Endocrinology, 2023, **164**, 1–15
https://doi.org/10.1210/endocr/bqad081
Advance access publication 24 May 2023
Mini-Review

Regulation of Alternative Splicing by Steroid Hormones

Florian Le Billan,[1,*] Gloria Umogbai,[1,*] and Carolyn L. Cummins[1]

[1]Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario M5S 3M2, Canada

Correspondence: Carolyn L. Cummins, PhD, Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, 144 College St, Rm 1101, Toronto, Ontario M5S 3M2, Canada. Email: carolyn.cummins@utoronto.ca.
*F.L.B. and G.U. contributed equally to this work.

Abstract

Steroid hormone signaling pathways are critical for organismal development and act through binding to nuclear receptors (NRs) driving transcriptional regulation. In this review, we summarize evidence for another—underrated—mechanism of action for steroid hormones: their ability to modulate the alternative splicing of pre–messenger RNA. Thirty years ago, pioneering studies used in vitro transfection of plasmids expressing alternative exons under the control of hormone-responsive promoters in cell lines. These studies demonstrated that steroid hormones binding to their NRs affected both gene transcription and alternative splicing outcomes. The advent of exon arrays and next-generation sequencing has allowed researchers to observe the effect of steroid hormones at the whole-transcriptome level. These studies demonstrate that steroid hormones regulate alternative splicing in a time-, gene-, and tissue-specific manner. We provide examples of the mechanisms by which steroid hormones regulate alternative splicing including 1) recruitment of dual-function proteins that behave as coregulators and splicing factors, 2) transcriptional regulation of splicing factor levels, 3) the alternative splicing of splicing factors or transcription factors that feed-forward regulate steroid hormone signaling, and 4) regulation of elongation rate. Experiments performed in vivo and in cancer cell lines highlight that steroid hormone–mediated alternative splicing occurs both in physiological and pathophysiologic states. Studying the effect of steroid hormones on alternative splicing is a fruitful avenue for research that should be exploited to discover new targets for therapeutic intervention.

Key Words: nuclear receptor, coregulator, splicing factor, estrogen, androgen, glucocorticoid

Abbreviations: A3/5SS, alternative 3′ or 5′ splice site; ACTH, adrenocorticotropic hormone; AP, alternative promoter; AR, androgen receptor; ARGLU1, arginine and glutamate rich protein 1; AS, alternative splicing; CE, cassette exon; COBRA1, cofactor of BRCA1; D2R, dopamine receptor; DDX5/17, DEAD-box helicase 5 or 17; Dex, dexamethasone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; E2, estradiol; ER, estrogen receptor; ESRP1/2, epithelial splicing regulatory protein 1 or 2; GR, glucocorticoid receptor; hnRNP, heterogeneous nuclear ribonucleoprotein; IR, intron retention; MR, mineralocorticoid receptor; mTOR, mechanistic target of rapamycin; NCoR, nuclear receptor co-repressor; NR, nuclear receptor; P4, progesterone; PCR, polymerase chain reaction; PR, progesterone receptor; pre-mRNA, pre–messenger RNA; qPCR, quantitative polymerase chain reaction; RNAP II, RNA polymerase II; RNA-seq, RNA sequencing; RRM, RNA recognition motif; RT-PCR, reverse transcription–polymerase chain reaction; STREX, stress-axis regulated exon; T, testosterone; TF, transcription factor.

Since the cloning of the first nuclear receptors (NRs) more than 35 years ago, we have come to understand their role in transcriptional regulation through a variety of experimental approaches. NRs can bind directly to their regulatory regions on DNA in proximal and distal enhancers, and consequently recruit coregulators to either induce or repress transcription (1, 2). Additionally, through tethering, NRs can bind to other transcription factors (TFs) that contact DNA to regulate target genes. This is an important mechanism by which transrepression of genes by the glucocorticoid receptor (GR) occurs (3). Many NRs act in response to the binding of a ligand, such as steroid hormones. When the NR resides in the cytoplasm, hormone binding promotes the nuclear translocation and DNA binding of the receptor. While pioneering studies mapped the functional regulatory regions where NRs were bound in gene promoters, more recent genome-wide studies have revealed that many NR binding sites are found at enhancers long distances from the transcription start site (4). The interactions at such distances are facilitated by DNA looping where protein-protein interactions between NRs, TF complexes, and RNA polymerase II (RNAP II) are crucial players (5).

Studies have found that the effect of steroid hormones transcends their transcriptional effects. Specifically, the role of steroid hormones in the posttranscriptional messenger RNA (mRNA) regulation of alternative splicing has been explored. Alternative splicing (AS) refers to the mechanism by which protein diversification, despite a limited gene number, is achieved. Before an mRNA is translated, its precursor form (pre-mRNA) undergoes modifications, executed by a ribonucleoprotein complex called the spliceosome, which consists of 5 small nuclear RNAs (U1, U2, U4, U5, U6) associated with splicing factors. In metazoans, splicing modifications include mutually exclusive exons, alternative promoter (AP), alternative polyadenylation, alternative 3′/5′ splice sites (A3SS; A5SS), intron retention (IR), and cassette exon (CE) events (Fig. 1) (6). The complexity of an organism affects how many of their genes are alternatively spliced, as it has been shown that 95% of transcripts containing multiple exons undergo AS in human cells (7). Indeed, 52% to 80% of

Received: 26 February 2023. **Editorial Decision:** 19 May 2023. **Corrected and Typeset:** 6 June 2023

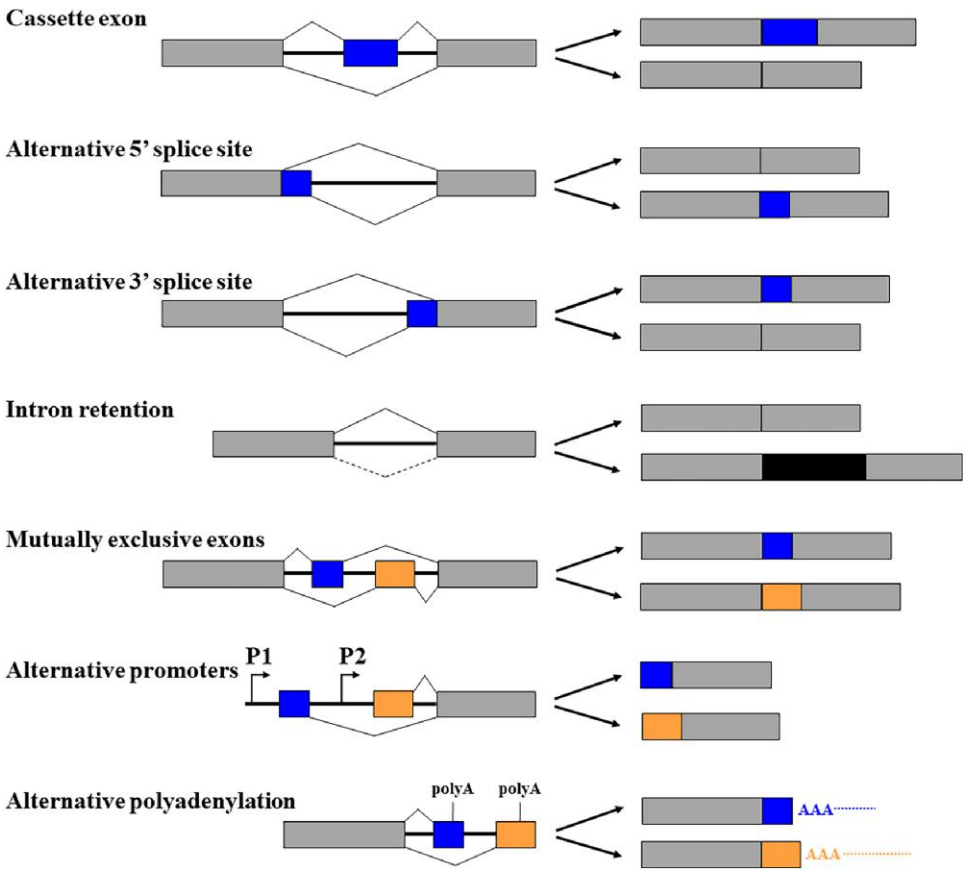

Figure 1. Types of messenger RNA alternative splicing events regulated by steroid hormones. Exons are represented by boxes, introns by lines. Constitutive exons are in gray, alternative exons are in blue or orange. Alternative promoters are annotated P1 and P2, alternative polyadenylation signals are annotated polyA, and the poly(A) tail is represented by AAA.

alternatively spliced transcripts have been characterized as tissue specific, with the majority found in the myocardium and neurons (8, 9). Likewise, the specific splicing events that occur are contingent on the type and pathophysiological status of the tissue. For example, the progression of some cancers including prostate cancer has been shown to be driven by the action of androgens on AS (10). However, to date, AS is a less known consequence of steroid hormone action. This narrative review aims to explore the current state of knowledge about the effect of steroid hormones on AS and the mechanisms by which steroid hormones have been shown to modulate mRNA diversification.

Methodology

Medline, PubMed, JSTOR, and Scopus bibliographic databases were used. Search concepts were narrowed down to "steroid hormones" AND "alternative splicing"; truncations applied where necessary (eg, steroid hormone*, and alternat* splic*); and keywords derived from each search concept were used to narrow down article search. For the search concept "steroid hormones," sample key words included "glucocorticoids" OR "androgens," and for the search concept "alternative splicing" sample keywords included "RNA processing" OR "trans-splicing." Title and abstract screening were performed to ascertain the eligibility of the study. Consequently, to expand the search reach, forward and backward searching was performed with the tools Connected Papers, and SCIntilla based Text Editor (SciTE) (11). Titles and abstracts of more than 500 articles were screened for review, and 84 extracted for meeting the eligibility requirements. Employing the data review tool SysRev (https://sysrev.com/u/5078/p/72871), 3 reviewers (F.L.B., G.U., C.L.C.) screened articles based on 2 criteria: 1) are steroid hormones and AS discussed? 2) are the AS events occurring because of hormone action?. Discordances were resolved after thorough discussion, and consensus was achieved to include 56 out of 84 articles for data abstraction. Most of the articles described in vitro and in vivo experiments designed to provide insight into the mechanisms of steroid hormone action, and some mini-reviews were included that discussed broader concepts.

