THEMATIC REVIEW

Adrenarche: a cell biological perspective

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Abstract

Adrenarche is a cell biological and endocrinological puzzle. The differentiation of the zona reticularis in childhood in humans requires special techniques for study because it is confined to humans and possibly a small number of other primates. Despite the rapid progress in the definition of adrenocortical stem/progenitor cells in the mouse, the factors that cause the differentiation of adrenocortical cells into zonal cell types have not been identified. There are, however, many candidates in the Wnt, Hedgehog, and other families of signaling molecules. A suitable system for identifying authentic stem cells, capable of differentiation into all zones, has yet to be developed. It is proposed here that the *in vitro* differentiation of pluripotent cells, combined with appropriate *in vitro* and *in vivo* methods for validating authentic adrenocortical stem cells, is a promising approach to solving these questions.

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Background and introduction

The phenomenon of adrenarche has always presented a puzzle for both the cell biologist and the endocrinologist. Adrenarche is defined as the time in the human life history, at about 6–7 years of age, when the adrenal androgens again begin to increase in concentration in the circulation (Fig. 1). The first phase of life when these steroids, DHEA and DHEAS, are found at high concentrations in the plasma is in the fetus. After birth, levels decline very rapidly, and the period from birth to adrenarche is a time of very low levels of these steroids. The return of adrenal androgens in the circulation results from the development of the innermost zone of the human adrenal cortex, the zona reticularis (Rainey *et al.* 2002, Hui *et al.* 2009), which does not secrete cortisol or other biologically active glucocorticoids or mineralocorticoids but instead secretes the weak androgens DHEA and DHEAS (Endoh *et al.* 1996) as well as small quantities of testosterone (Nakamura *et al.* 2009*a*). DHEAS has a much lower rate of clearance from the circulation than DHEA (Haning *et al.* 1991), thus leading to very high levels of this steroid following adrenarche and into young adulthood. Fairly frequently, adrenarche occurs in children at an earlier age than the average (premature adrenarche). Premature adrenarche has been associated with endocrine and metabolic abnormalities, including insulin resistance, obesity, and low birth weight, but the associations are relatively inconsistent and how they influence adrenarche is unknown (Williams *et al.* 2012).

Despite the well-described phenomenon of adrenarche, the function of DHEA(S) has remained an almost complete mystery. Campbell (2006) has provided a very thorough review of the potential significance of adrenarche in human biology. He convincingly links DHEAS to increased brain development, extended life span, and decreased sexual dimorphism. These ideas indicate that the significance of adrenarche lies in the evolution of the life history of the human species (Hochberg 2010). It is beyond the scope of this review to consider the function of DHEA(S) in human biology, and those who want to learn more about these ideas are referred to these excellent reviews. Moreover, the senescent decline in circulating DHEAS, associated with the involution of the zona reticularis, is also beyond the scope of this review, but this topic has been covered in detail elsewhere (Hornsby 2004, 2005).

The main cell biological questions regarding the zona reticularis are as follows: how does the zone develop at adrenarche; what gives it its unique properties; and how is the size of the zone controlled? Having stated these issues, one must then immediately confront the considerable problems facing the cell biologist who wishes to study adrenarche and the zona reticularis.

This paper is one of three papers that form part of a thematic review section on Adrenarche. The Guest Editor for this section was Ian Bird, University of Wisconsin, USA.

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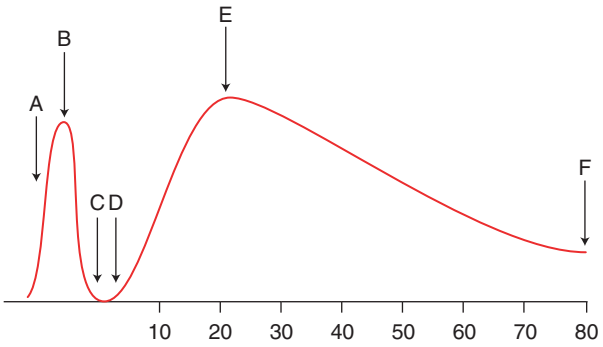

Figure 1 Phases of plasma DHEAS concentrations over the life span in humans. Before birth (A), the fetal zone of the adrenal cortex secretes large amounts of DHEA(S); following birth (B), the fetal zone rapidly involutes (C). DHEAS levels remain very low until 6–7 years of age (D, adrenarche), when plasma DHEAS concentrations begin to rise coincident with the development of the zona reticularis in the adrenal cortex. The achievement of the peak concentration of plasma DHEAS in young adulthood (E) is followed by a progressive decline in adrenal secretion of DHEA(S) (F). The ordinate represents age in years; the scale is expanded before 10 years of age. Reproduced with permission from Hornsby PJ 2004 Aging of the human adrenal cortex. *Science of Aging Knowledge Environment* **2004** RE6.

