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Primary Cultures and Cell Lines for *In Vitro* Modeling of the Human Adrenal Cortex

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The human adrenal cortex is a complex endocrine organ that produces mineralocorticoids, glucocorticoids and androgens. These steroids are produced in distinct cell types located within the glomerulosa, fasciculata and reticularis of the adrenal cortex. Abnormal adrenal steroidogenesis leads to a variety of diseases that can cause hypertension, metabolic syndrome, infertility and premature adrenarche. The adrenal cortex can also develop steroid-producing adenomas and rarely adrenocortical carcinomas. *In vitro* cell culture models provide important tools to study molecular and cellular mechanisms controlling both the physiologic and pathologic conditions of the adrenal cortex. In addition, the presence of multiple steroid-metabolizing enzymes within adrenal cells makes it a model for defining possible endocrine disruptors that might block these enzymes. The regulation and dysregulation of human adrenal steroid production and cell division/tumor growth can be studied using freshly isolated cells but this requires access to human adrenal glands, which are not available to most investigators. Immortalized human adrenocortical cell lines have proven to be of considerable value in studying the molecular and biochemical mechanisms controlling adrenal steroidogenesis and tumorigenesis. Current human adrenal cell lines include the original NCI-H295 and its substrains: H295A, H295R, HAC13, HAC15, HAC50 and H295RA as well as the recently established MUC-1, CU-ACC1 and CU-ACC2. The current review will discuss the use of primary cultures of fetal and adult adrenal cells as well as adrenocortical cell lines as *in vitro* models for the study of human adrenal physiology and pathophysiology.

Keywords: adrenal; cell lines; primary monolayer culture; steroidogenesis
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Introduction

The human adrenal gland is composed of an outer cortex, a major site for steroid hormone production, and an inner medulla where catecholamines are produced. The human adrenal cortex contains three functionally distinct regions, the zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR). The ZG synthesizes aldosterone (mineralocorticoid); the ZF produces cortisol (glucocorticoid) and the ZR secretes dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S) (adrenal androgens). To maintain this functional zonation, cellular renewal in the adrenal cortex occurs through centripetal and/or zonal models as extensively reviewed elsewhere (Xing et al. 2015; Hammer and Basham 2021). Steroid production in each zone is preferentially regulated by different circulating factors that include angiotensin II (Ang II) and potassium (K^+) for the ZG, adrenocorticotropic hormone (ACTH) for the ZF and ACTH plus other unknown factors for the regulation of the ZR. Disruption of adrenal steroid production can result in diseases of steroid insufficiency or excess. Diseases include Addison's disease (adrenal steroid insufficiency), primary aldosteronism (mineralocorticoid excess), Cushing's syndrome (glucocorticoid excess) as well as adrenal androgen excess, which can cause premature adrenarche in children or hirsutism and infertility in women. Disruption of adrenal steroid production can also result from exposure to some therapeutics or exogenous endo-

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crine-disrupting agents.

In vitro cell culture models provide one avenue for researching the complex signaling pathways that act to determine adrenal steroid production in both normal physiology and disease. Although human adrenal primary culture has been shown to be a useful experimental tool, limited access to fresh adrenal tissue and technical difficulties in cell isolation and culture have prevented broad-based use of these cells. To overcome the problems with primary adrenal cells, many groups have attempted to establish cell lines from adrenocortical tumors. This approach has been somewhat successful leading to adrenal cell lines from several species. We have previously reviewed the overall development of these models (Rainey et al. 1994, Rainey et al. 2004b). The H295R cell line and its substrains have been widely used by many laboratories. Several other adrenocortical cancer-derived cell lines are also currently available. Herein, we provide details with regard to the isolation, development and utility of human adrenocortical primary cultures and available cell lines.

Primary Cultures of Human Adrenal Cells

Fetal adrenal primary cultures

The human fetal adrenal (HFA) cortex is characterized by the presence of a histologically unique fetal zone that accounts for more than 80% of the fetal gland and is the main source for steroid precursors that are used by the placenta to produce estrogens (Rainey et al. 2004a). Functionally, the fetal zone is most similar to the adult ZR as judged by its production of DHEA and DHEA-S. However, the fetal zone also produces large amounts of other sulfated $\Delta 5$ steroids (pregnenolone sulfate and 17α-hydroxypregnenolone sulfate). The outer zone of the HFA is called the neocortex, or the definitive zone, and is believed to represent the cortical stem cell population that will give rise to the adult adrenal cortex. The most rapid growth of the HFA occurs during the last six weeks of gestation and is mainly due to an enlarging fetal zone. At birth the adrenal is almost as big as the fetal kidney and is equal to the size of an adult adrenal gland but 50% of the gland's size is lost over the first two weeks of neonatal life as the fetal zone undergoes apoptosis (Mesiano and Jaffe 1997, Rainey et al. 2004a).

Monolayer culture of human fetal adrenal cells was first described in the 1960s and early 1970s. These studies occurred in parallel to the easier-to-obtain non-human mammalian adrenal cell culture studies that included development of a mouse adrenal cell line, Y-1 (Buonassisi et al. 1962; Kowal 1969,1970; Grower and Bransome 1970). Early HFA cell cultures focused on *in vitro* growth conditions and regulation of steroid hormone synthesis (Stark et al. 1965; Milner and Villee 1970; Kahri and Halinen 1974). Interestingly, early HFA cells were also used to examine the toxicological effects of o,p1-DDD (later to become the adrenal cancer treatment drug mitotane) (Komissarenko et al. 1971a, b). The availability of primary cultures of HFA cells allowed researchers to establish the role of ACTH and its signaling cascade (cyclic AMP; cAMP) in the regulation of steroid enzyme activity and steroid hormone production (Kahri and Halinen 1974; Roos 1974; Goodyer et al. 1976; Kahri et al. 1976; Turchin and Mellina 1976; Baird et al. 1978; Voutilainen 1979; Voutilainen and Kahri 1979). Following the isolation and subsequent cloning of the genes encoding steroidogenic enzymes, HFA cells were used to define the mechanisms controlling transcription of the enzymes needed for steroid hormone synthesis which included ACTH as well as insulin-like growth factor II (IGF-2) (Ohashi et al. 1983; Mason et al. 1986; Di Blasio et al. 1987; Voutilainen and Miller 1988; Doody et al. 1990; Lebrethon et al. 1994; Mesiano et al. 1997). Primary HFA cell cultures were also used to define the relative roles of lipoproteins and de novo synthesis of the cholesterol substrate needed for steroid hormone production (Carr and Simpson 1981; Mason and Rainey 1987). Because of fetal zone expansion seen during development, fetal adrenal cells also acted as a model to examine the growth factors that most impact adrenal cell division. Fibroblast growth factor (FGF) and epidermal growth factor (EGF) were shown to significantly increase cell growth rates (Crickard et al. 1981; Hornsby et al. 1983). With the appropriate addition of these growth factors, cultures of HFA cells could be used to isolate cell clones that could be maintained for months *in vitro*, although ACTH response and the steroids released did change with time in culture (McAllister and Hornsby 1987; Yang et al. 2001).