Results

Technological Advances Have Revolutionized the Ability to Study Alternative Splicing

Before the sequencing of the human genome, researchers interested in studying AS were limited to the analysis of individual genes that had been shown to have alternative isoforms discovered by classic techniques of Northern blotting or nuclease protection assays. Newer technologies including exon splicing arrays and next-generation sequencing rapidly expanded the numbers of genes known to be alternatively spliced and opened the possibility of examining genome-wide changes on AS in response to steroid hormones. To capture the

technological evolution of the field, we summarized the publications that were included in our search results into 2 tables. Table 1 organizes the AS events that have been studied at the single-gene level in response to steroid hormone treatment. A subset of these is described in "Results". Table 2 summarizes the genome-wide effect of hormone treatment on AS in different cell systems. While there are understandably fewer examples of studies that have explored genome-wide regulation of AS by hormone signaling compared to single-gene analyses, this number is expected to increase as RNA sequencing (RNA-seq) costs continue to decrease. It is important to note that to quantitatively characterize AS events in human- or mouse-derived samples, sequencing depth must be increased to above 60 M reads per sample (as opposed to 20-30 M reads for regular RNA-seq). This necessity continues to pose a financial barrier to the widespread adoption of AS analyses.

Single-gene studies that uncovered a role for steroid hormones in the regulation of known AS events are summarized in Table 1. These splicing events were characterized using a variety of models: in vitro culture of cell lines, ex vivo culture of primary cells and tissues, and in vivo rodent studies. Most publications examined CE events using reverse transcription–polymerase chain reaction (RT-PCR) and approximately half demonstrated substantial differences in AS (with changes > 2-fold). Remarkably, AS can be modulated by steroid hormones on a relatively short time scale (ie, 30 minutes-4 hours). However, as shown in Tables 1 and 2, most studies (87%) were performed with hormone treatment longer than 4 hours.

One gene that has been studied extensively for its regulation is the calcium-activated potassium channel (*Slo* aka *Kcnma1* (49-52)) as there is a stress-axis regulated exon (STREX) that affects its function. The regulation of the physiological stress response, crucial for survival, is mediated in part through catecholamine secretion by the adrenal gland. Glucocorticoid treatment (24 hours) modulates the expression of the STREX isoform and excitability of this potassium channel in adrenal chromaffin cells, which contributes to a faster adapted response to stress by enhancing secretion of catecholamines (49). In primary cultures of chromaffin cells from hypophysectomized rats, the STREX exon shows decreased inclusion compared to nonhypophysectomized rats (49). This is reversed by treatment with adrenocorticotropin (ACTH), which increases corticosterone levels (49). In castrated rats, the absence of testosterone (T) leads to an increase in ACTH and consequently of corticosterone, resulting in an increase in STREX inclusion in the adrenal glands, but a decrease in the pituitary glands (51). Treatment with T reverses these effects in both tissues (51). In primary culture of adrenal medullae from rats, corticosterone has no effect on STREX inclusion at low concentration, while at high concentration STREX inclusion is decreased in a GR-dependent manner (52). This in vitro response to glucocorticoids was also observed in primary cultures of bovine chromaffin cells, in which a decrease in inclusion of STREX was found with dexamethasone (Dex, synthetic glucocorticoid) treatment (50). This collection of individual studies on *Slo* AS highlights the species-specific, tissue-specific, and receptor-dependent aspects of AS regulation by steroid hormones.

Studies examining the role of steroid hormones and AS on a more global basis are summarized in Table 2. These types of studies capture a variety of splicing events (RNA-seq) and allow the identification of unexpected mechanisms of drug resistance in cancer. For example, the first-line treatment for estrogen-dependent breast cancer is the estrogen receptor (ER) antagonist tamoxifen. Using exon arrays, it was shown that estradiol (E2) regulates the AS of the fibroblast growth factor receptor FGFR2, which has been directly implicated in tamoxifen resistance (21). In androgen-dependent prostate cancer, androgens profoundly alter the transcriptome-wide AS at 2 levels: directly, and by activating 2 splicing factors epithelial splicing regulatory proteins (ESRP) 1 and 2. The androgen-mediated overexpression of ESRP1 leads to an increase in splice variants for the ribosomal protein S24 (*RPS24*) and the actin-binding protein filamin B (*FLNB*), which are directly correlated to decreased survival rate and increased tumor severity. Interestingly, both *RPS24* and *FLNB* dysregulated splice events can be reversed by treatment with the androgen antagonist bicalutamide (62).

Steroid Hormones Can Affect Alternative Splicing in a Short Time Frame

A few studies demonstrated that steroid hormones can significantly affect AS in as short a time frame as 1 hour (31, 54, 57). This time frame suggests that this function of steroid hormones can occur without newly translated proteins being involved. For example, binding of the mineralocorticoid receptor (MR) within the AP of *WNK1* occurs as fast as 30 minutes after murine renal cell exposure to aldosterone (57). This promotes the expression of WNK1 kidney specific isoform, which stimulates sodium transporter activity (57). In the case of 4-hour Dex treatment of N2a neural cells, there was very little overlap in the identity of the Dex-mediated transcriptionally regulated or alternatively spliced genes (Fig. 2) (45). Therefore, steroid hormones promote dual-functional outcomes on transcription and splicing and these can occur in a relatively short time frame and on distinct gene sets.

Mechanisms by which steroid hormones exert these effects on AS may include steroid hormone–mediated recruitment of specific dual-function coregulators, the AS of coregulators and splicing factors themselves, and other indirect roles influencing AS such as transcription speed and splicing factor or steroid hormone receptor protein levels (Fig. 3). Examples for each of these mechanisms are described next.

Transcriptional Coregulators Have Dual Roles in Transcriptional Regulation and Alternative Splicing

Transcriptional coregulators interact with other TFs to function in chromatin remodeling, recruitment of RNAP II, and as transactivators and/or transrepressors (66). Over the past 2 decades, an increasing body of evidence demonstrated their concomitant involvement in AS regulation, defining them as transcriptional/splicing factors or "coupling" proteins (67), a terminology that yields some confusion since genes that are transcriptionally upregulated are frequently distinct from those that are alternatively spliced (see Fig. 2). The concept of proteins that could "couple" transcriptional activation with AS on the same gene was perpetuated in early experiments using artificial minigene systems. By design, these minigene assays containing alternative exons were created to be responsive to steroid hormones (eg, by incorporating the MMTV promoter). Using these artificial minigene systems, several "coupling proteins' were identified. These include the heterogeneous nuclear ribonucleoproteins (hnRNPs); the DEAD-box RNA helicases p68/DDX5 and p72/DDX17;

Table 1. Summary of steroid-regulated splicing events sorted by gene of interest

Gene	Hormone treatment (duration)	In vitro or in vivo model	Technology[a]	Splice event	Effects	Reference
AE2 (*SLC4A2*)	10^{-4} M Dex and/or 10^{-4} M UDCA (6, 24, 72 h)	Normal human cholangiocytes; hepatocyte lineage PLC/PRF/5 cells	qPCR	Alternative promoters	*AE2b* variants 1 and 2, not *AE2a*, upregulated by Dex + UDCA-activated GR interacting with HNF1 in 6 h after PLC/PRF/5 cells, after 24 h in cholangiocytes	Arenas et al 2008 (12)
APP	2 × 10 mg T propionate, or 2 g E2 (5 d)	Gonadectomized AKR mice	RT-PCR and Southern blot	Cassette exons	*APP* 695 isoform only: upregulated by T in old males and adult females, downregulated by T in old females, upregulated by E2 in adult males and females, downregulated by E2 in old females; no significant changes observed for *APP* 751 and *APP* 770 isoforms	Thakur and Mani 2005 (13)
Bcl-X (*Bcl2l1*)	10^{-8} M synthetic progestin R5020, 10^{-8} M Dex, 10^{-7} M P4 (2 h)	Rat endometrial RENTROP cells	RNase protection assay; RT-PCR	A5SS	Bcl-X_L/Bcl-X_S ratio (long over short isoform ratio) upregulated by R5020 and P4 in a PR-dependent manner, and by Dex	Pecci et al 1997 (14)
CD44 minigene	10^{-8} M P4 (24 h)	HeLa cells	Radiolabeled RT-PCR	Cassette exons	Inclusion/skipping ratio downregulated by P4-activated PR in presence of CoAA and TRBP	Auboeuf et al 2004 (15)
	10^{-8} M R1881 (16 h)	HEK293 cells	RT-PCR	Cassette exons	Inclusion/skipping ratio downregulated by R1881-activated AR in presence of DDX5	Clark et al 2008 (16)
	10^{-8} M E2	HEK293T cells; MCF-7 cells	RT-PCR and agarose gel	Cassette exons	Inclusion/skipping ratio upregulated by E2-activated p-Ser-118 ERα in presence of DDX17, downregulated by E2-activated p-Ser-118 ERα in presence of SF3A1	Masuhiro et al 2005 (17)
	10^{-8} M R1881	HEK293 cells; LNCaP cells	RT-PCR	Cassette exons	Inclusion/skipping ratio downregulated by R1881-activated AR in presence of Sam68	Rajan et al 2008 (18)
	10^{-8} M DHT, 10^{-9} M R1881 (24 h)	T47D cells; HEK293T cells	Radiolabeled RT-PCR	Cassette exons	Inclusion/skipping ratio downregulated by R1881-activated AR, not DHT-activated AR; inclusion/skipping ratio upregulated by R1881- or DHT-activated AR in presence of COBRA1	Sun et al 2007 (19)
	10^{-9} M P4, 5×10^{-8} M Dex, or 10^{-9} M E2 (24 h)	HeLa cells	Radiolabeled RT-PCR	Cassette exons	Inclusion/skipping ratio downregulated by P4-activated PR, by Dex-activated GR, or by E2-activated ERα in presence of CoAA; Inclusion/skipping ratio upregulated by E2-activated ERα/β in presence of DDX17	Auboeuf et al 2002 (20)
	E2 (24 h)	HEK293 cells; MCF-7 cells	qPCR	Cassette exons	Inclusion/skipping ratio upregulated by E2-activated ERα; Inclusion/skipping ratio downregulated by E2-activated ERα in presence of AKT	Bhat-Nakshatri et al, 2013 (21)
CD163	10^{-8} M fluticasone-17-propionate (1, 2, 3, 4, 6, 8, 24 h)	Human monocytes	RT-PCR and agarose gel	Cassette exons	Splice variants A, AC1 and AC2, not E1, upregulated by fluticasone propionate after 2 h	Högger et al 1998 (22)
Crp1 (*Andpro*)	0.5 mg T or 0.5 mg T propionate injection (6, 12, 18, 24 h)	Female and castrated male Wistar rats	Northern blot	Alternative polyadenylation	Prostate: longer poly(A) tail isoform promoted by T and T propionate in castrated males after 6 and 12 h Lachrymal glands: longer poly(A) tail isoform promoted by T and T propionate in females and castrated males after 6 and 12 h	Winderickx et al 1990 (23) Vercaeren et al 1992 (24)
CT/CGRP	5.10^{-7} M Dex (5 d)	Neuronal CA77 thyroid C-cells; TT thyroid C-cells	Northern blot	Alternative 3′ splice site	*CGRP/CT* ratio downregulated by Dex	Russo et al 1992 (25)