First, true adrenarche is confined to humans and a few closely related species, although a complete description is lacking in all primate species with the exception of humans (Campbell 2006). Even chimpanzees may significantly differ from humans in the biology of infancy, childhood, juvenility, and adolescence (Hochberg 2010). Other primates, such as the marmoset, also develop specialized inner zones of the adrenal cortex that secrete androgens, but the extent to which primates other than the great apes resemble humans is uncertain (Pattison *et al.* 2009). Mice and other rodents, in the absence of appropriate genetic modifications, do not provide a valid model for adrenarche. Therefore, models that address the questions of adrenarche and DHEA(S) production at the cell biological level must involve *in vitro* systems (cell culture) together with cell transplantation models in immunodeficient mice.

Biology of adrenocortical stem/progenitor cells

The nature of adrenocortical stem/progenitor cells remains relatively undefined in both primates and rodents, although dramatic progress has been made in the last decade. A recent review suggests that a consensus is being reached on the most likely location and characteristics of adrenocortical stem cells (Wood & Hammer 2011). The major roadblock to further progress is the lack of a cell transplantation assay that could be used to characterize adrenocortical stem cells, similar to the bone marrow repopulation assays used to define hematopoietic stem cells (Iscove & Nawa 1997). Differentiated adrenocortical cells are capable of being transplanted and forming functional vascularized tissue, and therefore, functional tissue formation *per se* does not provide an assay to define an adrenocortical stem cell (see below). On the other hand, properly zoned tissue has not yet been shown to be formed by transplantation of differentiated adrenocortical cells; therefore, a simple proposition is that we should expect an authentic adrenocortical stem cell to be capable of forming properly zoned adrenocortical tissue following cell transplantation. In the case of human cells, zonation ought to include zona reticularis as well as zona fasciculata and zona glomerulosa.

In the absence of a cell transplantation assay for authentic stem cells, most knowledge on adrenocortical stem cells has been provided by the study on mice, including a variety of genetic mutants in this species (Kim *et al.* 2009). The concept that adrenocortical cells are 'born' in the capsule/zona glomerulosa, and, as they age, successively become zona fasciculata cells and then zona reticularis cells, is supported by a variety of older and newer evidence. Important studies using chimeric and transgenic animals (Iannaccone & Weinberg 1987, Morley *et al.* 1996) showed that clonal expansion of cells forms centripetal stripes, supporting this concept. While this developmental pattern is often described as 'centripetal migration,' it is not clear whether the cells truly migrate (i.e. the marked cells move past other cells) or whether the whole tissue is pushed by the pressure of cell division, similar to the movement of cells within the epidermis. More recently, fate-mapping experiments have confirmed these views (Huang *et al.* 2010). A Cre recombinase/floxed gene strategy was used to mark single cells within the capsule, which later expanded to clusters. Subsequently, these marked cells were found at progressively deeper locations within the cortex. Additionally, fate-mapping studies in which CYP11B2-Cre mice were crossed with reporter lines to mark zona glomerulosa cells show that zona fasciculata cells arise from the zona glomerulosa (Freedman *et al.* 2011). CYP11B2 is a marker for differentiated zona glomerulosa cells because it is the terminal enzyme in the aldosterone biosynthetic pathway (Mornet *et al.* 1989).