As noted earlier the HFA is composed of two distinct steroid-producing zones, the definitive and fetal zone. Multiple groups were able to isolate and culture the two zones. Cells from both zones were shown to be ACTH-sensitive with the definitive zone producing more cortisol and the fetal zone cells producing more DHEA (Gill et al. 1980; Simonian and Gill 1981). Interestingly, cultured fetal zone and definitive zone cell cultures were also instrumental in demonstrating a direct role for corticotropin-releasing hormone (CRH) in the regulation of both DHEA and cortisol (Sirianni et al. 2005a, b). More recent studies have taken advantage of improved methodologies to define the broad-based changes in gene expression seen in cultured HFA cultured cells treated with ACTH (Xing et al. 2010). This study compared ACTH- regulated genes in the HFA vs. adult adrenal cells showing that while both cell types shared ACTH-responsive genes, there were distinct genes induced in each cell population.

While monolayer HFA cell culture has provided considerable insight into the regulation of fetal adrenal steroid production and gene expression, its lack of the three-dimensional structure normally seen within the gland remains a concern. This issue may be addressed in part by taking freshly isolated cells and expanding them as organoids, which has recently been done using HFA cells (Poli et al. 2019). The reports describing the preliminary attempts to form "organoid adrenals" are promising and may provide a

model that can help better study formation of the adrenocortical zones seen within the fetal and adult glands.

Adult adrenal primary cultures

The difficulty in obtaining normal adult adrenal glands has limited the role of primary cultures as a model system. Early studies isolated adrenal cells from glands obtained following adrenalectomy to decrease circulating steroids in women with breast cancer (a therapy no longer used) (O'Hare et al. 1974). Adult adrenal glands have also been obtained following adrenalectomy in conjunction with surgery for renal cell carcinoma or by removal of the adrenocortical tissue from patients undergoing adrenalectomy for pheochromocytoma (Armato et al. 1975). In addition, adrenals can be obtained from deceased renal transplant donors (Xing et al. 2011), which has been the approach taken by our laboratory. This approach can be adapted to many referral centers that have renal transplant programs. Using these donor adrenals, we have developed a successful adrenocortical cell isolation protocol for both ZG and ZF cells. The details of our protocol are included in the Appendix for this manuscript. A photomicrograph of isolated human primary adrenal cells in culture is shown in Fig. 1. Early cultures show retention of lipid droplets (as seen within the adrenal ZF cells) and the ability to respond to ACTH and cAMP agonists, including forskolin. Freshly isolated cells can be frozen as aliquots and stored for later use for new or replicate experiments. The ability to freeze cells alleviates part of the logistical problems of having to isolate and immediately execute experiments with the isolated cells.

In contrast to the HFA, adult adrenal glands have the distinctive ZG, ZF and ZR zones to consider when isolating cells for primary culture. Most researchers use protocols that do not selectively isolate a specific zone and therefore use a mixed adrenocortical cell population. That said, because the ZF and ZR constitute most of the adult adrenal, primary cultures would primarily consist of cells from these zones. Primary cultures of adult adrenocortical cells were first described by the adrenal researchers O'Hare, Hornsby and Neville (O'Hare et al. 1974). They reported maintenance of primary cells in culture for several months and suggested the retention of ACTH response by demonstrating the conservation of steroid production and morphologic "rounding" response seen in other adrenal cells in culture (Fig. 1) (Rainey et al. 1983). Their research was closely followed by Armato et al. (1975) which examined the proliferative activity of adult human adrenal cells. With the cloning of the steroidogenic enzymes, human adrenal cell cultures were used to study the factors regulating not only steroidogenesis but the enzymes needed to produce steroids. ACTH, through cAMP signaling pathway, was found to be the major hormonal enhancer of the transcripts needed to raise steroidogenic enzyme levels (l'Allemand et al. 1996; Kristiansen et al. 1997; Xing et al. 2010). The actions of ACTH have been shown to be increased in the presence of high insulin levels, IGF-1 or IGF-2 (l'Allemand et al. 1996; Kristiansen et al. 1997; Fottner et al. 1998).

Concerns regarding the isolation of mixed cell populations of ZG, ZF and ZR can be somewhat addressed by targeted methodologic approaches that enrich a specific zone. This includes mechanical sectioning methods that enrich ZG, as well as antibody targeted purification of ZG cells. Mechanical dissection to selectively isolate ZG cells has proven to yield Ang II-responsive, aldosterone-producing population of cells (Natarajan et al. 1988; Gu et al. 1994; Payet et al. 1994). Enriched ZG cell cultures have proven useful to study signaling properties of Ang II as well as broad-based transcriptional effects of Ang II treatment (Gu et al. 1994; Payet et al. 1994). Rossi and colleagues (Caroccia et al. 2010) described a procedure for ZG cell isolation using immunomagnetic beads conjugated with antibodies to the ZG cell antigen CD56. Although yet to be widely used, this method was shown to increase ZG markers including aldosterone synthase (CYP11B2) expression and aldosterone production.

Unlike the regulation of ZG and ZF steroid production, the mechanisms controlling ZR DHEA production remain to be clarified. Thus, isolating pure or semi-pure popula-

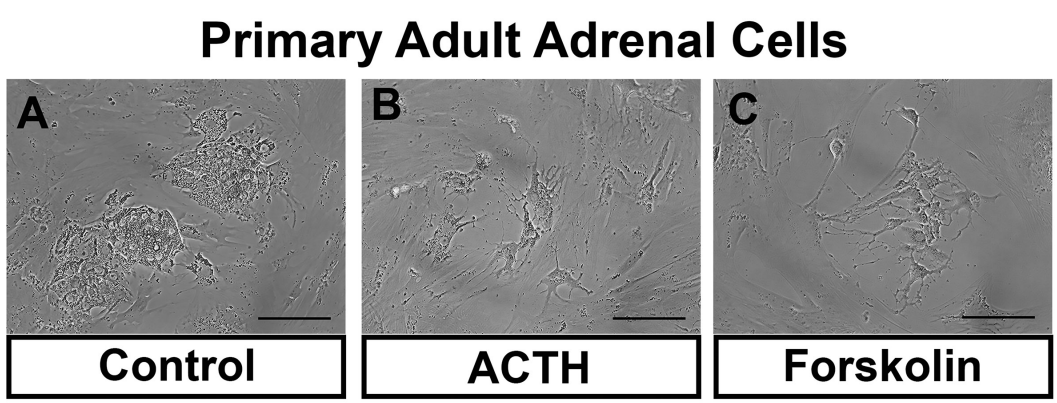

Fig. 1. Phase contrast photomicrographs of primary cultured human adrenocortical cells. Untreated cells (A), 24 h ACTH treatment (100 nM) (B), and 48 h Forskolin treatment (10 μM) (C). The treatment with ACTH or forskolin resulted in the morphological changes in cultured primary adrenocortical cells. The treated cells showed shrunk and rounded cell morphology. Scale bar is 100 μm.