(*continued*)

Table 1. Continued

Gene	Hormone treatment (duration)	In vitro or in vivo model	Technology[a]	Splice event	Effects	Reference
CT/CGRP minigene	10^{-8} M P4 or 10^{-9} M E2 (24 h)	HeLa cells	Radiolabeled RT-PCR	Alternative 3' splice site	*CGRP/CT* ratio upregulated by P4-activated PR, E2-activated ERα, E2-activated ERβ, in presence of CAPERα or CAPERβ	Dowhan et al 2005 (26)
D2R (dopamine receptor, *Drd2*)	10^{-8} M E2, P4, or T (6, 12, 24, 48 h)	PRL-secreting MMQ cells	Radiolabeled RT-PCR	Cassette exons	Exon 6 inclusion/skipping ratio upregulated by E2-activated ER and T after 12 h, downregulated by E2 + P4 after 6 h	Guivarc'h et al 1998 (27)
	42 ± 8 pg/mL E2 and 0.68 ± 0.09 ng/mL T via silastic implants (96 h)	Castrated Wistar male rats	Radiolabeled RT-PCR; single-cell PCR	Cassette exons	Pituitary gland: exon 6 inclusion/skipping ratio downregulated by T Olfactory tubule: exon 6 inclusion/skipping ratio upregulated by E2 and T hypothalamus: exon 6 inclusion/skipping ratio downregulated by E2	Guivarc'h et al 1995 (28)
Egf	200 µg T propionate injection (3, 5, 7 d)	Female and castrated male Balb/c mice	3' rapid amplification of cDNA ends (3' RACE)	Alternative polyadenylation	Long poly(A) tail isoforms downregulated by T propionate in submaxillary glands, not kidney	Sheflin et al 1996 (29)
FHL-1 (*CFH*)	10^{-7} M Dex (48 h)	U251 glioblastoma cells; HT1080 fibrosarcoma cells; T37D mammary carcinoma cells	RT-PCR	Mutually exclusive exons	Exon X-including *FHL-1* isoform, not exon XI-including *FH* isoform, upregulated by Dex	Friese et al 1999 (30)
GHR	10^{-5}, 10^{-6} or 10^{-7} M Dex (1, 16 h, 7 d)	Human hepatoma HuH7 cells	qPCR; FACS	Cassette exons	*GHR/GHRtr* ratio downregulated by Dex after 1 h and 7 d in a dose-dependent manner; *GHR/GHRtr* ratio upregulated by Dex after 16 h	Vottero et al 2003 (31)
gr	High stocking stress (6, 33 h)	*Salmo salar*	RT-PCR	Cassette exon; A3SS; mutually exclusive exons	*gr-1a*, *gr-1b*, *gr-2*, *gr-2a* and *gr-2b* isoforms, but not *gr-1a2* isoform, upregulated by 33 h high stocking stress in head kidney and gill tissue	Romero et al 2020 (32)
Ido1	10 mg/kg Dex (5 h)	Male C57BL/6J mice	qPCR	Alternative promoters	*Ido1* FL downregulated by Dex in peripheral tissues (lungs, spleen, liver); *Ido1*-v1 (class 1) upregulated by Dex in astrocytes and microglia; *Ido1*-v2 (class 2): absent expression	Dostal et al 2018 (33)
INSR	10^{-12} M to 10^{-6} M Dex (6 d)	HepG2 cells	RT-PCR	Cassette exons	Exon 11 inclusion/skipping ratio upregulated by Dex in a dose-dependent manner	Norgren et al 1993 (34) Norgren et al 1994 (35)
	10^{-10} M to 10^{-6} M Dex (8, 24, 96 h)	HepG2 cells	qPCR followed by PAGE	Cassette exons	Exon 11 inclusion/skipping ratio upregulated by Dex in a dose- and time-dependent manner	Kosaki and Webster 1993 (36)
LIG4	10^{-8} M R1881 (24 h)	LNCaP cells	qPCR	Alternative promoter	Promoter 1 repressed and promoter 2 activated by R1881	Munkley et al 2018 (37)
MAT2A	10^{-8} M R1881 (24 h)	LNCaP cells	qPCR	Alternative 3' splice site	Isoform 1 upregulated and isoform 2 downregulated by R1881	Munkley et al 2018 (37)
NCOR	2.5×10^{-7} M Dex (48 h)	3T3-L1 cells	RT-PCR	Cassette exons	*NCoRδ*, not *NCoRω*, upregulated by Dex only in cells undergoing adipocyte differentiation	Snyder et al 2015 (38)
NF1	10^{-6} M Dex (6, 12, 18, 24 h)	PC12 cells	RT-PCR	Cassette exons	Exon 23a inclusion/skipping ratio downregulated by Dex after 24 h	Metheny and Skuse 1996 (39)

(*continued*)

Table 1. Continued

Gene	Hormone treatment (duration)	In vitro or in vivo model	Technology[a]	Splice event	Effects	Reference
Nr3a1 (ERα)	0.05 or 5 μg E2/rat, or 1 mg P4/rat (1 injection/d for 4 d; last injection 24 h before killing)	Wistar female rats	RT-nested PCR	Cassette exons	ERα downregulated with high-dose and low-dose E2; ∑3 isoform downregulated with low-dose E2 alone or combined with P4	Varayoud et al 2005 (40)
NR3C1 (GR)	10^{-9} to 10^{-6} M DHEA (4, 16, 18 h)	THP1 human monocytic cells	qPCR; Western blot	Mutually exclusive exons	GRβ, not GRα, upregulated by 10^{-7} M DHEA after 4 or 18 h; GRβ protein, not GRα, upregulated by 10^{-7} and 10^{-8} M DHEA after 16 h	Pinto et al 2015 (41)
	10^{-7} M DHEA (18 h) 10^{-7} cortisol (6 h)	THP1 human monocytic cells	RT-PCR; qPCR; Western blot	Mutually exclusive exons	GRβ, not GRα, upregulated by 10^{-7} M DHEA in presence of SRSF9; GRα upregulated and GRβ downregulated by 10^{-7} M cortisol in presence of SRSF3	Buoso et al 2017 (42)
NR3C2 (MR)	10^{-6} to 10^{-11} M aldosterone; 10^{-7} M Dex; 10^{-8} to 10^{-10} M cortisol (24 h)	Human renal H5 cells	Luciferase assay; EMSA; dNase footprint assay	Alternative promoters	Promoter P1 upregulated by aldosterone in a dose- and MR-dependent manner, by Dex in a GR-dependent manner, by cortisol in a dose- and MR or GR-dependent-manner; Promoter P2 upregulated by aldosterone in a dose- and MR-dependent manner	Zennaro et al 1995 (43) Zennaro et al 1996 (44)
Numb	10^{-7} M Dex (4 h)	Neuro-2a cells	RT-PCR	Cassette exons	Exon 3 inclusion/skipping ratio upregulated by Dex	Magomedova et al 2019 (45)
PNMT	10^{-5} M Dex (4 d)	PC12 cells	RT-PCR	Intron retention	Intron 2 retention downregulated by Dex	Unsworth et al 1999 (46)
	10 mg/kg Dex (6 h)	C57BL/6J mice	RT-PCR	Intron retention	$PNMT_{tr}$ isoform, not $PNMT_{WT}$, downregulated by Dex in atria and ventricle	Bao et al 2002 (47)
Rush1	3 mg/kg/d P4, or 2 mg/d PRL, or 0.05 mg/d E2Bz (7, 10 d)	Estrous rabbits	RT-PCR	Alternative 3′/5′ splice sites	*Rush1α*/*Rush1β* ratio upregulated by P4, P4 + PRL, or PRL + E2Bz	Hayward-Lester et al 1996 (48)
Slo (*Kcnma1*)	Hypophysectomy; 4 U/100 g ACTH injected subcutaneously daily (15 d)	Adrenal chromaffin tissue from rats	RT-PCR and agarose gel	Cassette exons	STREX inclusion downregulated by hypophysectomy, upregulated by ACTH-induced corticosterone increase	Xie and McCobb 1998 (49)
	2×10^{-5} M Dex or 2×10^{-5} M DHEA (24 h)	Bovine chromaffin cells	RT-PCR and agarose gel	Cassette exons	STREX inclusion downregulated by Dex-activated GR, upregulated by DHEA	Lai and McCobb 2002 (50)
	2-2.5 $\times 10^{-9}$ g/mL T by silastic capsules: 1 for age 3 wk, 2 for age 8 wk	Male and female Sprague Dawley rats	RT-PCR	Cassette exons	STREX inclusion downregulated by T in adrenal glands of castrated males, upregulated by T in pituitary glands of castrated males	Mahmoud and McCobb 2004 (51)
	5×10^{-7} M or 2×10^{-5} M Cort (24 h)	Male Sprague Dawley rats	RT-PCR and agarose gel	Cassette exons	STREX inclusion upregulated by 5×10^{-7} M Cort-activated MR and GR, downregulated by 2×10^{-5} M Cort-activated MR and GR in anterior pituitary cells; STREX inclusion downregulated by Cort-activated GR in a dose-dependent manner in adrenal chromaffin cells	Lai and McCobb 2006 (52)
	6.25 or 50 μg/kg E2 (2×/d, 4, 6 d) 3 mg/kg P4 (2×/d, 4 d) 40 μg/kg E2 (2×/d, 2 d) then combined with 3 mg/kg P4 (2×/d, 4 d) 6.25 μg/kg E2 and 625 μg/kg ICI 182 780 (ICI) (2×/d, 4 d) 625 μg/kg ICI (2×/d, 4.5 d)	Female Sprague Dawley rats	qPCR	Cassette exons	STREX inclusion downregulated by E2-activated ER, upregulated by P4	Zhu et al 2005 (53)

(*continued*)