Hypotheses concerning the establishment and maintenance of zonation

Hypotheses regarding the initiation of adrenarche generally fall into two groups. The first group comprises hypotheses that relate the development of the zona reticularis to growth and development of the adrenal cortex. The second group comprises hypotheses that suggest that specific hormone(s) act on some form of precursor cell type (either stem cells or differentiated zona fasciculata cells) to cause the development of the zona reticularis. The concept of an adrenal androgen-stimulating hormone was popular during some periods of the past (Anderson 1980, Parker & Odell 1980), but over the subsequent decades, no such hormone was ever defined. Premature adrenarche is occasionally caused by a pituitary tumor (Iwatani *et al.* 1992), but the secreted tumor products have not been shown to involve a specific adrenarche-stimulating

hormone. The available data are more consistent with the concept that the hormonal influences on adrenarche act via stimulation of the growth of the adrenal gland, causing it to reach a critical size (depth of the cortex) rather than the existence of a unique hormone. This 'critical size' hypothesis is also consistent with observations that adrenarche fails to occur in patients with glucocorticoid-treated 21-hydroxylase deficiency (Brunelli *et al*. 1995) and in familial glucocorticoid deficiency (Weber *et al*. 1997), conditions in which the adrenal gland would be expected to be atrophic.

The arrangement of the blood vessels in the adrenal cortex, together with the observed centripetal movement of