tions of ZR cells could help define signaling pathways that control DHEA production. There have also been reports of the successful isolation of ZR cells using Percoll gradient centrifugation (Hyatt et al. 1983) and by visual macrodissection of adrenal glands using the ZR's distinct dark red color compared to the adjacent gold-colored ZF (Endoh et al. 1996; Casson et al. 2007). These studies have shown a clear role for the cAMP pathway in the activation of ZR steroidogenesis but factors that work with ACTH to increase DHEA production remain to be defined.

The NCI-H295-Derived Adrenocortical Carcinoma Cell Lines and Related Strains

The parental NCI-H295 cell line

In 1980, an adrenocortical carcinoma (ACC) was identified by computer-assisted tomography scan in a 48-year-old black woman who presented with weight loss, acne, facial hirsutism, edema, diarrhea and a recent cessation of menses (Gazdar et al. 1990). The excised tumor was 14 X 13 X 11 cm and was used to establish the original NCI-H295 cell line. Tumor tissue was finely minced and the resulting suspension was maintained in multiple serum-containing culture media and one serum-free culture medium for a one-year period (Gazdar et al. 1990). Successful culture of floating cell aggregates was accomplished in RPMI 1640 medium supplemented with hydrocortisone, insulin, transferrin, 17β-estradiol and sodium selenite (serum-free HITES medium). Cytogenetically the H295 cell is highly aneuploid, hypertriploid with 30% of the cells containing a modal chromosome number of 62. There are no Y or Y-like chromosomes.

The primary physiologic regulators of steroid hormone production in the human adrenal gland are the peptide hormones ACTH and Ang II as well as circulating levels of K^+. The original description of NCI-H295 cells did not report on the hormonal responsiveness of these cells (Gazdar et al. 1990). Subsequently it was shown this population of NCI-H295 cells was unable to respond to ACTH or Ang II with increased levels of steroidogenic enzyme transcripts (Staels et al. 1993). A later study demonstrated a modest but significant increase in NCI-H295 aldosterone production following Ang II treatment (Wang et al. 2012) (Table 1).

Expression of genes encoding the cholesterol side-chain cleavage enzyme (*CYP11A1*), 17α-hydroxylase/17,20-lyase (*CYP17A1*) and 21-hydroxylase (*CYP21A2*) together with 11β-hydroxylase (*CYP11B1*) and *CYP11B2* were first studied in the NCI-H295 cells (Winqvist et al. 1992; Staels et al. 1993). The transcripts encoding these enzymes accumulated in response to agonists (8-bromo-cAMP, forskolin, cholera toxin and 3-isobutyl-1-methylxanthine) which activate the protein kinase-A pathway. Consistent with normal adrenocortical tissue, stimulating the protein kinase-C pathway using phorbol esters resulted in decreased *CYP11A1* and *CYP17A1*, but accumulation of *CYP21A2*. In a similar manner, 8-bromo-cAMP increased *CYP11B1*, *CYP11B2* and, unexpectedly, aromatase (*CYP19A1*) mRNA levels.

With regard to NCI-H295 steroid hormone biosynthesis, the clinical features of the patient with the adrenocortical cancer predicted that multiple steroids, which normally arise from each of the adrenal zones, would be produced. Gas chromatography-mass spectroscopy and radioimmunoassay were initially used to identify the production of steroids by these cells. Gazdar and coworkers found that the NCI-H295 cells produced a broad spectrum of steroid products (30 steroids) under basal conditions of synthesis and that the steroid profile was greatly influenced by serum conditions (Gazdar et al. 1990). Many of the steroids fall outside of the typical adrenal steroid hormones (aldosterone, cortisol, DHEA). One unusual glucocorticoid precursor that is elevated in most patients with ACC and is produced by NCI-H295 cells is 11-deoxycortisol (Kikuchi et al. 2000; Taylor et al. 2017; Suzuki et al. 2020). This cell line is presently available from the American Type Culture Collection as ATCC CRL-10296 and will slowly grow in suspension with loosely attached cell aggregates as shown in Fig. 2A.

The NCI-H295 Substrains: H295A, H295R, H295RA, HAC13, HAC15 and HAC50

NCI-H295R (H295R) cells

Multiple substrains have been adapted from the NCI-H295 cell line using alternative growth conditions to

Table 1. Characteristics of human adrenocortical cell lines.

Cell line	Ang II	K^+	dbcAMP or forskolin	ACTH	Steroid production
NCI-H295	Modest	Not determined	+	−	M, G, A
H295R	+	+	+	Poor	M, G, A
H295RA	+	Not determined	+*	+	M, G, A
H295A	−	Not determined	+	−	M, G, A
HAC15	+	+	+	Modest	M, G, A
MUC1	Not determined	Not determined	Not determined	Not determined	G
CU-ACC1	−	Not determined	+	−	G
CU-ACC2	−	Not determined	−	−	G

M, mineralocorticoids; G, glucocorticoids; A, adrenal androgens; +, indicates the ability to enhance steroidogenesis after agonist treatment; −, indicates absence of response to the agonist; *, unreported but observed in our laboratory.