Table 1. Continued

Gene	Hormone treatment (duration)	In vitro or in vivo model	Technology[a]	Splice event	Effects	Reference
Taiman	10^{-6} M JH (1, 2, 4, 8 h)	A. *Aegypti* mosquitoes	RT-PCR; Western blot	Mutually exclusive exons; cassette exons	AaTai-A/B isoforms upregulated and AaTai C/D isoforms downregulated by JH after 1 h in a dose-dependent manner	Liu et al 2018 (54)
Tdo2	10 mg/kg Dex (5 h)	Male C57BL/6J mice	qPCR	Alternative promoters	*Tdo2-FL* upregulated by Dex in brain, astrocytes, and peripheral tissues; *Tdo2-v1/v2* (class 2) upregulated by Dex in peripheral tissues in a tissue-specific manner	Dostal et al 2018 (33)
TSC2	10^{-8} M R1881 (24 h)	LNCaP cells	qPCR; Western blot	Alternative promoter	*TSC2* isoform A, not full-length *TSC2*, upregulated by R1881	Munkley et al 2014 (55)
TSHβ	10^{-6} M, 10^{-7} M, or 10^{-8} M Dex (12, 24, 48 h)	Peripheral blood leukocytes of Hashimoto thyroiditis patients	Western blot; RT-PCR and agarose gel	Intron retention	Intron 2 retention upregulated by Dex in a dose- and time-dependent manner	Liu et al 2012 (56)
VEGF	10^{-8} M P4 (4 h)	T47D cells	Radiolabeled RT-PCR	Cassette exons	Exons 6 + 7 inclusion/skipping ratio downregulated by P4 in presence of CAPERα	Dowhan et al 2005 (26)
WNK1	10^{-9} M aldosterone (0.5, 2, 4, 24 h) 5×10^{-9} M Dex (4 h)	Murine M1 cells	RT-PCR	Alternative promoter	Kidney-specific WNK1 isoform, not long isoform, upregulated by aldosterone after 30 min and by Dex after 4 h	Náray-Fejes-Tóth et al 2004 (57)
ZNF678	10^{-8} M R1881 (24 h)	LNCaP cells	qPCR	Cassette exons	Inclusion/skipping ratio downregulated by R1881	Munkley et al 2018 (37)

Abbreviations: ACTH, adrenocorticotropic hormone; AR, androgen receptor; cDNA, complementary DNA; DDX5, DEAD-box helicase 5; DDX17, DEAD-box helicase 17; Dex, dexamethasone; DHEA, dehydroepiandrosterone; E2, estradiol; E2Bz, estradiol benzoate; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; FACS, fluorescence-activated cell sorting; FL, full-length; GHR, growth hormone receptor; GR, glucocorticoid receptor; JH, juvenile hormone; MR, mineralocorticoid receptor; P4, progesterone; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PR, progesterone receptor; PRL, prolactin; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; STREX, stress-axis regulated exon; T, testosterone; TSH, thyroid stimulating hormone; VEGF, vascular endothelial growth factor.

[a]RT-PCR: one-step, gene-specific reverse transcription with end-point PCR visualized on a gel.

and the NR coactivators CAPERα/RBM39 and CAPERβ/RBM23, first characterized as transcriptional coregulators and later characterized as splicing factors because of their homology to U2AF65 (68, 69).

The splicing of the *CD44* reporter minigene by E2 was examined in HeLa cells when cotransfected with ERα or ERβ and either p72/DDX17 or the hnRNP-like proteins CoAA/RBM14 or TLS/FUS (20). Quantification of *CD44* splice variants by RT-PCR showed that, while TLS did not affect splicing, p72/DDX17 decreased and CoAA increased the CE skipping to inclusion ratio (ie, the relative ratio of transcript without the alternative exon to that with the alternative exon included; see Fig. 1) in a E2-dependent and ER isoform-specific manner (20). Moreover, the interaction between ERα and DDX17 was shown to be dependent on the phosphorylation of ERα at serine 118 (17). The phosphorylation of ERα Ser118 is regulated by MAPK (mitogen-activated protein kinase) signaling activated by the epithelial growth factor (17). This suggests that the crosstalk of several signaling pathways is necessary for the interaction between ERα and the dual function factor DDX17 to mediate E2-modulated AS (17).

CAPERα and CAPERβ were found to have high structural similarity with splicing factors U2AF2/U2AF65 and Poly(U)-binding-splicing factor PUF60, implicating their involvement in the spliceosome complex (68). Indeed, the use of a minigene strategy revealed that CAPERα and CAPERβ participate in the regulation of both transcriptional targets and AS in response to E2 and progesterone (P4) (26). Both CAPERα and CAPERβ contain 3 RNA recognition motifs (RRMs), RRM1 being implicated in NR-driven transcriptional regulation, and RRM2 and RRM3 in the regulation of steroid hormone–modulated AS. These proteins were shown to increase progesterone receptor (PR) activation and ER-dependent *CGRP/CT* splicing products ratio (26). CAPERα causes a significant increase in *VEGF*-121/180 mRNA ratio when knocked out (26). Expression profiles showed that, while U2AF2 and PUF60 are ubiquitous components of the spliceosome, CAPERα and CAPERβ have tissue-specific expression (26). Some studies have proposed the recruitment model of coregulator-mediated coupling of transcription and splicing, which suggests that CAPERα and CAPERβ recruit splicing factors to the transcription complex (70).

Cofactor of BRCA1 (COBRA1) acts as a transrepressor in response to steroid hormone, and modulates AS of androgen receptor (AR)-regulated genes (19). A glutathione S-transferase (GST)-pulldown assay confirmed interactions of COBRA1 and AR, and *CD44* minigene assays demonstrated that expression of the transrepressor led to a 3-fold reduction in skipping for the splice event (19). It was suggested that proteins such as COBRA1 that promote exon inclusion may be altering AS by influencing the rate of RNAP II activity, likely slowing it down, and/or promoting interactions with spliceosome components (71, 72). In the human prostate cancer cell line LNCaP, coimmunoprecipitation experiments revealed that, in the presence of androgens, the interaction between DDX5 and AR was enhanced. Furthermore DDX5 was found to potentiate AR-driven downregulation of the *CD44* minigene inclusion/skipping ratio, while acting as a transcriptional coactivator (16).

As summarized here, the use of transfected minigene reporters like the MMTV-*CD44* minigene was crucial in demonstrating that through "coupling" proteins transcription and AS could both be altered in the presence of steroid hormones. Identification of some of the specific proteins involved in both mechanisms reinforced the concept that a "coupling" of these processes was occurring. However, genome-wide studies discussed later in this review demonstrated that these proteins may be better described as "dual-function" proteins. In other words, rather than supporting the idea that they are necessarily functionally coupled, many NR coregulators have been shown to interact with the transcription complex and the spliceosome independently (see Figs. 2 and 3) (21, 45, 59, 60, 62, 63).

Regulating the Splicing of Coregulators

Coregulators with dual functions are crucial for modulating both transcription and AS of NR signaling pathways, either through the recruitment of splicing factors or by controlling the kinetics of transcriptional elongation (69). The value of this concerted role for coregulators makes their own regulation particularly influential (73), and steroid hormones have been observed to influence the splicing of specific coregulators directly and indirectly.

Dex was observed to modulate the splicing of the NCoRω corepressor transcript in favor of the NCoRδ isoform during 3T3-L1 cell adipogenesis (38). This isoform switch by Dex no longer occurs once the cells have differentiated into mature adipocytes. In the rabbit endometrium, A3SS/A5SS of the transcript encoding the helicase-like TF RUSH was shown to be regulated by P4 (48). RUSH acts in chromatin remodeling and regulates the tissue-specific expression of the uteroglobin gene in rabbits. Full-length *Rush1α* isoform was favored when P4 was combined with prolactin, while truncated *Rush1β* was predominant when cells were sequentially treated with P4 and E2. Tissue-specific regulation of *Rush1* AS by P4 defines uteroglobin abundance, which is itself implicated in P4 binding (48).

Altogether, regulation of the splicing of both transcription factors and splicing factors can act as an additional layer of automodulation of the steroid hormone's signaling pathways.

Regulating Splicing Factor Levels

An indirect way by which steroid hormones modulate AS is through the regulation of splicing factors levels. In prolactin-secreting MMQ clonal rat cells, E2 and T were found to favor the splicing of the long isoform of the dopamine receptor (D2R) transcript, while P4 counteracted these effects, stabilizing the ratios between the long and the short Δ6 isoforms (27). When MMQ cells were treated with the translation inhibitor cycloheximide, the effect of these sex hormones on *Drd2* isoform ratio was abolished (27). It was posited that at the transcriptional level, E2, T, and P4 were involved in modulating the genetic expression of factors that were involved in transregulating *Drd2* splicing (27). Indeed, splicing factors may regulate the choice of the splice site leading to preferential production of the long isoform (27). While the specific proteins were not alluded to, it was investigated that the modulation of *Drd2* splicing was an indirect effect of the hormone's presence (27). Tissue-specific effects were observed in long/short variant ratios of *Drd2*, which differed in response to T and E2 treatment in the anterior pituitary gland, olfactory tubule, hypothalamus, substantia nigra, and striatum (28).

In HepG2 human hepatoma cells, it was discovered that exon 11 inclusion/exclusion is the main event by which the insulin receptor *INSR* transcript is spliced, the exon 11 exclusion being enhanced in response to Dex in a dose-dependent (35, 36) and time-dependent manner (36). Although they

Table 2. Studies investigating steroid hormone effect on transcriptome-wide alternative splicing

