Structural variants of sex steroid hormone receptors in the testis: from molecular biology to physiological roles

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Abstract
Introduction
Sex steroid hormones, namely androgens and oestrogens, regulate multiple reproductive and non-reproductive processes by interacting with their cognate specific receptor protein and regulating the expression of target genes. Classical sex steroid hormone receptors (SSHRs) belong to the nuclear receptor superfamily, which represents the largest known family of transcription factors in eukaryotes. A common feature of sex steroid hormone receptors is the incidence of alternative splicing, a process that generates multiple variants from a single mRNA precursor molecule. This process facilitates the production of functionally distinct proteins and contributes to proteome diversity in higher eukaryotes. Herein we will critically review current information about the diversity of oestrogen receptors and androgen receptors variants, discussing their structural features and physiological functions.

Conclusion
The identification of structural variants of both ERs and ARs in healthy testis and a broad range of vertebrate species, highlights the newly revealed complexity of SSRHs signalling mechanisms in this tissue.

Introduction
Sex steroid hormones, namely androgens and oestrogens, regulate multiple reproductive and non-reproductive processes by interacting with their cognate specific receptor protein and regulating the expression of target genes. Classical sex steroid hormone receptors (SSHRs) belong to the nuclear receptor superfamily, which represents the largest known family of transcription factors in eukaryotes. In addition, SSRHs are present in a variety of species ranging from nematodes to humans.

For several years, the existence of a single oestrogen receptor (ER) was generally accepted. The discovery of a second type of ER (ERβ) in rats, humans and other species profoundly changed the outlook of ER signalling, stimulating a new wave of research on ERs that was aimed at discovering their specific roles and interactions. While the two ER subtypes (ERα and ERβ) have a typical steroid receptor structure, they are encoded by genes located on different chromosomes, sharing only 41%–65% of overall amino acid identity depending on the species being considered. Nevertheless, both receptors exhibit similar binding affinity to 17β-oestradiol and display binding characteristics recognised for ER proteins; specifically, relative binding affinity for natural and synthetic oestrogens in addition to anti-oestrogenic compounds is higher than the binding affinity for androgens, progestogens or corticosteroids. However, selective modulators functioning as agonists or antagonists have been identified for ERα and ERβ proteins.

Discussion
The authors have referenced some of their own studies in this review. These referenced studies have been conducted in accordance with the Declaration of Helsinki (1964) and the protocols of these studies have been approved by the relevant ethics committees related to the ins-
Critical review

Steroid hormone receptor structure and general mechanism of action

All steroid receptors share common structural features with the following functional domains encoded by distinctly separate exons 1–8 (Figure 1): amino-terminal domain (NTD or A/B region), DNA-binding domain (DBD or C region), hinge region (D region) and ligand binding domain (LBD or E region). The NTD, which contains the transcription activation function 1 (AF1), is the most variable region. In contrast, DBD is the most conserved domain being characterised by the presence of two zinc-fingers, which are responsible for binding receptors to DNA at specific nucleotide sequences called hormone response elements (HREs). DBD is linked to LBD through the hinge region, which is a poorly conserved domain that often contains the nuclear localisation signal. LBD, which is less conserved than DBD, is responsible for ligand binding, dimerisation and interaction with heat shock proteins, in addition to containing the ligand-dependent transactivation function 2 (AF2). The F region of yet unknown function is only present in ERs.

Supporting their structural similarities, SSHRs modulate numerous and specific responses in a large variety of cells that share a general mechanism of action, which has been clarified in detail through molecular biology research.

A simplified model of steroid action in target cells is presented in Figure 2. After biosynthesis, steroid hormones reach target cells via the bloodstream and due to their lipophilic nature, they cross the cell membrane by simple diffusion, being retained with high affinity and specificity by their nuclear receptors. In the absence of the hormones, the receptors are mostly located in the cytoplasm and are transcriptionally inactive due to being associated with heat shock proteins (HSPs). The most widely accepted theory states that ligand-binding promotes a set of conformational modifications in the receptor structure, which causes the displacement of inhibitory HSPs and facilitates receptor translocation to the nucleus. Upon binding to the hormone response elements of target genes, hormone–receptor complexes induce remodelling of chromatin and interact with transcriptional machinery, thereby activating or repressing the transcription of target genes.