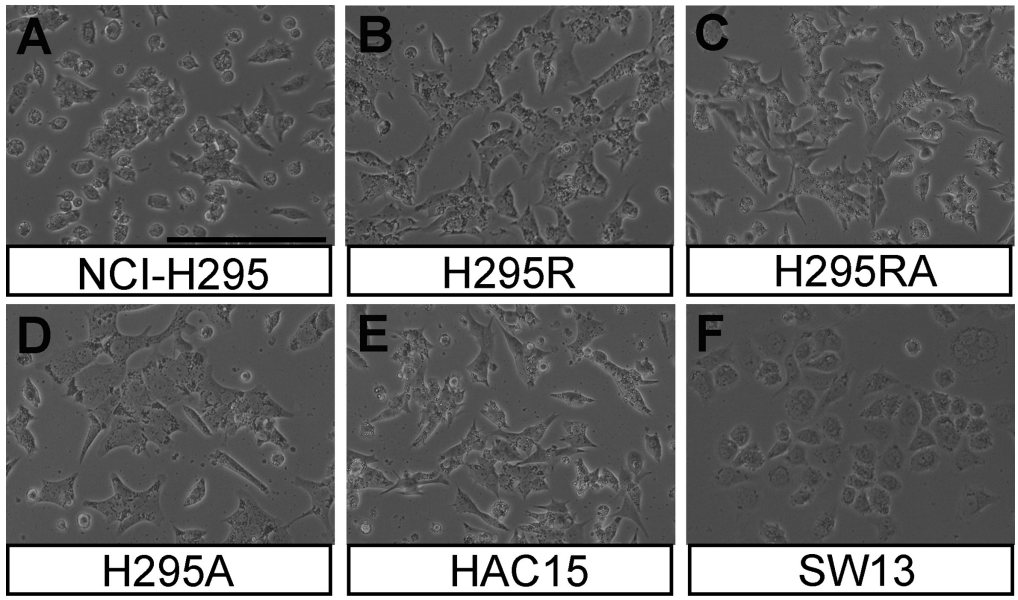

Fig. 2. Photomicrographs of NCI-H295 and its derivative cell lines. SW13 is also shown as a comparison. Scale bar is 200 μm.

encourage substrate attachment and shorter cell cycle times. Initially a strain was developed over a three-month period, during which culture medium was changed every three days and unattached cells were discarded, selecting cells for their attachment to plastic culture dishes. After characterization, this adapted strain was later designated as NCI-H295R (H295R) to differentiate it from the original NCI-H295 cells. In comparison to the parent NCI-H295 cell line, H295R (strain 1) cells grow as an adherent monolayer with a population doubling time reduced from five to two days. The selected cells had good steroidogenic response to Ang II and agonist of the cAMP pathway (forskolin), but poor response to ACTH (Bird et al. 1993a; Rainey et al. 1993). The limited response to ACTH was partly explained by low expression level of ACTH receptor (MC2R) in H295R cells (Parmar et al. 2008).

Because of difficulty in US importation of the original H295R selection medium serum substitute, Ultroser, cells were selected to grow in a commercially available serum substitute (Nu-Serum type I). These cells have decreased responses to Ang II and K^+ but continue to respond to agonists of the protein kinase-A signaling pathways (Rainey et al. 1994). In an attempt to develop a strain of H295R cells that would grow in commercial serum and retain Ang II and K^+ responsiveness, a series of sera were tested. Growth of cells in DMEM/F12 medium with 5-10% supplementation with Cosmic Calf Serum was found to maintain cell growth and responses to Ang II and K^+ (Wang and Rainey 2012). This strain of H295R (strain 3) is advantageous because the growth medium is considerably less expensive as no supplementation with ITS-plus is needed (Fig. 2B).

H295R cells have been shown to be able to produce an array of steroids even under basal conditions (Bird et al. 1993a; Rainey et al. 1993; Xing et al. 2011). Treatment with agonists selectively promotes the synthesis of certain zone-specific steroid hormones (Table 1). Ang II is the primary hormonal regulator within the renin-angiotensin-aldosterone system (RAAS), and it acts on the ZG by binding to type 1 Ang II (AT1) receptors to increase the production of aldosterone. The H295R cell has proven to be a useful model to study Ang II-regulated aldosterone production (Bird et al. 1993a; Nogueira et al. 2007, 2009; Otani et al. 2008). Only AT1 receptor antagonists have a significant inhibitory effect on these cells, while type 2 Ang II (AT2) receptor antagonists have little impact on Ang II stimulation, demonstrating that H295R cells respond almost exclusively to the AT1 receptors (Bird et al. 1993a, b; Holland et al. 1993). Subsequent studies also revealed AT1 receptor coupled with the expression of phosphoinositidase C increased inositol phosphates in H295R cells (Bird et al. 1993b). The H295R cell line has been used to study the regulation of AT1 receptor expression. These cells exhibit high levels of AT1 receptor mRNA as well as [^{125}I] Ang II binding (Bird et al. 1993a, 1994, 1995; Wang et al. 2012). Expression of AT1 receptor transcript and binding appears to be affected in parallel (Bird et al. 1994, 1995). Thus, the H295R cell line has been widely used to define the mechanisms regulating adrenal cell responsiveness to Ang II and the ZG physiology.

The other major physiologic regulator of adrenal aldosterone production is extracellular K^+ levels. K^+ increases intracellular calcium levels in H295R cells which appears to be the mechanism to increase aldosterone biosynthesis (Rainey et al. 1994; Pezzi et al. 1996). In addition, there is evidence of an intra-adrenal renin / angiotensin system, in which K^+ stimulation increases the production of both Ang I and Ang II (Hilbers et al. 1999). The H295R cell line is used by many laboratories as a model to study the mecha-

nisms of K^+ regulation of adrenal steroid production (Clyne et al. 1997; Nogueira et al. 2010).

Treatment of the H295R cell strain with agonists working through the cAMP pathway produce a pattern of steroids approaching those of the ZF and ZR. Steroids produced during treatment with forskolin include cortisol, 11β-hydroxyandrostenedione, DHEA, DHEA-S, corticosterone, 11-deoxycortisol and androstenedione (Rainey et al. 1993). Studies have also demonstrated that transcripts encoding the *StAR* (steroidogenic acute regulatory protein) gene as well as the five forms of cytochrome P450 known to be involved in adrenal steroidogenesis (*CYP11A1*, *CYP17A1*, *CYP21A2*, *CYP11B2* and *CYP11B1*) are also detectable in the H295R cell strain (Bird et al. 1993a; Rainey et al. 1993; Denner et al. 1996; Zenkert et al. 2000; Wang et al. 2012; Rege et al. 2015).

One of the additional advantages of cell lines over primary cultures is the ability to transfect and transduce cells to express wild type or mutated genes of interest. The ability of the H295R to produce aldosterone has made this model an attractive choice to examine the effects of gene mutations suspected of causing primary aldosteronism (Tauber et al. 2014; Stindl et al. 2015; Daniil et al. 2016; Xie et al. 2016; Maniero et al. 2017; Scholl et al. 2018; Fernandes-Rosa et al. 2018; Gurtler et al. 2020). Notably, H295R cells harbor an activating mutation in the *CTNNB1* gene (p.S45P), which encodes β-catenin (Tissier et al. 2005) and a large deletion in the *TP53* gene (Cerquetti et al. 2008). The activating *CTNNB1* mutation has been observed in adrenocortical adenomas as well as ACC (Tissier et al. 2005; Maharjan et al. 2018).