Hormone treatment (duration)	In vitro model	Technology	Splice events[a]	Effects	Reference
10^{-8} M E2 (6 or 24 h)	MCF-7 cells	Affymetrix Exon arrays	Alternative promoters	121 alternative promoters differentially regulated by E2; 16 validated by RT-PCR (including *NET1*)	Dutertre et al 2010 (58)
10^{-10a} M E2 (3 h)	MCF-7 cells	Microarray (AS events covering a possible 10 659 AS events of 893 apoptosis-related genes)	175 cassette exons; 87 A3SS; 75 A5SS; 24 intron retentions	463 AS events regulated by E2; 4 validated by qPCR (*AXIN-1*, *CASP7*, *FGFR2*, *FAS*)	Bhat-Nakshatri et al 2013 (21)
10^{-8} M E2 (10 h) 10^{-8} M DHT (24 h)	MCF-7 cells; LNCaP cells	Affymetrix Exon arrays	Various; cassette exons	65/462 AS events regulated by E2 in a DDX5- and DDX17-dependent manner; 371/1573 AS events regulated by DHT in a DDX5- and DDX17-dependent manner	Samaan et al 2014 (59)
10^{-8} M DHT (24 h)	LNCaP cells	Affymetrix Exon arrays and RNA-seq (150 bp paired-end reads, 45-80 M/reads/sample)	Exon Arrays: 84% cassette exons, 8% A5SS, 6% A3SS; RNA-Seq: mutually exclusive exons and cassette exons	Affymetrix Exon arrays: 869 AS events regulated by DHT; 7 validated by RT-PCR (including *IDH1*) RNA-Seq: 198 AS events induced by DHT	Shah et al 2020 (60)
10^{-8} M R1881 (24 h)	LNCaP cells	Affymetrix Exon arrays	108 cassette exons, 144 A5SS, 73A3SS	325 AS events regulated by R1881; 7 validated by RT-PCR (*NDUFV3*, *ZNF121*, *PDE4D*, *TACC2*, *TSC2*, *RIMS1*, *WEE1*)	Rajan et al 2011 (61)
10^{-8} M R1881 (24 h)	LNCaP cells	RNA-seq (paired-end reads)	56 alternative promoters, 12 cassette exons, 4 A3SS, 1 Intron retention	73 AS events regulated by R1881: 17 validated (eg, *LIG4*, *ZNF678*, *MAT2A*)	Munkley et al 2018 (37)
10^{-8} M R1881 (48 h)	LNCaP cells	RNA-seq (75 bp paired-end reads, 76-91 M reads/sample)	Cassette exons	37 AS events regulated by R1881: validation of 6 exon inclusions (*FLNB*, *GRHL1*, *ITGA6*, *MAP3K7*, *MINK1*, *NUMB*) and 6 exon exclusions (*CTNND1*, *DOCK7*, *FN1*, *MAGI1*, *MYH10*, *RPS24*)	Munkley et al 2019 (62)
10^{-8} M R1881 (24 h)	LNCaP cells; LAPC4 cells; 22Rv1 cells	RNA-seq (125 bp paired-end reads, 16-22 M reads/sample)	41% cassette exons, 21% alternative polyadenylation, 15% tandem TSS, 8% A5SS, 7% A3SS, 4% intron retention	In LNCaP: 1943 upregulated and 2018 downregulated AS events In LAPC4: 2872 upregulated and 2728 downregulated AS events In 22Rv1: 1834 upregulated and 1796 downregulated AS events 2 validated AS events: *FOLH1* and *MDH1*	Germain et al 2020 (63)
10^{-8} M R1881 (24 h)	LNCaP cells; VCaP cells	RNA-seq (LNCaP: 200 paired-end reads, 216-269 M reads/sample; VCaP: 150 bp paired-end reads, 44-70 M reads/sample)	In decreasing order: cassette exons, mutually exclusive exons, A3SS, A5SS, intron retention	In LNCaP: 508 AS events regulated by R1881 In VCaP: 1091 AS events regulated by R1881	Rana et al 2021 (64)
10^{-7} M Dex (4 h)	Neuro2a cells	RNA-seq (100 bp paired-end reads, 178-234 M reads/sample)	50% cassette exons, 27% A3SS, 23% A5SS	426 AS events regulated by Dex: 208 upregulated (eg, *Numb*), 218 downregulated	Magomedova et al 2019 (45)
	GC-resistant vs GC-sensitive childhood ALL samples and ex vivo culture	RNA-seq (100 bp single-end reads, 22 ± 5 M reads/sample)	In both B-cell precursor-ALL and T cell–ALL, in decreasing order: cassette exons, intron retentions, A3SS or A5SS	In BCP-ALL: 1035 AS events associated with 777 genes. 13 validated AS events, including *SOD1, ARPC2*, and *PSMD11* In T-ALL: 932 AS events associated with 722 genes. 19 validated AS events, including *CDK4*, *SRSF3*, and *THOC6*	Sciarrillo et al 2020 (65)

Abbreviations: ALL, acute lymphoblastic leukemia; AS, alternative splicing; DHT, 5-dihydrotestosterone; GC, glucocorticoid; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RNA-seq, RNA sequencing; RT-PCR, reverse transcription–polymerase chain reaction.
[a]A5SS: alternative 5' splice site; A3SS, alternative 3' splice site.

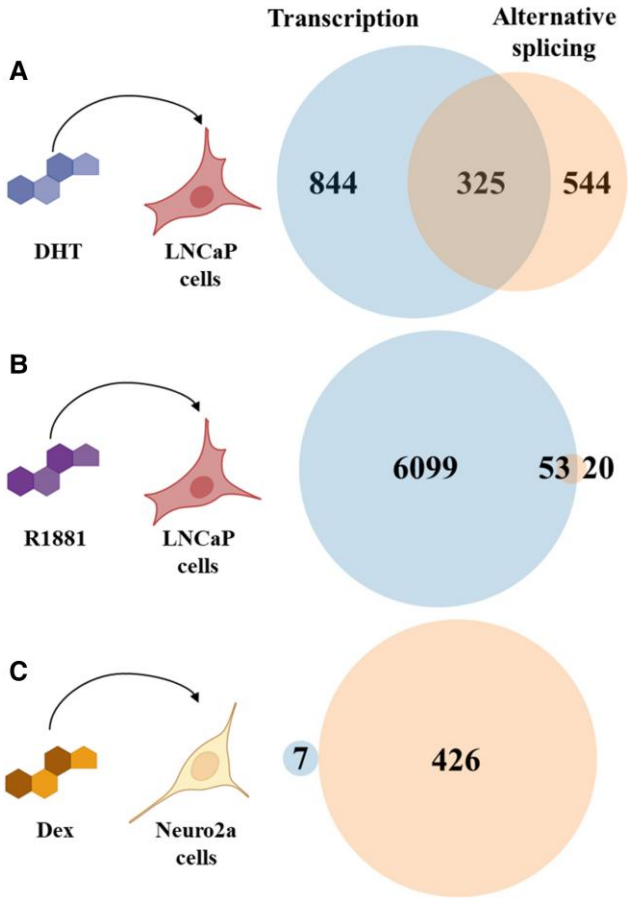

Figure 2. Steroid hormones regulate distinct pre-messenger RNA (pre-mRNA) sets at the transcriptional and the alternative splicing levels. Sets of pre-mRNA, as identified by Affymetrix Exon arrays or RNA sequencing that are differentially regulated by A, 10 nM dihydrotestosterone (DHT) 24 hours (60); B, 10 nM R1881 24 hours (37); or C, 10 nM dexamethasone (Dex) 4 hours (45), at the transcriptional level (blue) or at the alternative splicing level (orange). Transcriptome-wide studies highlighted a limited overlap between the 2 gene sets, suggesting that depending on the cell system and hormone, dual function factors are differentially important for both mechanisms. Created in part with BioRender.com.

did not determine this experimentally, Norgren et al (35) proposed that these effects may be occurring through the indirect modulation of an "exclusion or inclusion factor."

At the transcriptome level, 2 important splice factor proteins, ESRP1 and ESRP2, have been studied for the role AR plays in their regulation in prostate cancer cell progression. ESRP2 transcription is upregulated in prostate cancer carcinoma, specifically in a metastatic subgroup, and in prostate cancer in vitro models (LNCaP and CWR22Rv1 cells) (60). In these models, it was shown that ESRP2 was essential for prostate cancer cell invasiveness (60). In patients, the expression of ESRP2 is mainly upregulated by AR and decreased by androgen deprivation therapy (62). In vitro experiments performed on LNCaP cells showed that androgen-dependent AS was mediated through ESRP2. R1881 is a potent synthetic androgen agonist. ESRP2 is a direct genomic target for R1881-activated AR (demonstrated by chromatin immunoprecipitation) and is transcriptionally upregulated (62). Performing RNA-seq on LNCaP cells in which ESRP2 mRNA was silenced, Munkley et al (62) demonstrated that ESRP2, and its close paralog ESRP1, regulate the splicing of a large subset of androgen-dependent AS events. Furthermore, treatment with AR antagonist casodex, or RNA silencing of AR in prostate cancer samples, inhibits the transcriptional upregulation of *ESRP2*, leading to a reverse modulation of androgen-dependent AS events (62). Treatment with antiandrogenic drugs casodex and enzalutamide, or with the androgen dihydrotestosterone (DHT), has a major effect on AS events (2127, 167, and 869 differentially regulated AS events, respectively), with CE, alternative 3′/5′ splicing, IR, and mutually exclusive exons being the most represented event types, as identified by Affymetrix Exon array or RNA-seq (60). Interestingly, silencing of AR in human prostate cancer MDA-pCa-2b cells profoundly modified the basal transcriptome, suggesting that AR itself contributes to the differential regulation of transcription and AS (60).

Speed of Transcription Indirectly Affects Alternative Splicing

Transcription elongation rate affects alternative splicing decisions in metazoans and yeast (74, 75). In prostate cancer cells, DHT-activated AR increases the efficiency of transcriptional elongation (76). As described earlier, agonist-activated AR increases CD44 exon skipping, and this is reversed in the presence of COBRA1 (19). Interestingly, COBRA1, both an NR corepressor and a splicing factor (19), was also characterized as a subunit of the negative elongation factor complex (77, 78). Sun et al (19) hypothesized that the interaction of COBRA1 with AR may promote a slower elongation rate by the RNAP II, which would affect the recruitment and activity of the spliceosome, thus allowing a differential AS output. Modulation of the transcription elongation was also proposed as an underlying mechanism for the regulation of AS by hormone-activated ER and PR (26).

Indirect or Direct Modulation of Nuclear Receptor Protein Levels

Steroid hormones also play a role in directly affecting receptor protein levels. Investigation of the effects of steroid hormones on ERα protein levels in ovariectomized rats showed that on treatment with high-dose E2 there was a downregulation of ER mRNA levels, and 3-exon skipping (Σ3) isoforms. When rats were treated with P4 there was a reduction of ERα mRNA expression, and no effect was seen when animals were administered a low dosage of E2. In contrast, a combination of both hormones (high-dose E2 and P4) caused a high expression of the Σ3 isoform (40). This tissue-specific dose dependency could indicate a regulatory feedback loop in which steroid hormones are involved in modulating the effects of the hormone presence in the uterus.

The MR gene *NR3C2* contains two 5′ alternative exons, *1α* and *1β*, under the control of APs P1 and P2, respectively (43). In human renal H5 cells, P1 and P2 were shown to both be induced by Dex in a GR-dependent manner, while only P2 is induced by aldosterone in a dose- and MR-dependent manner (44). Interestingly, P2 is induced by cortisol through both GR and MR in a synergistic and dose-dependent manner (44).