Steroid hormone receptors may also establish protein–protein interactions with other sequence-specific transcription factors or establish cross-talk with signal transduction pathways that are activated by extracellular signals via membrane receptors [e.g., the G-protein coupled receptor (GPCR)] and the activation of protein kinase cascades. These represent the so-called non-genomic actions.

Figure 1: Schematic of the structural and functional domains of steroid hormone receptor proteins (a) and the coding of exons 1–8 in relation to each functional domain of human ER (b) and AR (c) genes. The highly conserved regions, domain C and E containing DNA-binding and ligand-binding domains, respectively, are indicated by shaded boxes. Empty boxes represent the divergent regions A/B, D and F. Note that ER is the only steroid receptor where the F domain is present. The function of each domain is indicated. AF, transcriptional activation function; NLS, nuclear localisation signal; HSP, heat shock protein.
Molecular mechanism of the action of steroid hormone receptors.

1. Through the fine tuning of all these different mechanisms, steroid hormone receptors may exert particular and tissue-specific effects that depend on genetic, cellular and physiological circumstances.

2. Nevertheless, the manner in which SSHRs function in healthy and deregulated reproductive processes has not been completely resolved. There is much more to learn about steroid hormone actions in testicular cells that regulate spermatogenesis; specifically, knowledge remains lacking about the set of steroid activated pathways of steroid hormone action and have been connected with rapid effects, because they do not depend on the activation of RNA transcription or translation into protein and usually involve the activation of protein kinase cascades, mobilisation of intracellular calcium, increased cyclic AMP (cAMP) concentrations and modulation of nitric oxide release, amongst others. Through the fine tuning of all these different mechanisms, steroid hormone receptors may exert particular and tissue-specific effects that depend on genetic, cellular and physiological circumstances.

3. Furthermore, the manner in which SSHRs function in healthy and deregulated reproductive processes has not been completely resolved. There is much more to learn about steroid hormone actions in testicular cells that regulate spermatogenesis; specifically, knowledge remains lacking about the set of steroid activated pathways of steroid hormone action and have been connected with rapid effects, because they do not depend on the activation of RNA transcription or translation into protein and usually involve the activation of protein kinase cascades, mobilisation of intracellular calcium, increased cyclic AMP (cAMP) concentrations and modulation of nitric oxide release, amongst others. Through the fine tuning of all these different mechanisms, steroid hormone receptors may exert particular and tissue-specific effects that depend on genetic, cellular and physiological circumstances.

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5. Alternatively, in the nucleus, hormone-bound receptors may interact with transcription factors, which in turn bind to their responsive elements on the DNA, thereby controlling gene expression. Hormone independent mechanisms may involve steroid-hormone receptor phosphorylation and activation, which is triggered by protein-kinase cascade in response to growth factors binding to their membrane receptor. Phosphorylated steroid receptors enter the nucleus and bind to DNA, regulating gene expression. Steroid hormones may also bind membrane located steroid receptors, triggering the activation of protein-kinase cascades that result in the phosphorylation of transcription factors that bind to their own response elements in the genome, thereby controlling gene expression. There are also steroid hormone effects that do not depend on transcription and protein synthesis, which are usually mediated by intracellular secondary messengers produced in response to the activation of G-protein coupled receptors by steroids. TF, transcription factor; cAMP, cyclic AMP; PKA, protein kinase A; PLC, phospholipase C; IP3, Inositol 1,4,5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C.

Figure 2: Molecular mechanism of the action of steroid hormone receptors. After entering the cell, steroid hormones bind to their specific intracellular receptors, and hormone–receptor complexes are translocated to the nucleus, where they bind to DNA as dimmers modulating gene expression. Alternatively, in the nucleus, hormone-bound receptors may interact with transcription factors, which in turn bind to their responsive elements on the DNA, thereby controlling gene expression. Hormone independent mechanisms may involve steroid-hormone receptor phosphorylation and activation, which is triggered by protein-kinase cascade in response to growth factors binding to their membrane receptor. Phosphorylated steroid receptors enter the nucleus and bind to DNA, regulating gene expression. Steroid hormones may also bind membrane located steroid receptors, triggering the activation of protein-kinase cascades that result in the phosphorylation of transcription factors that bind to their own response elements in the genome, thereby controlling gene expression. There are also steroid hormone effects that do not depend on transcription and protein synthesis, which are usually mediated by intracellular secondary messengers produced in response to the activation of G-protein coupled receptors by steroids. TF, transcription factor; cAMP, cyclic AMP; PKA, protein kinase A; PLC, phospholipase C; IP3, Inositol 1,4,5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C.

genes and signalling mechanisms involved in both classical- and membrane-mediated effects. Detailed research on the mechanism of steroid actions in each particular cell type of the testis may lead to the development of new and more efficient options for the prevention and treatment of male reproductive disorders.