Finally, it is worth noting that the U.S. Environmental Protection Agency Endocrine Disruptor Screening Program and the Organization for Economic Co-operation and Development (OECD) have adopted the H295R cells as a model to help define endocrine disrupting compounds / chemicals. There is growing evidence for a need to identify endocrine bioactive pesticides and petroleum-based chemicals with the potential of contaminating habitats, food and water supplies. Endocrine disruption can occur through direct interaction with hormone nuclear receptors either by receptor activation or antagonizing activity of the natural ligand. However, many of these compounds have the potential of also inhibiting synthesis or metabolism of steroid hormones. *In vitro* cell models have proven to be one of the mechanisms to better define and screen for endocrine disruptors that act on steroid-metabolizing enzymes. The human adrenal H295R line has recently been used to develop a high-throughput testing model to ascertain chemical effects on steroid synthesis (HT-H295R) (Karmaus et al. 2016). The utility of the system results from the broad steroid metabolome secreted by H295R cells, which includes mineralocorticoids, glucocorticoids, progestins, androgens and estrogens (Botteri Principato et al. 2018; Pinto et al. 2018; Haggard et al. 2018, 2019). This allows the cells to act as a "Tier 1" steroidogeneses screen that has been validated using a set of recommended international test guidelines.

The H295R cell strain grown in Nu-Serum (strain 2) is available from the American Type Culture Collection as ATCC CRL-2128.

H295RA cells

As noted above the H295R adrenocortical cell line has only modest ACTH responsiveness and limited MC2R expression. Adrenal cell ACTH response is not only controlled by receptor expression, but also expression of MC2R accessory protein (MRAP) (Metherell et al. 2005). Our group tested the hypothesis that increasing MRAP expression in H295R would increase ACTH responsiveness (Nanba et al. 2016). Lentiviral particles containing human MRAP-open reading frame were generated and transduced in H295R cells. 18 clones were isolated for characterization. The most ACTH-responsive steroidogenic clone was named H295RA (Fig. 2C). Successful induction of MRAP increased protein and mRNA levels for MC2R in H295RA cells. Treatment with ACTH significantly increased aldosterone, cortisol and DHEA production in H295RA cells. ACTH also significantly increased transcript levels for all of the steroidogenic enzymes required to produce aldosterone, cortisol and DHEA, as well as *MC2R* mRNA. Treatment of H295RA cells with ACTH also acutely increased cAMP production and cellular protein levels for total and phosphorylated StAR. Importantly, H295RA cells retain response to Ang II for enhanced aldosterone production (Nanba et al. 2016) (Table 1). This genetically modified ACC cell line provides an ACTH-responsive human line that could be useful for development of MC2R targeted inhibitors or to study downstream signaling systems for adrenal cell regulation of steroid production. The cells are currently being used by multiple laboratories to study steroid hormone production and enzyme expression (Engels et al. 2017; Mangelis et al. 2019). The H295RA cells are available commercially from Applied Biological Materials (Richmond, Canada).

H295A cells

Another strain of cells designated NCI-H295A has been described which also grows as a monolayer (Rodriguez et al. 1997) (Fig. 2D). The method for isolation of the NCI-H295A strain was similar to H295R strains and relied on the selection of a population of cells that grew as a monolayer while removing non-attached cells with medium changes. In contrast to the H295R cells, the H295A cell strain did not show a significant increase of steroid production when stimulated by Ang II (Samandari et al. 2007) (Table 1). The lack of response to Ang II in H295A cells is consistent with lower expression level of AT1 receptor compared with that in H295R cells (Samandari et al. 2007; Wang et al. 2012). However, the H295A cells have proven useful in defining the mechanisms regulating the transcription of steroid-metabolizing

enzymes.

Both the H295R and H295A cell lines have also been shown to be useful model systems to better define the mechanisms controlling transcription of the steroid-metabolizing genes. Fusion genes containing the 5'-flanking DNA from *CYP11B1*, *CYP11B2*, *CYP17A1* and *HSD3B2* (3β-hydroxysteroid dehydrogenase) have been studied using these cell lines (Holland et al. 1995; Clyne et al. 1997; Leers-Sucheta et al. 1997; Rodriguez et al. 1997). The H295A cells are available from the originating laboratory (Dr. Walter Miller, UCSF).

HAC cells

In 2008, Parmar et al. (2008) isolated clonal populations of cells from primary cultures of an adrenal carcinoma in order to develop a novel human adrenocortical carcinoma (HAC) cell line. Although these clones were thought to be novel cell lines, subsequent single-nucleotide polymorphism (SNP) array analysis indicated that the clones represented contaminating H295R cells. Three of the clones, HAC13, HAC15 (Fig. 2E) and HAC50, however, exhibited high responses to Ang II and K^+ treatment, and had a modest ACTH response through increases in cortisol production and steroidogenic enzyme expression (Table 1). Compared to the NCI-H295, H295A and H295R cell strains which were a mixed population of tumor cells, the HAC cell lines were clonally derived which may provide more stable steroidogenic phenotypes with time in culture. Like the H295R cell line, these cells have also proven useful for transgenic testing of the somatic gene mutations causing primary aldosteronism (Monticone et al. 2012; Oki et al. 2012; Hattangady et al. 2016; Reimer et al. 2016; Nanba et al. 2020). The HAC15 clonal cell line is available commercially from the American Type Culture Collection as ATCC CRL-3301 and Applied Biological Materials (Richmond, Canada). The HAC13 and HAC50 are also available from Applied Biological Materials (Richmond, Canada).

MUC-1 cells

Hantel et al. (2016) used a xenograft approach to develop the MUC-1 adrenal cancer cell line. A neck metastasis tissue of ACC from a 24-year-old man who initially reported with a left adrenal mass of 22 cm was used. While the patient had no clinical symptoms of overt Cushing's syndrome, urinary steroid analysis indicated elevated precursor steroids noted to be of a malignant adrenal profile. The tumor pieces were directly implanted into ten athymic nude mice and two of the xenografts showed aggressive growth of patient-individual tumor models. Genetic analysis of the tumor tissue showed a somatic mutation in *TP53* (a frameshift deletion: Hg19 positions: 7574003 on Chr 17: G is deleted). One xenograft (MUC-1) was used to establish long-term primary cultures that sustained positive nuclear steroidogenic factor-1 (SF-1) and cytoplasmic HSD3B immuno-positivity. The cells also secreted cortisol but the potential for Ang II or ACTH response has not been reported (Table 1). Initial characterization studies did, however, examine therapeutic responsiveness upon treatment with the current systemic gold standard EDP-M (etoposide, doxorubicin, cisplatin and mitotane) (Berruti et al. 1998; Fassnacht et al. 2018) and demonstrated maintenance of the clinically observed drug resistance for MUC-1 exclusively. The MUC-1 cell line adds to our currently available human ACC cell culture models and, like the NCI-H295 line and its substrains and CU-ACC1 / 2, MUC-1 provides another xenograft model for whole animal studies. The MUC-1 cell line is available from the originating laboratory (Dr. Constanze Hantel, University Hospital Zurich).