In humans, GRα and GRβ are generated by AS of mutually exclusive exons 9α and 9β of *NR3C1* (79). Treatment of THP1 human monocyte cells with dehydroepiandrosterone (DHEA) selectively upregulates the dominant negative isoform of GR (GRβ), both at the mRNA and protein levels (41). Subsequent studies showed that DHEA does so by increasing the expression of the serine and arginine-rich splicing

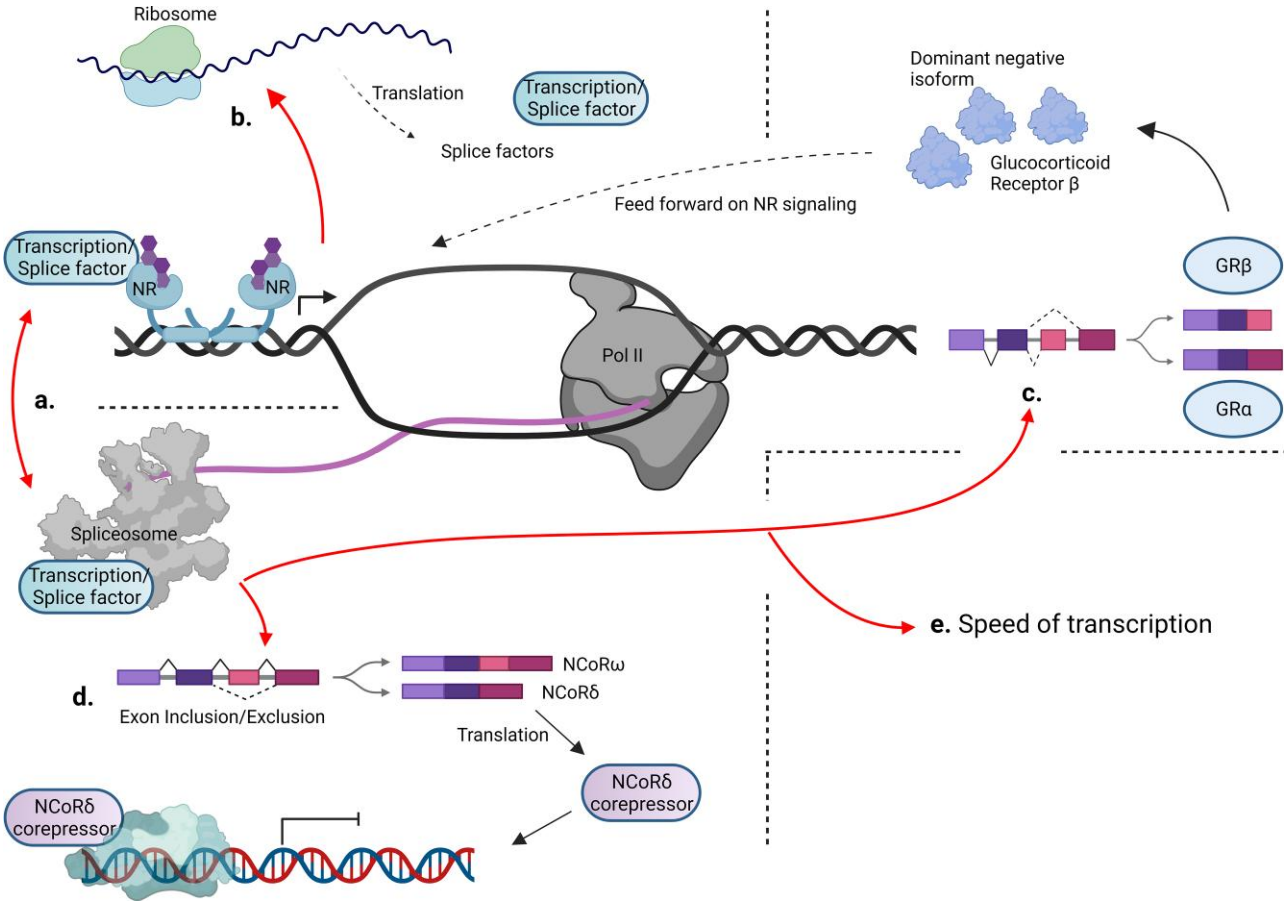

Figure 3. Mechanisms of regulation of alternative splicing (AS) by steroid hormones. A, Transcription coregulators/splicing factors recruited by steroid hormone–nuclear receptor (NR) complex can play a dual function in transcription and AS. B, Expression levels of splicing factors can be modulated by steroid hormone-NR mediated transcriptional activation. C, Steroid hormones directly affect receptor levels and transcriptional activity, as observed for the splice variant glucocorticoid receptor GRβ, which acts as a dominant negative isoform for the main glucocorticoid receptor, GRα. D, Ligand-NR complexes through their dual function capability modulate the splicing of coregulators such as the nuclear receptor co-repressor (NCoR) corepressor. E, The elongation rate for RNA polymerase II can affect exon inclusion because splice sites in alternative exons may require more time to be recognized by the spliceosome. Figure created with BioRender.com.

factor SRSF9, which favors *NR3C1* splicing into GRβ. In contrast, cortisol upregulates SRSF3, which promotes the expression of GRα (42).

High-throughput Sequencing of Messenger RNA Identifies Steroid Hormone Effects on Alternative Splicing at the Whole-Transcriptome Level

High-throughput technologies have allowed the identification and quantification of numerous transcriptional and AS events in one or multiple samples in parallel. MCF-7 breast cancer cells treated with E2 and analyzed by microarray (directed to apoptosis-related genes) found 463 AS events regulated by the hormone (21). In this study, it was found that approximately 67% of genes that were alternatively spliced after a 3-hour treatment were not changed at the transcriptional level (21). From the analysis, a selection of E2-regulated AS events (*AXIN-1*, *FGFR2*, *FAS*) whose dysregulation could potentially affect breast cancer therapy efficacy were validated by quantitative PCR (21). In a separate study using an Affymetrix Exon array, E2-treated MCF-7 cells were shown to regulate 121 APs in a DDX5- and DDX17-dependent manner at loci in close proximity to binding motifs for ERα and CCCTC-binding factor (CTCF) (58).

Next-generation sequencing of mature transcripts (RNA-seq) has become a standard experimental approach to quantify all RNA species in cells. The identification of transcripts that are alternatively spliced is possible with sufficient read depth (>60 M reads per sample). To evaluate the effect of glucocorticoids on AS in neurons, RNA-seq was performed on mouse neural N2a cells and revealed 426 Dex-responsive splicing events (208 upregulated, 218 downregulated) (45). The GR coregulator arginine and glutamate rich-1 (ARGLU1) was found to be a crucial dual partner of Dex-dependent GR-regulated transcription and AS (45). ARGLU1 was shown to contribute to the Dex-dependent AS of 398 transcripts (93% of Dex-regulated AS events) (45). ARGLU1 coimmunoprecipitated with splicing factors PUF60, U2AF2, and JMJD6, supporting its role in AS.

In prostate cancer models established in vitro and in vivo, whole-transcriptomic analysis deepened understanding of the androgen and AS effect on tumor progression. Affymetrix Exon array applied to R1881-treated LNCaP cell transcripts provided the first set of 325 AS events regulated by androgens: 108 CEs, 144 A5SS, 73 A3SS, and 5 APs (61). One of the R1881-differentially regulated APs is upstream of the tumor-suppressor *TSC2* coding gene, and increases expression of a truncated isoform A (TSC2A) unable

to inhibit mTOR (mechanistic target of rapamycin) signaling (61). mTOR itself is a crucial regulator of protein synthesis and cell growth, and the androgen-driven switch promoting TSC2A contributes to prostate cancer cell proliferation (55, 61). RNA-seq performed on transcripts from LNCaP cells treated with AR agonist R1881 identified 73 androgen-dependent AS events, from which 53 were also regulated at the transcriptional level (37). A total of 48 out of 73 AS events induced a protein isoform change, among which were the prostate cancer–relevant targets PrLZ, TACC2, NDUFV3, MAT2, and CNNM2. Interestingly, 11 of 73 AS events were related to a switch from a coding to a noncoding RNA isoform or untranslated mRNA, including APs for *RLN1* and *RLN2* encoding the peptide hormones relaxins, which are upregulated during prostate cancer. In addition, several AS events were found to correlate with prostate cancer severity (Gleason score) when analyzed in a prostate adenocarcinoma transcriptomic data bank, such as *OSBPL1A*, *CLK3*, and *TSC22D3* (37).

Discussion

In this review, we illustrated the diversity of ways steroid hormones have been shown to affect mRNA AS, and thus protein diversification. This consequence of hormone action is understudied but will hopefully become a standard step in RNA-seq analyses to allow researchers to decipher the consequences of AS on protein function and ultimately pathophysiological processes. Primarily acting through their NR, steroid hormones promote gene- and tissue-specific recruitment of dual-function transcription/splicing factors, hence modulating the binding and activity of the transcription machinery and the spliceosome on DNA and pre-mRNA, respectively. This steroid hormone–driven molecular assembly regulates major cellular processes, as well as the hormonal signal itself, through a feed-forward loop controlling the level and stability of the NR, TFs, and splicing factors. Other mechanisms that we did not mention that have been described for the regulation of AS output include microRNA and RNA binding proteins that control pre-mRNA stability. These factors are themselves targets of steroid hormones and have been proposed to represent an additional way steroid hormones may regulate AS (69, 80, 81).

Errors in AS affect the progression of many genetic diseases including myotonic dystrophy, familial dysautonomia, and Menkes disease (82, 83). In this review, studies focusing on hormone-driven cancers, including breast cancer, prostate cancer, and leukemia, highlight the importance of investigating this critical step of pre-mRNA processing in understanding disease pathophysiology more broadly (84-86).

Most of the early knowledge of steroid hormone action on AS came from studies that employed artificial systems like minigene assays, which may not represent in vivo cellular processes. On one hand, these artificial systems are highly versatile, reproducible, easy to use, quantitative, and allow for the isolated analysis of a single allele (87). On the other hand, there is an inherent experimental bias that comes from studying the effect of a hormone on the expression of a heterologous gene (often downstream of a hormone response element) in comparison to what is derived from the endogenous context. Another challenge in the field is deciphering whether a splicing event will affect protein function under normal or pathophysiological contexts. The recent substantial advances in protein-folding prediction models will help move this aspect of biology forward (88, 89). Bulk RNA-seq data can provide only the average AS patterns and gene expression in cell populations (7, 90, 91). Single-cell RNA sequencing methods are able to detect specific AS events within a particular cell of interest (90). The very recent development of dedicated statistical tools, such as SCATS (92) and SpliZ (93), strengthens single-cell RNA sequencing's power to resolve alternatively spliced transcripts in a cell-type–specific manner. Therefore, we suggest that applying the newest technologies to the regulation of alternative splicing by steroid hormones will profoundly increase our understanding of its implication in challenging pathophysiological contexts.