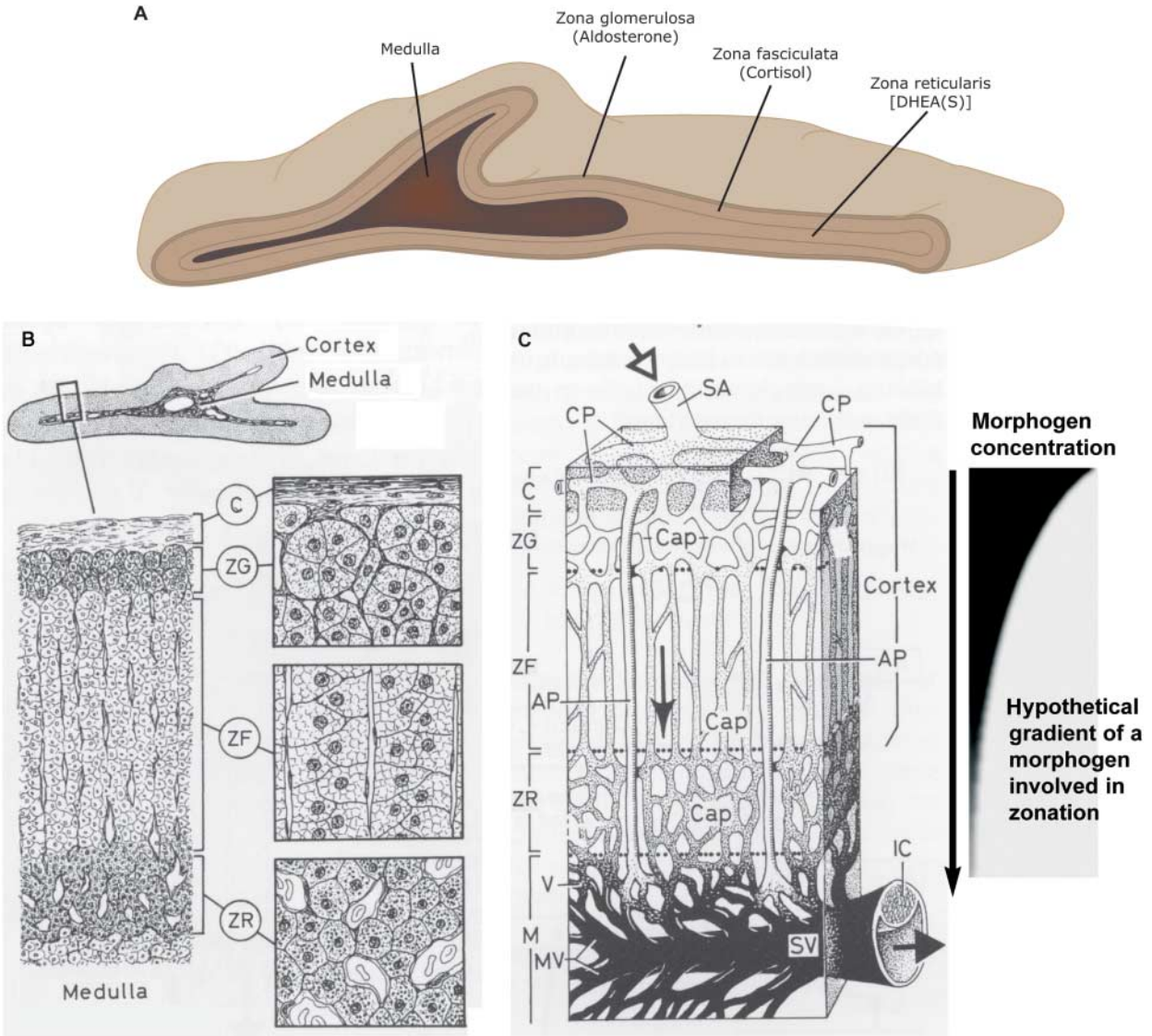

Figure 2 The zonation of the adult human adrenal cortex. (A) The anatomy of the adult gland. Before adrenarche, the overall structure is similar to that illustrated, but there is no zona reticularis or only a very small zone. After adrenarche, the cortex increases in size and the zona reticularis develops. (B) The histological structure of the zones in the adult (C, capsule; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis). (C) The blood vessels of the adrenal cortex; the direction of blood flow is indicated. At the right, a model for a gradient of a morphogen involved in zonation. The morphogen is hypothesized to originate in the capsule or in the zona glomerulosa and to diminish in concentration with increasing distance from the site of origin, i.e. within the zona fasciculata and zona reticularis. Cells in these zones are hypothesized to respond to the varying concentration of the morphogen. (C, capsule; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; M, medulla; SA, adrenal artery; CP, capsule plexus; AP, medullary artery; Cap, capillaries; V, veins; MV, medullary veins; SV, adrenal vein; IC, smooth muscle in the wall of the adrenal vein). The diagram to the right of panel C represents a hypothetical gradient of a morphogen involved in zonation. Panels A, B, and C reproduced with permission from Hornsby PJ 2004 Aging of the human adrenal cortex. *Science of Aging Knowledge Environment* **2004** RE6.

adrenocortical cells within the gland, strongly suggests that the differentiation of adrenocortical cells into zones is regulated by the gradient of a morphogen (Fig. 2). In that case, the differentiation of the zona reticularis occurs when the depth of the cortex is adequate to create an appropriate concentration of the morphogen. This hypothesis would make the relationship of adrenocortical stem cells and their differentiated progeny similar to that of cells in the colon (Brabletz *et al.* 2009, van der Flier & Clevers 2009, Zeki *et al.* 2011). In that stem cell system, the originating cells in the crypt differentiate under the influence of morphogen gradients as they move toward the lumen of the colon. Many of the morphogens involved in the colon, including those in the Hedgehog, Wnt, and Notch families, have also been strongly implicated in the biology of adrenocortical stem cells (Kim *et al.* 2009). Figure 2 illustrates the specific hypothesis that the source of the morphogen is in the capsule/zona glomerulosa compartment of the adrenal cortex. An opposite hypothesis would have the source of the morphogen in the inner part of the cortex, but the flow of blood from zona glomerulosa to zona reticularis suggests that a morphogen is more likely to have a higher concentration on the arterial side of the capillary bed and a lower concentration on the venous end. An older hypothesis suggested that the concentration gradient might be composed of a substance made by all steroidogenic cells, which would also then create an opposite direction gradient (Hornsby 1987). A model in which there are different source cells and responding cells is simpler than one in which the same cells that secrete the compound also respond to it. The former model has already been proposed in the adrenal cortex with respect to Sonic Hedgehog (Shh) signaling (King *et al.* 2009). It should be noted that Shh *per se* is not likely to be the morphgen involved in zonation because the absence of Shh in the mouse does not prevent zonation, although the cortex is hypoplastic (Ching & Vilain 2009).

If, in fact, the differentiation of adrenocortical cells into zonal cells occurs in response to a gradient, there are few clues as the nature of the gradient. It was observed some time ago that zona glomerulosa cells have a high tendency to adopt the both functional and morphological features of zona fasciculata cells when placed in culture (Crivello *et al.* 1982), thereby suggesting that the maintenance of the cellular characteristics of the zona glomerulosa cell requires exposure to factor(s) not present in cell culture. The cells then 'default' to zona fasciculata characteristics. Candidate factors are Wnt-related molecules, such as WNT4 and DKK3 (Suwa *et al.* 2003, Chen & Hornsby 2006, Kim *et al.* 2009). On the other hand, there is no evidence that cells of the zona fasciculata differentiate spontaneously in culture to cells of the zona reticularis. As indicated above, the morphogen gradient hypothesis (Fig. 2) indicates that zona reticularis cells differentiate in response to a very low concentration of morphogen when, at adrenarche, the cortex has expanded to the extent that the morphogen concentration is now lower on the venous end of the capillary bed than it was at younger ages. However, this is difficult to reconcile with the observation that the zona fasciculata rather than the zona reticularis is the 'default' characteristic adopted by the adrenocortical cells in culture.