CU-ACC1 and CU-ACC2 cell lines

Two adrenocortical cancer cell lines designated CU-ACC1 and CU-ACC2 were developed through intermediate patient-derived mouse xenografts (Kiseljak-Vassiliades et al. 2018a). The CU-ACC1 cell line was derived from a perinephric metastasis in a patient whose primary adrenal tumor secreted mineralocorticoids. At the time of metastasis, no evidence of hyperaldosteronism was observed. The CU-ACC2 cell line was derived from a liver metastasis in a Lynch syndrome patient whose primary adrenal tumor showed no evidence of hormonal excess. Short tandem repeat (STR) profiling confirmed consistent matches between human samples and mouse models. Mutation analysis showed CU-ACC1 to have an activating *CTNNB1* mutation (c.G100A, p.G34R) and CU-ACC2 to have a *TP53* mutation (c.G337A, p.G245S). RNA sequencing and immunohistochemistry demonstrated expression of adrenal cortex markers including SF-1 as well as steroidogenic enzymes required for synthesis of adrenal steroids. The CU-ACC1 line produced multiple steroid hormones including cortisol which increased in response to forskolin but not ACTH or Ang II treatment (Table 1). In contrast the CU-ACC2 produced only small amounts of cortisol with no steroidogenic response to ACTH, Ang II or forskolin treatment (Table 1). The initial characterization studies also showed that both cell lines could be efficiently transfected with expression plasmid DNA as well as transduced with lentivirus.

The CU-ACC1 and CU-ACC2 cell lines have recently been used to better define and test drug targets for the treatment of adrenocortical cancer. Using their new ACC models along with H295R cells, Kar et al. (2019) demonstrated that mitotic PDZ-binding kinase (PBK) is a promising therapeutic target. ACC express high levels of PBK and its expression correlates with poor survival. ACC cell PBK silencing decreased cell proliferation, clonogenicity and anchorage-independent growth possibly through inhibition of pAkt, pp38MAPK and pHistone H3, which would slow cell cycling. The small-molecule inhibitor, HITOPK032, also modulated pAkt and pHistone H3 and induced apoptosis and supports the therapeutic potential of PBK-targeting in ACC patients. The CU-ACC1 and CU-ACC2 lines have also been used to examine the role of the maternal embry-

onic leucine zipper kinase (MELK) in ACC (Kiseljak-Vassiliades et al. 2018b). MELK is overexpressed in ACC compared with normal adrenal samples and its expression in tumors correlated with shorter survival. CU-ACC1 and CU-ACC2 were compared with H295R cell models. All models responded to an MELK inhibitor, OTSSP167, by decreasing rates of cell proliferation, colony formation and cell survival with relative sensitivity of each ACC cell line based upon the level of MELK overexpression. Finally, these cells were recently used to investigate the role of ferroptosis, an iron-dependent cell death mechanism, with the goal of using this type of programmed cell death as an ACC drug target (Weigand et al. 2020). They were found to highly express glutathione peroxidase 4 (GPX4) and long-chain-fatty-acid CoA ligase 4 (ACSL4) genes, key factors in the initiation of ferroptosis. All three cell models (H295R, CU-ACC1 and CU-ACC-2) were highly susceptibility to the GPX4 inhibitor RSL3. Interestingly, RSL3-induced ferroptosis was completely reversed in adrenal cortex cells by inhibition of steroidogenesis with ketoconazole but not by blocking the final step of cortisol synthesis with metyrapone. While more research is needed this report suggested that the ferroptosis pathway may be a target for steroidogenically active ACC.

Together these studies demonstrate the utility of the CU-ACC1 and CU-ACC2 cells as models to improve the search for effective adrenocortical cancer treatment targets and drugs. The potential for the cells to provide a model for certain aspects of normal adrenal physiology, steroidogenesis and cell signaling have yet to be exploited. The CU-ACC1 and CU-ACC2 cell lines are available from the originating laboratory (Dr. Katja Kiseljak-Vassiliades, University of Colorado School of Medicine). STR profiles of aforementioned cell lines are shown in Table 2.

Other Adrenal Cell Models

There are several other adrenal cell lines that include SW13 (Fig. 2F) (Leibovitz et al. 1973), CAR47 (Nesterova et al. 2008), ACT-1 (Ueno et al. 2001), RL-251 (Schteingart et al. 2001), pediatric adrenocortical adenoma derived cell line (Almeida et al. 2008) and SV40 transformed adrenal cell lines (Hornsby et al. 1989). Of note, the SW13 cell line, possibly derived from a neuroendocrine carcinoma in the adrenal gland, is not an adrenocortical model since the cells produce no steroids and do not have detectable levels of steroidogenic enzymes (Wang et al. 2012).

Although these cells have some limitations in terms of steroid-producing abilities and / or stability of cell characteristics over passages, if available they may provide useful tools as models to study adrenal tumorigenesis or screen therapeutics for ACC.

Conclusion

Human adrenocortical cell lines represent a crucial tool for molecular and cellular studies that cannot practically be done in animal models or primary cultures. Currently available human cell lines can produce mineralocorticoids, glucocorticoids and adrenal androgens, respond to Ang II, ACTH and K^+ and act as a screening tool for chemical endocrine disruptors and potential cancer therapies. Importantly the reliance on one ACC line (the NCI-H295) for *in vitro* research has been resolved by development of multiple ACC-derived models including MUC-1, CU-ACC1 and CU-ACC2. Finally, the significance of adrenocortical cell interactions with stromal and immune cells as well as specific zonal microenvironments created by autocrine and paracrine factors remains an open area of research. Co-culture of freshly isolated adrenal cells / cell lines with other cellular components may be required to address certain aspects of adrenal biology and diseases.

Conflict of Interest

The authors declare no conflict of interest.

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Table 2. Comparison of short tandem repeat (STR) profiling among adrenocortical cell lines.