Acknowledgments

We thank Dr Lilia Magomedova and Michael Saikali for their careful editorial review of the manuscript.

Funding

This work was supported by the Novo Nordisk-Banting and Best Diabetes Centre Fellowship (to F.L.B.), the Amgen Scholars Summer Program (to G.U.), and the Natural Sciences and Engineering Research Council of Canada (RGPIN-2020-07212 to C.L.C.).

Disclosures

The authors have nothing to disclose.

Data Availability

Original data analyzed during this study are included in this published article or in the data repositories listed in "References".

References

1. Beato M, Candau R, Chavez S, Mows C, Truss M. Interaction of steroid hormone receptors with transcription factors involves chromatin remodelling. *J Steroid Biochem Mol Biol*. 1996;56(1-6 Spec No):47-59.
2. Kininis M, Kraus WL. A global view of transcriptional regulation by nuclear receptors: gene expression, factor localization, and DNA sequence analysis. *Nucl Recept Signal*. 2008;6:e005.
3. Ratman D, Vanden Berghe W, Dejager L, *et al*. How glucocorticoid receptors modulate the activity of other transcription factors: a scope beyond tethering. *Mol Cell Endocrinol*. 2013;380(1-2):41-54.
4. Le Dily F, Beato M. Signaling by steroid hormones in the 3D nuclear space. *Int J Mol Sci*. 2018;19(2):E306.
5. Mangelsdorf DJ, Thummel C, Beato M, *et al*. The nuclear receptor superfamily: the second decade. *Cell*. 1995;83(6):835-839.
6. van den Hoogenhof MMG, Pinto YM, Creemers EE. RNA splicing: regulation and dysregulation in the heart. *Circ Res*. 2016;118(3):454-468.
7. Pan Q, Shai O, Lee LJ, Frey BJ, Blencowe BJ. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat Genet*. 2008;40(12):1413-1415.
8. Wang ET, Sandberg R, Luo S, *et al*. Alternative isoform regulation in human tissue transcriptomes. *Nature*. 2008;456(7221):470-476.
9. Rodriguez JM, Pozo F, di Domenico T, Vazquez J, Tress ML. An analysis of tissue-specific alternative splicing at the protein level. *PLoS Comput Biol*. 2020;16(10):e1008287.

10. Munkley J, Livermore K, Rajan P, Elliott DJ. RNA splicing and splicing regulator changes in prostate cancer pathology. *Hum Genet*. 2017;136(9):1143-1154.
11. Nicholson JM, Uppala A, Sieber M, Grabitz P, Mordaunt M, Rife SC. Measuring the quality of scientific references in Wikipedia: an analysis of more than 115M citations to over 800 000 scientific articles. *FEBS J*. 2021;288(14):4242-4248.
12. Arenas F, Hervias I, Uriz M, Joplin R, Prieto J, Medina JF. Combination of ursodeoxycholic acid and glucocorticoids upregulates the AE2 alternate promoter in human liver cells. *J Clin Invest*. 2008;118(2):695-709.
13. Thakur MK, Mani ST. Estradiol regulates APP mRNA alternative splicing in the mice brain cortex. *Neurosci Lett*. 2005;381(1-2):154-157.
14. Pecci A, Scholz A, Pelster D, Beato M. Progestins prevent apoptosis in a rat endometrial cell line and increase the ratio of bcl-XL to bcl-XS. *J Biol Chem*. 1997;272(18):11791-11798.
15. Auboeuf D, Dowhan DH, Li X, *et al*. CoAA, a nuclear receptor coactivator protein at the interface of transcriptional coactivation and RNA splicing. *Mol Cell Biol*. 2004;24(1):442-453.
16. Clark EL, Coulson A, Dalgliesh C, *et al*. The RNA helicase p68 is a novel androgen receptor coactivator involved in splicing and is overexpressed in prostate cancer. *Cancer Res*. 2008;68(19):7938-7946.
17. Masuhiro Y, Mezaki Y, Sakari M, *et al*. Splicing potentiation by growth factor signals via estrogen receptor phosphorylation. *Proc Natl Acad Sci U S A*. 2005;102(23):8126-8131.
18. Rajan P, Gaughan L, Dalgliesh C, *et al*. The RNA-binding and adaptor protein Sam68 modulates signal-dependent splicing and transcriptional activity of the androgen receptor. *J Pathol*. 2008;215(1):67-77.
19. Sun J, Blair AL, Aiyar SE, Li R. Cofactor of BRCA1 modulates androgen-dependent transcription and alternative splicing. *J Steroid Biochem Mol Biol*. 2007;107(3-5):131-139.
20. Auboeuf D, Hönig A, Berget SM, O'Malley BW. Coordinate regulation of transcription and splicing by steroid receptor coregulators. *Science*. 2002;298(5592):416-419.
21. Bhat-Nakshatri P, Song EK, Collins NR, *et al*. Interplay between estrogen receptor and AKT in estradiol-induced alternative splicing. *BMC Med Genomics*. 2013;6(1):21.
22. Högger P, Dreier J, Droste A, Buck F, Sorg C. Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). *J Immunol*. 1998;161(4):1883-1890.
23. Winderickx J, Hemschoote K, De Clercq N, *et al*. Tissue-specific expression and androgen regulation of different genes encoding rat prostatic 22-kilodalton glycoproteins homologous to human and rat cystatin. *Mol Endocrinol*. 1990;4(4):657-667.
24. Vercaeren I, Winderickx J, Devos A, Peeters B, Heyns W. An effect of androgens on the length of the poly(A)-tail and alternative splicing cause size heterogeneity of the messenger ribonucleic acids encoding cystatin-related protein. *Endocrinology*. 1993;132(3):2496-2502.
25. Russo AF, Lanigan TM, Sullivan BE. Neuronal properties of a thyroid C-cell line: partial repression by dexamethasone and retinoic acid. *Mol Endocrinol*. 1992;6(2):207-218.
26. Dowhan DH, Hong EP, Auboeuf D, *et al*. Steroid hormone receptor coactivation and alternative RNA splicing by U2AF65-related proteins CAPERalpha and CAPERbeta. *Mol Cell*. 2005;17(3):429-439.
27. Guivarc'h D, Vincent JD, Vernier P. Alternative splicing of the D2 dopamine receptor messenger ribonucleic acid is modulated by activated sex steroid receptors in the MMQ prolactin cell line. *Endocrinology*. 1998;139(10):4213-4221.
28. Guivarc'h D, Vernier P, Vincent JD. Sex steroid hormones change the differential distribution of the isoforms of the D2 dopamine receptor messenger RNA in the rat brain. *Neuroscience*. 1995;69(1):159-166.
29. Sheflin LG, Brooks EM, Keegan BP, Spaulding SW. Increased epidermal growth factor expression produced by testosterone in the submaxillary gland of female mice is accompanied by changes in poly-A tail length and periodicity. *Endocrinology*. 1996;137(5):2085-2092.
30. Friese MA, Hellwage J, Jokiranta TS, *et al*. FHL-1/reconectin and factor H: two human complement regulators which are encoded by the same gene are differently expressed and regulated. *Mol Immunol*. 1999;36(13-14):809-818.
31. Vottero A, Kimchi-Sarfaty C, Kratzsch J, Chrousos GP, Hochberg Z. Transcriptional and translational regulation of the splicing isoforms of the growth hormone receptor by glucocorticoids. *Horm Metab Res*. 2003;35(1):7-12.
32. Romero A, Vega M, Santibáñez N, *et al*. Salmo salar glucocorticoid receptors analyses of alternative splicing variants under stress conditions. *Gen Comp Endocrinol*. 2020;293:113466.
33. Dostal CR, Gamsby NS, Lawson MA, McCusker RH. Glia- and tissue-specific changes in the kynurenine pathway after treatment of mice with lipopolysaccharide and dexamethasone. *Brain Behav Immun*. 2018;69:321-335.
34. Norgren S, Zierath J, Galuska D, Wallberg-Henriksson H, Luthman H. Differences in the ratio of RNA encoding two isoforms of the insulin receptor between control and NIDDM patients. The RNA variant without exon 11 predominates in both groups. *Diabetes*. 1993;42(5):675-681.
35. Norgren S, Li LS, Luthman H. Regulation of human insulin receptor RNA splicing in HepG2 cells: effects of glucocorticoid and low glucose concentration. *Biochem Biophys Res Commun*. 1994;199(1):277-284.
36. Kosaki A, Webster NJ. Effect of dexamethasone on the alternative splicing of the insulin receptor mRNA and insulin action in HepG2 hepatoma cells. *J Biol Chem*. 1993;268(29):21990-21996.
37. Munkley J, Maia TM, Ibarluzea N, *et al*. Androgen-dependent alternative mRNA isoform expression in prostate cancer cells. *F1000Res*. 2018;7:1189.
38. Snyder CA, Goodson ML, Schroeder AC, Privalsky ML. Regulation of corepressor alternative mRNA splicing by hormonal and metabolic signaling. *Mol Cell Endocrinol*. 2015;413:228-235.
39. Metheny LJ, Skuse GR. NF1 mRNA isoform expression in PC12 cells: modulation by extrinsic factors. *Exp Cell Res*. 1996;228(1):44-49.
40. Varayoud J, Ramos JG, Monje L, Bosquiazzo V, Muñoz-de-Toro M, Luque EH. The estrogen receptor alpha sigma3 mRNA splicing variant is differentially regulated by estrogen and progesterone in the rat uterus. *J Endocrinol*. 2005;186(1):51-60.
41. Pinto A, Malacrida B, Oieni J, *et al*. DHEA modulates the effect of cortisol on RACK1 expression via interference with the splicing of the glucocorticoid receptor: DHEA and effect of cortisol on RACK1 expression. *Br J Pharmacol*. 2015;172(11):2918-2927.
42. Buoso E, Galasso M, Ronfani M, *et al*. Role of spliceosome proteins in the regulation of glucocorticoid receptor isoforms by cortisol and dehydroepiandrosterone. *Pharmacol Res*. 2017;120:180-187.
43. Zennaro MC, Keightley MC, Kotelevtsev Y, Conway GS, Soubrier F, Fuller PJ. Human mineralocorticoid receptor genomic structure and identification of expressed isoforms. *J Biol Chem*. 1995;270(36):21016-21020.
44. Zennaro MC, Le Menuet D, Lombès M. Characterization of the human mineralocorticoid receptor gene 5′-regulatory region: evidence for differential hormonal regulation of two alternative promoters via nonclassical mechanisms. *Mol Endocrinol*. 1996;10(12):1549-1560.
45. Magomedova L, Tiefenbach J, Zilberman E, *et al*. ARGLU1 is a transcriptional coactivator and splicing regulator important for stress hormone signaling and development. *Nucleic Acids Res*. 2019;47(6):2856-2870.
46. Unsworth BR, Hayman GT, Carroll A, Lelkes PI. Tissue-specific alternative mRNA splicing of phenylethanolamine *N*-methyltransferase (PNMT) during development by intron retention. *Int J Dev Neurosci*. 1999;17(1):45-55.