Although from time to time an influence of the medulla and chromaffin cells on adrenocortical zonation has been proposed (Bornstein *et al.* 1994), this is difficult to reconcile with the arrangement of the cortex and the medulla over much of the adult human adrenal gland (Fig. 2). In the flatter edges of the gland (alae), there is no medulla; the cortex folds on itself with a central raphe (Dobbie & Symington 1966). Therefore, this anatomical arrangement almost certainly excludes a major role for the medulla in determining the properties of the zona reticularis.

Cell transplantation studies

The better characterization of authentic adrenocortical stem/progenitor cells, as cells capable of differentiation into all adrenocortical zones, requires an appropriate cell transplantation model, using immunodeficient mice or other suitable hosts. This is particularly applicable with respect to the differentiation of the zona reticularis because such an experimental system must be able to use human cells. The reader is referred to earlier reviews for a review of the topic of adrenocortical cell transplantation in immunodeficient mice (Hornsby 2001, Cardoso *et al.* 2010). No cell transplantation study has yet shown the capacity of a single cell to give rise to fully zoned functional adrenocortical tissue. Nevertheless, the appropriate combination of *in vitro* (cell culture) and *in vivo* (cell transplantation) studies will be key to determining the mechanism of the differentiation of the zona reticularis.

Potential studies on differentiation of pluripotent cells

Even though the differentiation of the zona reticularis occurs in postnatal life, issues regarding its biology are essentially of development. Because no population of cells isolated from the human adrenal cortex (or other species) has yet been shown to have the expected properties of stem/progenitor cells, new methods are needed to investigate the biology of the zona reticularis and of adrenarche. This is particularly true in view of the considerable difficulties, or actual impossibility, of investigating the biology of adrenarche in mouse models, despite the value of mouse studies for adrenocortical stem/progenitor cells in general (Kim *et al.* 2009).

An alternative strategy is to use pluripotent cell types of human origin (such as human embryonic stem cells) and to study the differentiation of such pluripotent cells into adrenocortical cells *in vitro*. Presumably, the pathway of differentiation from pluripotent cell to fully differentiated adrenocortical cell includes an intermediate stage of adrenocortical stem cell. This approach has proved to be

very productive for investigating the differentiation of pluripotent cells into neural cell lineages; hypotheses concerning the pathways of commitment to the neural lineages and fate decisions within specific neural cell types have been directly tested by directed differentiation in cell culture (Han *et al.* 2011). These studies used both extracellular ligands involved in cell signaling and a variety of chemical inhibitors of intracellular pathways. This approach should be highly applicable for defining and characterizing human adrenocortical stem cells. For steroidogenic cells, progress using this approach has been limited, although valuable insights have been generated from purely *in vitro* studies, as reviewed below. Future studies should extend to testing of cells generated *in vitro* by cell transplantation in immunodeficient mice, or by the use of innovative *in vitro* techniques such as seeding cells into a decellularized matrix derived from the intact adrenal gland (Allen *et al.* 2010).

In addition to these two more conventional approaches – the addition of ligands for signaling pathways and small molecule inhibitors – a third approach is the 'forced' expression of proteins that may alter differentiation. This can be used as a way to coax pluripotent cells to differentiate in a specific direction, e.g. the expression of *PAX4* in human embryonic stem cells can produce cells with characteristics of pancreatic islet cells (Blyszczuk *et al.* 2003). Alternatively, forced expression can be used in various forms of transdifferentiation, a classical example being the effects of the skeletal muscle transcription factor *MyoD* (*MyoD1*) in fibroblasts (Choi *et al.* 1990). These approaches typically require permanent genetic modification of the starting cell type and continued expression of the transcription factor. On the other hand, true reprogramming from one lineage to another (e.g. from fibroblasts to neurons, as has now been demonstrated: Vierbuchen *et al.* (2010)) requires only the temporary expression of the reprogramming factors, for a time sufficient to accomplish lineage switching in the cells. Therefore, the recent demonstration of the usefulness of mRNA as a reprogramming agent provides an ideal vector for these types of studies, as no permanent genetic modification occurs (Warren *et al.* 2010). Indeed, the authors of this method already demonstrated the usefulness of temporary expression of a transcription factor (*MyoD*) for directed differentiation (Warren *et al.* 2010). Transfection of mRNA, together with external ligands and small molecule inhibitors, provide powerful tools for investigating directed differentiation of pluripotent cells. The application of these concepts to study the differentiation of pluripotent cells into adrenocortical stem cells should be very productive.