	NCI-H295	H295A	H295R	HAC15	H295RA	MUC1	CU-ACC1	CU-ACC2
Amelogenin	X	X	X	X	X	X	X	X
CSF1PO	10, 12	10	10, 12	10, 12	10, 12	12	12	11, 12
D13S317	13	13	13	13	13	9	9, 13	13
D16S539	11	11	11	11	11	11, 14	9, 12	11, 12
D5S818	12	12	12	12	12	11	12	11, 12
D7S820	9, 12	9, 12	9, 12	9, 12	9, 12	8, 10	8	8
TH01	9.3	9.3	9.3	9.3	9.3	9.3	7	9.3
TPOX	8	8	8	8	8	8	9, 11	8
vWA	17, 18	17, 18	17, 18	17, 18	17, 18	16, 17	18	16, 18, 20

Results of STR profiling of NCI-H295 cell line and its derivatives are shown. STR profiles of MUC1, CU-ACC1, and CU-ACC2 were adopted from Pinto et al. 2019.

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Appendix

Primary human adrenal cell culture protocol

Primary human adrenal cell cultures can be prepared from a normal adrenal gland, adrenocortical carcinoma, or an adrenal adenoma that is decapsulated, minced into small pieces, and incubated in Dulbecco's modified Eagle's medium/F-12 (DMEM/F12) containing 0.1% collagenase/0.01% DNase I. An alternative protocol uses adrenal slices prepared with a handheld microtome (see below). Digestion and mechanical dispersion are carried out through repeated 1 hour incubations at 37°C. Following isolation, cells can either be frozen or immediately plated in DMEM/F12 medium supplemented with 10% Cosmic Calf Serum (CCS) and antibiotics.

I. Reagent Preparations

1. Washing Medium (WM):

- DMEM/F12 — Gibco #11330-032

The medium has to be at the same temperature as the tissue (room temperature, 37°C, 4°C, depending on the timing and handling of the sample).

2. Collagenase Solution (500 mL):

Reconstitute Collagenase/Dispase and DNase I with sterile Nuclease-Free Water (Qiagen #129114).

- 495 mL — DMEM/F12 — Gibco #11330-032
- 5 mL (50 U/mL) — Pen/Strep (5000 U/mL) — Gibco #15070-063
- 10 µL (1 µg/mL) — Gentamicin (50 mg/mL) — Gibco #15750-060
- 500 mg — Collagenase/Diapase — Roche #11097113011
- 50 mg — DNase I — Roche #10104159001

Mix and filter through a 0.22 µm filter.

After filtration add 0.5% sterile CCS to decrease cell death during isolation.

- 2.5 mL (0.5%) — CCS — Hyclone/Fisher Scientific #SH3008703

Aliquot 40 mL into 50 mL sterile centrifuge tubes and store at -20°C.

3. Inactivation Medium / Growth Medium 4 (GM4) (500 mL):

- 445 mL — DMEM/F12
- 5 mL (50 U/mL) — Pen/Strep (5000 U/mL)
- 10 µL (1 µg/mL) — Gentamicin (50 mg/mL)
- 50 mL (10%) — CCS

4. Digestion Medium

- 1:2 ratio of Washing Medium: Collagenase Solution

5. Growth Medium 1 (GM1) for the first 4 days of culture (50 mL):

- 49.9 mL — GM4
- 0.1 mL (0.1 mg/mL) — Kanamycin (50 mg/mL) — Sigma #K0254

Make this medium fresh each time.
Swirl the Kanamycin stock before using as there is often precipitate at the bottom of the container.

6. Low Serum Medium (500 mL)

- 495 mL DMEM/F12
- 5 mL (50 U/mL) Pen/Strep (5000 U/mL)
- 10 µL (1 µg/mL) Gentamicin (50 mg/mL)
- 0.5 mL (0.1%) CCS

7. Other required supplies for primary adrenal cell culture:

Set these up in the tissue culture hood. Keep them on i
about 15 min before starting.

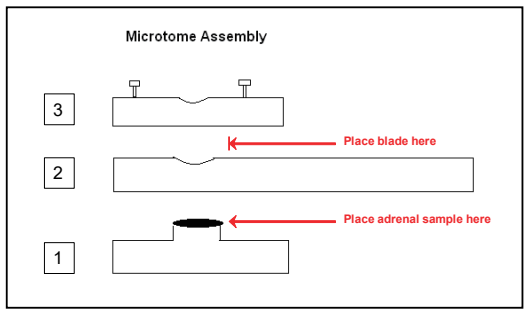
- Metal tray with ice
- Biohazard bag
- Sterile Petri dishes
- Waste container
- Sterile forceps, scissors, and scalpel
- Sterile T25 or T75 flask and 50 mL vials
- (For zone isolation) Microtome, blade, and blade h
- Stadie-Riggs Handheld Tissue Slicer (Microtome)

(Thomas Scientific): wipe with 70% ethanol (EtOH) and
with UV light in the hood for 15 min—Do not autoclave
UV for long periods. The autoclave will warp the plastic
periods under UV will cause the plastic to crack).

- Falcon cell strainer, 100 µm pore size

Other materials not in the hood.

- 70% EtOH for cleaning

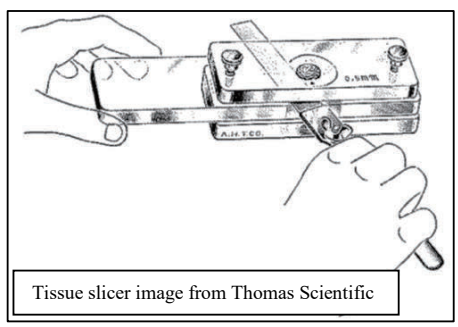


II. Isolation Method

1. Set water bath to 37˚C. Wipe down hood with 70% EtOH and set out supplies.

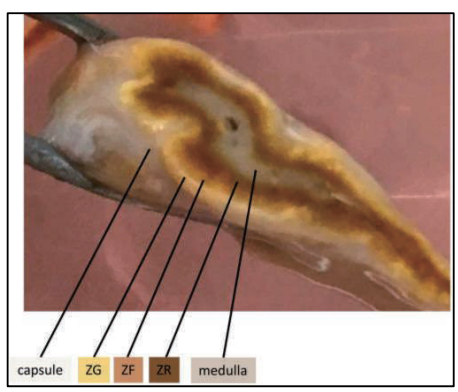
2. Prepare tissue
Aspirate or decant carrier medium from the container with the tissue. Wash well with Washing Medium (WM) at least 3 times to remove blood cells.

3. Transfer tissue to a sterile plastic Petri dish with WM.
Keep on ice. Trim away any fat or connective tissue and discard (into a separate Petri dish, then into a biohazard bag).