47. Bao X, Kennedy B, Enns R, *et al.* A truncated mouse phenylethanolamine *N*-methyltransferase splice variant with dominant-negative activity. *Ann N Y Acad Sci*. 2002;971:89-91.
48. Hayward-Lester A, Hewetson A, Beale EG, Oefner PJ, Doris PA, Chilton BS. Cloning, characterization, and steroid-dependent post-transcriptional processing of RUSH-1 alpha and beta, two uteroglobin promoter-binding proteins. *Mol Endocrinol*. 1996;10(11):1335-1349.
49. Xie J, McCobb DP. Control of alternative splicing of potassium channels by stress hormones. *Science*. 1998;280(5362):443-446.
50. Lai GJ, McCobb DP. Opposing actions of adrenal androgens and glucocorticoids on alternative splicing of *Slo* potassium channels in bovine chromaffin cells. *Proc Natl Acad Sci U S A*. 2002;99-(11):7722-7727.
51. Mahmoud SF, McCobb DP. Regulation of *Slo* potassium channel alternative splicing in the pituitary by gonadal testosterone. *J Neuroendocrinol*. 2004;16(3):237-243.
52. Lai GJ, McCobb DP. Regulation of alternative splicing of Slo K^+ channels in adrenal and pituitary during the stress-hyporesponsive period of rat development. *Endocrinology*. 2006;147(8):3961-3967.
53. Zhu N, Eghbali M, Helguera G, Song M, Stefani E, Toro L. Alternative splicing of Slo channel gene programmed by estrogen, progesterone and pregnancy. *FEBS Lett*. 2005;579(21):4856-4860.
54. Liu P, Fu X, Zhu J. Juvenile hormone-regulated alternative splicing of the *taiman* gene primes the ecdysteroid response in adult mosquitoes. *Proc Natl Acad Sci U S A*. 2018;115(33):E7738-E7747.
55. Munkley J, Rajan P, Lafferty NP, *et al.* A novel androgen-regulated isoform of the TSC2 tumour suppressor gene increases cell proliferation. *Oncotarget*. 2014;5(1):131-139.
56. Liu C, Li L, Ying F, Xu C, Zang X, Gao Z. A newly identified TSHβ splice variant is involved in the pathology of Hashimoto's thyroiditis. *Mol Biol Rep*. 2012;39(12):10019-10030.
57. Náray-Fejes-Tóth A, Snyder PM, Fejes-Tóth G. The kidney-specific WNK1 isoform is induced by aldosterone and stimulates epithelial sodium channel-mediated Na^+ transport. *Proc Natl Acad Sci USA*. 2004;101(50):17434-17439.
58. Dutertre M, Gratadou L, Dardenne E, *et al.* Estrogen regulation and physiopathologic significance of alternative promoters in breast cancer. *Cancer Res*. 2010;70(9):3760-3770.
59. Samaan S, Tranchevent LC, Dardenne E, *et al.* The Ddx5 and Ddx17 RNA helicases are cornerstones in the complex regulatory array of steroid hormone-signaling pathways. *Nucleic Acids Res*. 2014;42(4):2197-2207.
60. Shah K, Gagliano T, Garland L, *et al.* Androgen receptor signaling regulates the transcriptome of prostate cancer cells by modulating global alternative splicing. *Oncogene*. 2020;39(39):6172-6189.
61. Rajan P, Dalgliesh C, Carling PJ, *et al.* Identification of novel androgen-regulated pathways and mRNA isoforms through genome-wide exon-specific profiling of the LNCaP transcriptome. *PLoS One*. 2011;6(12):e29088.
62. Munkley J, Li L, Krishnan SRG, *et al.* Androgen-regulated transcription of ESRP2 drives alternative splicing patterns in prostate cancer. *Elife*. 2019;8:e47678.
63. Germain L, Lafront C, Beaudette J, Karthik Poluri RT, Weidmann C, Audet-Walsh É. Alternative splicing regulation by the androgen receptor in prostate cancer cells. *J Steroid Biochem Mol Biol*. 2020;202:105710.
64. Rana M, Dong J, Robertson MJ, Basil P, Coarfa C, Weigel NL. Androgen receptor and its splice variant, AR-V7, differentially induce mRNA splicing in prostate cancer cells. *Sci Rep*. 2021;11(1):1393.
65. Sciarrillo R, Wojtuszkiewicz A, Kooi IE, *et al.* Glucocorticoid resistant pediatric acute lymphoblastic leukemia samples display altered splicing profile and vulnerability to spliceosome modulation. *Cancers (Basel)*. 2020;12(3):E723.
66. Stallcup MR, Poulard C. Gene-specific actions of transcriptional coregulators facilitate physiological plasticity: evidence for a physiological coregulator code. *Trends Biochem Sci*. 2020;45(6):497-510.
67. Kameoka S, Duque P, Konarska MM. $p54^{nrb}$ associates with the 5′ splice site within large transcription/splicing complexes. *EMBO J*. 2004;23(8):1782-1791.
68. Auboeuf D, Dowhan DH, Dutertre M, Martin N, Berget SM, O'Malley BW. A subset of nuclear receptor coregulators act as coupling proteins during synthesis and maturation of RNA transcripts. *Mol Cell Biol*. 2005;25(13):5307-5316.
69. Auboeuf D, Batsché E, Dutertre M, Muchardt C, O'Malley BW. Coregulators: transducing signal from transcription to alternative splicing. *Trends Endocrinol Metab*. 2007;18(3):122-129.
70. Rosonina E, Blencowe BJ. Gene expression: the close coupling of transcription and splicing. *Curr Biol*. 2002;12(9):R319-R321.
71. Jonkers I, Lis JT. Getting up to speed with transcription elongation by RNA polymerase II. *Nat Rev Mol Cell Biol*. 2015;16(3):167-177.
72. Chen M, Manley JL. Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. *Nat Rev Mol Cell Biol*. 2009;10(11):741-754.
73. Lou H, Gagel RF. Alternative ribonucleic acid processing in endocrine systems. *Endocr Rev*. 2001;22(2):205-225.
74. Fong N, Kim H, Zhou Y, *et al.* Pre-mRNA splicing is facilitated by an optimal RNA polymerase II elongation rate. *Genes Dev*. 2014;28(23):2663-2676.
75. Aslanzadeh V, Huang Y, Sanguinetti G, Beggs JD. Transcription rate strongly affects splicing fidelity and cotranscriptionality in budding yeast. *Genome Res*. 2018;28(2):203-213.
76. Lee DK, Duan HO, Chang C. Androgen receptor interacts with the positive elongation factor P-TEFb and enhances the efficiency of transcriptional elongation. *J Biol Chem*. 2001;276(13):9978-9984.
77. Narita T, Yamaguchi Y, Yano K, *et al.* Human transcription elongation factor NELF: identification of novel subunits and reconstitution of the functionally active complex. *Mol Cell Biol*. 2003;23(6):1863-1873.
78. Danko CG, Hah N, Luo X, *et al.* Signaling pathways differentially affect RNA polymerase II initiation, pausing, and elongation rate in cells. *Mol Cell*. 2013;50(2):212-222.
79. Kino T, Su YA, Chrousos GP. Human glucocorticoid receptor isoform β: recent understanding of its potential implications in physiology and pathophysiology. *Cell Mol Life Sci*. 2009;66(21):3435-3448.
80. Ehretsmann CP, Chandler LA, Bourgeois S. A nuclear post-transcriptional mechanism mediates the induction of fibronectin by glucocorticoids. *Mol Cell Endocrinol*. 1995;110(1-2):185-194.
81. Menotta M, Biagiotti S, Bianchi M, Chessa L, Magnani M. Dexamethasone partially rescues ataxia telangiectasia-mutated (ATM) deficiency in ataxia telangiectasia by promoting a shortened protein variant retaining kinase activity. *J Biol Chem*. 2012;287-(49):41352-41363.
82. Douglas AGL, Wood MJA. RNA splicing: disease and therapy. *Brief Funct Genomics*. 2011;10(3):151-164.
83. Tazi J, Bakkour N, Stamm S. Alternative splicing and disease. *Biochim Biophys Acta*. 2009;1792(1):14-26.
84. Sciarrillo R, Wojtuszkiewicz A, Assaraf YG, *et al.* The role of alternative splicing in cancer: from oncogenesis to drug resistance. *Drug Resist Updat*. 2020;53:100728.
85. Zhang Y, Qian J, Gu C, Yang Y. Alternative splicing and cancer: a systematic review. *Signal Transduct Target Ther*. 2021;6(1):78.
86. Sveen A, Kilpinen S, Ruusulehto A, Lothe RA, Skotheim RI. Aberrant RNA splicing in cancer; expression changes and driver mutations of splicing factor genes. *Oncogene*. 2016;35(19):2413-2427.
87. Fraile-Bethencourt E, Valenzuela-Palomo A, Díez-Gómez B, Caloca MJ, Gómez-Barrero S, Velasco EA. Minigene splicing assays identify 12 spliceogenic variants of *BRCA2* exons 14 and 15. *Front Genet*. 2019;10:503.

88. Jumper J, Evans R, Pritzel A, *et al*. Highly accurate protein structure prediction with AlphaFold. *Nature*. 2021;596(7873):583-589.
89. Varadi M, Anyango S, Deshpande M, *et al*. AlphaFold protein structure database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. *Nucleic Acids Res*. 2022;50(D1):D439-D444.
90. Chen G, Ning B, Shi T. Single-cell RNA-seq technologies and related computational data analysis. *Front Genet*. 2019 Apr 5;10:317.
91. Arzalluz-Luque Á, Conesa A. Single-cell RNAseq for the study of isoforms—how is that possible? *Genome Biol*. 2018;19(1):110.
92. Hu Y, Wang K, Li M. Detecting differential alternative splicing events in scRNA-seq with or without unique molecular identifiers. *PLoS Comput Biol*. 2020;16(6):e1007925.
93. Olivieri JE, Dehghannasiri R, Wang PL, *et al*; Tabula Sapiens Consortium. RNA splicing programs define tissue compartments and cell types at single-cell resolution. *Elife*. 2021;10:e70692.