasonkin *et al.* 2011). Mouse models with mutations of *Prop1* show low growth hormone (GH), thyroid-stimulating hormone (TSH), prolactin (PRL), LH and FSH levels (Tang *et al.* 1993). Mutations in *PROP1* also have been associated with multiple pituitary hormone deficiency in humans (Parks *et al.* 1999). Since *Prop1* is expressed in the adrenal at an extremely low level (Fragments Per Kilobase Million (FPKM) <1, preliminary RNAseq data from our laboratory), it is very unlikely that the X-zone phenotype found in *Prop1* KO mice is due to a direct effect on the X-zone.

Adrenocortical dysplasia (*Acd*)

The Acd^{acd} mouse strain (Jax Stock No. 001595) is a spontaneous autosomal recessive mutation that arose at the Jackson Laboratories (Beamer *et al.* 1994). These mice have reduced birth weight, slow growth and an abnormal adrenal cortex. Male and female Acd^{acd} mice exhibit no X-zone in their hypoplastic adrenals as well as enlarged cortical cells. Although the lack of the X-zone in Acd^{acd} mice could be due to the overall growth retardation and defects in the gonads, the absence of the cortical–medullary boundary suggests that telomere-binding protein TPP1, encoded by the *Acd* gene, may have a direct effect on adrenal cortical cells.

Sphingomyelin phosphodiesterase 1 (*Smpd1*) and ceramide synthase 2 (*CerS2*)

Smpd1 encodes for acid sphingomyelinase, which is responsible for breaking down sphingomyelin into phosphocholine and ceramide. The *Smpd1* KO results in a complete lack of the X-zone and the presence of an abnormal zone adjacent to the medulla (Carsia 2000). Interestingly, *Smpd1* is elevated in pheochromocytoma, which has a high incidence in aged *CerS2*-null mice (Thouënnon *et al.* 2007, Park *et al.* 2015). *CerS2* encodes an enzyme that synthesizes ceramide, so ablation of this gene, like mutation of *Smpd1*, should reduce ceramide levels. *CerS2*-null mice also have a severe accumulation of lipofuscin, or ceroid, in the X-zone as early as from 2 months of age (Park *et al.* 2015). Since *Smpd1* is downregulated in ceramidase-knockdown adrenocortical cells (Lucki *et al.* 2012*a*) and ceramidase represses SF1-dependnet gene transcription (Lucki *et al.* 2012*b*), components of the sphingolipid metabolic pathway seem to play roles in the X-zone.

AT-rich interaction domain 5B (*Arid5b*)

Arid5b, also known as *Desrt*, was first identified to encode a DNA-binding protein with a characteristic A-T-rich interaction domain (ARID). It is widely expressed in many tissues and KO mice show a reduction in the ZR (X-zone) thickness in 4-week-old females (Lahoud *et al.* 2001). Although the effect of KO on the X-zone in male mice was not reported, the thinner X-zone in females might be a secondary effect due to general growth retardation and/or gonadal defects including failure of ovulation and absence of corpora lutea.

Kisspeptin

Kisspeptin is expressed in all cortical zones including the inner fetal zone in human fetal adrenals (Katugampola *et al.* 2017). Although *Kiss1* and *Kiss1* receptor (*Kiss1r*) KO mice have a persistent X-zone (Berthon *et al.* 2018), the expression levels of *Kiss1* and *Kiss1r* are very low in adrenals with or without an X-zone (preliminary RNAseq data from our laboratory). The persistent X-zone may be secondary to the abnormal puberty in these two KO strains.

E3 ubiquitin-protein ligase (*Siah1a*)

Siah1a is expressed in many tissues including the adrenal cortex, and *Siah1a* KO mice show general growth retardation and cannot survive beyond P30 (Dickins *et al.* 2002, Scortegagna *et al.* 2017). Nevertheless, depletion of *Siah1a* results in increased expression of the SIAH1A substrate PIAS1, elevated aldosterone levels and a diminished X-zone at P21 (Scortegagna *et al.* 2017). It is unclear whether the diminished X-zone is due to early regression of the X-zone or inhibition of the differentiation of fetal cortical cells into X-zone cells.

Summary

The fetal zone in humans and the X-zone in mice are both transient cortical cells in the adrenal gland originating

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from the fetal stage. Although the X-zone is a unique structure found only in mouse adrenals, the sexual dimorphism of its regression and the ease of genetic manipulation of this model provides an opportunity to study the cell fate of aged cortical cells and the role of various gene products. In addition to sex-related factors, such as androgens, many other molecules are also involved in the formation, maintenance, and regression of the X-zone. It is interesting that many of these factors do not specifically express in the X-zone. Furthermore, many KO mouse models of X-zone-specific or highly expressed genes do not have X-zone phenotypes (Hershkovitz *et al.* 2007, Huang *et al.* 2015, Levasseur *et al.* 2017). Microarray analysis of castrated males, sham males, testosterone-treated females and vehicle-treated females has identified a group of genes that are differentially regulated in adrenals associated with the presence of the X-zone (El Wakil *et al.* 2013). Our laboratory is also using thyroid hormone-induced X-zone remodeling (Huang *et al.* 2015) as a tool to identify novel genes and pathways that control the cell fate of aged cortical cells. Studying the possible connection of these factors and the intrinsic regulation of fetal cortical cells together with consideration of the centripetal renewal of cortical layers will lead to new insights to the development of the adrenal cortex in both mice and human.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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