Pioneering experiments along these lines were reported by Milbrandt's group on the forced expression of *Sf-1* (*Nr5a1*) in mouse embryonic stem cells (Crawford *et al.* 1997). These cells became capable of converting cholesterol to progesterone, but their cellular identity was uncertain. In subsequent similar studies, mouse embryonic stem cells expressing *Sf-1* were differentiated via the formation of embryonic bodies and addition of retinoic acid, resulting in the generation of cells with some adrenocortical features (Jadhav & Jameson 2011). These authors concluded that 'further manipulation of differentiation conditions may allow the selection of specific steroidogenic lineages with characteristics of Leydig, granulosa, or various types of adrenocortical cells (reticularis, fasciculata, and glomerulosa)'.

An alternative set of experiments has involved mesenchymal stem cells (MSCs) as the starting cell type. Some evidence that MSCs could differentiate to steroidogenic cells was obtained via transplantation of bone marrow MSCs into the testis (Yazawa *et al.* 2006). Human and mouse MSCs, derived from bone marrow or fat, become capable of expressing steroidogenic genes and of synthesizing several steroids following forced expression of *SF-1* or *LRH-1* (*NR5A2*; Yanase *et al.* 2006, Tanaka *et al.* 2007, Gondo *et al.* 2008, Yazawa *et al.* 2009). Importantly, forced expression of *SF-1* in mesenchymal cells produced cells capable of secreting DHEA as well as other adrenocortical steroids (Tanaka *et al.* 2007, Gondo *et al.* 2008). In another interesting approach, it was shown that mouse embryonic stem cells carrying a tetracycline-regulated *Sf-1* gene could be differentiated to the mesenchymal cell lineage; then the addition of tetracycline to induce expression of *Sf-1* caused the cells to acquire steroidogenic features (Yazawa *et al.* 2011).

An important recent set of experiments was outlined, in which the authors expressed several factors in three cell types, to create cells with features of adrenocortical cells (Mazilu & McCabe 2011). They investigated the effects of forced expression of various combinations of five key transcription factors (*SF-1*, *DAX1* (*NR0B1*), *CITED2*, *PBX1*, and *WT1*) through adenoviral transduction in three cell types derived from the mesoderm: primary human dermal fibroblasts, primary human renal cortical epithelial cells, and human bone marrow MSCs. Although these experiments are ongoing, the authors drew some preliminary conclusions. At least under some circumstances, *DAX1* was needed, in addition to *SF-1*, to enable the expression of adrenal cortex-related genes in these mesodermal cells. The co-expression of *WT1, CITED2*, or *PBX1* also enhanced the expression of adrenocortical cell features.

It should be noted that these recent studies are based on the concept of transdifferentiation of mesenchymal cells into steroidogenic cells. However, the general concepts should be applicable to the differentiation of pluripotent cells into adrenocortical stem cells and differentiated adrenocortical cells. There is no lack of proposed and potential mediators of adrenocortical stem cell function, and there are at least 14 categories of mediators (transcriptional regulators, autocrine and paracrine factors, as well as hormones; Wood & Hammer (2011)). Another important clue regarding the differentiation of the zona reticularis cells is the finding that GATA6 and MED1 are involved in the regulation of DHEA(S) synthesis via transcription of genes expressed in the zona reticularis: *CYP11A1*, *CYP17* (*CYP17A1*), and *SULT2A1* (Nakamura *et al.* 2009*b*).

Summary

The difficulties encountered in a cell biological approach to adrenarche and the differentiation of the zona reticularis must be acknowledged. However, the time is ripe for an approach that takes advantage of recent advances in the study of the differentiation of pluripotent cells. These studies should include both *in vitro* assays and cell transplantation assays that enable proper definition of adrenocortical stem cells. A combinational approach involving ligands activating signaling pathways, small molecule inhibitors, and temporary expression of transcription factors can potentially yield the solution to the puzzles of the adrenarche and the zona reticularis.

Declaration of interest

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