Tissue slicer image from Thomas Scientific

4. Rinse again with WM.

5. Slice tissue
Handheld Microtome: Do not let adrenal tissue dry out when cutting.
Ensure the tissue pieces are dipped in WM before being placed in the microtome and slices are immediately placed back into WM.
Assemble the bottom two plates of the microtome and place a flat piece of tissue on the platform. Screw on the top plate and tighten the screws until the tissue is slightly flattened (do not crush the tissue).
Insert the blade and carefully shave off the ZG of the adrenal. Place sliced tissue in a separate Petri dish with WM. The ZG is lighter in color than the ZF. If only darker tissue remains on your tissue piece, you have shaved off all of the ZG. Dip the tissue piece in WM to keep from drying out, then flip the piece over and shave the ZG from the other side.
Once all of the ZG has been cut, mince the remaining tissue (ZF/ZR/medulla) with scissors if necessary. Transfer the ZG slices and ZF/ZR/medulla tissue to sterile T25 or T75 flasks along with 5 - 10 mL of WM from their respective Petri dishes.



Scissors: If isolating cells from the whole adrenal, place tissue in sterile Petri dish with about 10 mL WM and cut into approximately 1 cm^2 pieces with scissors. Transfer the tissue to a sterile T25 or T75 flask with 5 - 10 mL of WM from the Petri dish.

6. 1st Digestion
Digestion Medium is 1:2 ratio of WM/ Collagenase Solution
For tissue in T75 with 10 mL of WM, add 20 mL of Collagenase Solution (30 mL total).
For tissue in T25 with 5 mL WM, add 10 mL of Collagenase Solution (15 mL total).

7. Put flask in water bath (not completely submerged) and ensure it won't wiggle around. Make sure the cap of the flask doesn't touch the unsterile water. Shake at 90 rpm, 37°C, for 1 hour.

8. Collect Cells
Wipe or spray the outside of the flask with 70% EtOH and wipe with paper towel before bringing back to the hood.
Let tissue settle to bottom. Collect and filter the supernatant through sterile 100 µm pore size cell strainer into a 50 mL tube.

9. Rinse Cells
Add 5 or 10 mL of WM to the flask to wash tissue.

1st Wash – gently pipette tissue up and down a few times.
2nd Wash – pipette tissue a little more aggressively.
3rd and 4th Washes – intensively disperse the tissue by vigorously pipetting up and down and crushing with a pipette*.
*While still in the package, snap the end off of a 10 mL or 25 mL serological pipette and use for washing the tissue.

Collect and filter the supernatant through a 100 µm pore size cell strainer.
Pool with the supernatant from step 7.

10. Spin down the pooled supernatants [1500 rpm, 5 min].
Discard the supernatant and re-suspend the pellet in 10 mL Inactivation Medium.
Store the cells at room temperature in the hood.

11. Add 15 or 30 mL Digestion Medium to the remaining tissue in the flask.

12. 2nd - 4th Digestions
Repeat steps 7 - 10

13. Combine Cells
Combine cells from all digestions and washes.
Filter, pellet, and re-suspend in 10 mL Inactivation Medium.
If the pellet is really small, re-suspend in only 2.5 - 5 mL Inactivation Medium so cells are not too diluted to count.

14. Count Cells
Determine cell number using a Hemocytomer as the automated cell counter will count red blood cells.
Prepare a 1:1 aliquot of Trypan Blue (BIO-RAD #1450013) and the cell suspension (10 µL each) and pipette onto the Hemocytometer and examine under 40X magnification. Avoid counting red blood cells or any blue cells, which are damaged/dead cells.

III. Freezing / Unthawing Cells

Freeze cells for future use and/or culture for an agonist test. Culture cells for 7 - 10 days, then subculture and plate for the agonist experiment.

1. Freezing Method
For optimal results, cells should be freshly isolated or in the log phase of growth.

Suggested Freezing Medium:

- 50% Growth Medium
- 40% CCS (or Fetal Bovine Serum if it is used in the growth medium)
- 10% DMSO Sigma #D2650-100ML

After isolation of primary cells or trypsinization of cultured cells (at 70 - 80% confluence), gently pellet cells by centrifugation [5 min, ~1500 rpm]. Aspirate and discard supernatant.

Re-suspend pellet in Freezing Medium at a concentration of 5 x 10^6 - 1 x 10^7 cells/mL.

Aliquot into appropriate cryogenic storage vials, usually 1 mL tubes with proper labels in pencil, and place into a Mr. Frosty freezing container containing 100% isopropanol.

Freeze at a rate of cooling of -1˚C/min (4 - 8 hours in -80°C freezer) then transfer the cryovial to a liquid N_2 cryobank or -150°C freezer.

2. Thawing Method
Pre-heat an autoclaved beaker with sterile water to 37°C.
Remove cells from storage and immediately place in the 37°C water bath/beaker to quickly thaw. Rapid thawing decreases cell death during this process.

Transfer thawed cells into a 15 mL tube containing 8 mL of growth medium. Gently mix. Rinse out the cryovial with growth medium and add to the 15 mL tube.

Pellet cells by centrifugation [5 min, ~1500 rpm]. Aspirate and discard supernatant.

Gently re-suspend cells in growth medium and plate into appropriate growth vessel.

IV. Treating Cells

Thaw and culture cells for 7 - 10 days in T75 flasks, then subculture and plate for the experiments.

Example Agonist Experiment

- Culture freshly isolated or thawed cells in a T75 flask for 7 - 10 days prior to plating on multiwell dishes for agonist testing. For the first 4 days after isolation (or if using thawed cells frozen directly after isolation), culture with growth medium containing Kanamycin (GM1). Then change to growth medium without Kanamycin (GM4).

- Once the cells are approximately 75% confluent, subculture and plate on a 48-well dish (10K - 15K cells/well) with growth medium (GM4).

- After 2 - 3 days, change to Low Serum Medium (0.1% CCS). Incubate overnight.

- Start treatments the next day (mark the date/time on the plate). Label wells as Basal, ACTH, K^+, and Ang II. Change to Low Serum Medium containing agonists:
  - Basal No treatment
  - ACTH 10 nM
  - K^+ 18 mM
  - Ang II 10 nM

- Stop experiment at appropriate times. We normally test cells with a 24 hour incubation.

- Collect media and store at -20°C. Isolate DNA, RNA, and/or protein from the cells or store at -80°C (cells can be stored at -20 °C if they are only needed for DNA studies). Media steroid levels should be normalized to the amount of protein or DNA per well.

- Analyze media for steroids. Isolate RNA from cells for RT-qPCR for genes of interest. DNA and protein can be used to normalize steroid levels.