Alternatively spliced variants of ER and AR in the testis

Alternative splicing is a mechanism in which several mRNA transcripts are generated from a single gene giving rise to functionally distinct proteins. Thus, it plays a major role in the generation of proteomic and functional diversity in metazoans. Indeed, more than 50% of human genes undergo alternative splicing, which seems to be a process that is quite prevalent in SSHR genes. The process of alternative splicing involves several distinct mechanisms, including mainly the deletion of exons in addition to the use of ‘intronic’ exons and alternative splice sites (Figure 3).

Several variants of both ERα and ERβ (Table 1) have been found in the testis of vertebrates and, more recently, new exon-deleted ERα transcript variants have been isolated from piscine testis. Many of these transcripts seem to modulate oestrogenic actions and may play a role in the regulation of spermatogenesis. Some transcripts display cell-dependent expression pattern during spermatogenesis, either being absent or restricted to haploid cells. In addition, variants lacking an intact DBD or unaltered ligand-binding capability have been described as dominant negative receptors that block ERα, ERβ or both ERα and ERβ signalling pathways. An alternatively spliced isoform of ERβ lacking exon 5 and predicted to have part of LBD missing displayed ligand-independent nuclear localisation, inhibiting 17β-oestradiol-stimulated transactivation both in ERβ and ERα.
In contrast to the information available about ERs, data about alternatively spliced forms of AR is almost entirely associated with cancer and cases of androgen insensitivity syndrome. To date, limited knowledge about the presence of alternative forms of AR in non-pathological tissues, including testis, exists. However, a recent report by our research group, using a reverse transcription-polymerase chain reaction (RT-PCR) strategy, identified four alternatively spliced isoforms of AR in human testis: i) an isoform with the deletion of exon 2 introducing a premature stop codon, which is expected to result in a truncated protein mainly consisting of NTD, ii) an isoform with an in-frame deletion of 120 nucleotides of exon 4, iii) an isoform with an in-frame deletion of exon 3, which encodes the second zinc-finger in DBD and iv) an isoform lacking exon 2 but retaining 69 bp of intron 2, corresponding to the addition of 23 amino acids and a premature stop codon in the translated product. The partial retention of intron 2 resulted from the use of an alternative splicing site upstream of the standard site. This study also demonstrated the presence of some of these AR variants in evolutionarily distant vertebrate species and in a broad range of human tissues, suggesting a relevant physiological role for the putative protein isoforms that were generated. Moreover, this was the first report to clearly demonstrate that the occurrence of AR splice variants is not restricted to pathological tissues. It is worth noting that until 2011, only one AR variant had been identified in human testis. Table 2 summarises the information about the identified testicular AR splice variants, splicing mechanisms and features of the putative proteins.

Although the precise role of AR variants in the context of androgen signalling in testicular cells requires exploration in future studies, cellular and molecular studies have characterised the function of similar alternative forms of AR found in tissues other than testis. Many of these truncated AR isoforms, even with deletion of part or entire LBD, are constitutively active, serving as ligand-independent transcription factors and being able to promote the transcription of AR-dependent genes. Jagla et al. described a variant with aberrant retention of intron 2 and insertion of 23 amino acids, which, even though devoid of genomic actions, exhibits a cytoplasmic function affecting the activity of transcription factors NF-κB and AP-1. Another curious variant without DBD was described as being localised at the cell membrane, in addition to being required for the optimal transcriptional activity of AR. The AR45 variant, which was detected in the testis, lacks the entire region encoded by exon 1 and generates a protein with DBD, hinge region, and LBD, which are preceded by a short N-terminal region composed of seven amino acid encoded by a short alternative exon (exon 1B). This variant seems to act as a negative regulator of classical AR function, and its mechanism of AR inhibition was suggested to involve the formation of AR–AR45 heterodimers. Moreover, in addition to their identification and structural characterisation, it has been shown that some AR variants have relevant physiological functions in regulating prostate cancer cell growth and in the development of androgen-resistant phenotype.

Recent findings on the identification of new AR and ER variants in the testis have demonstrated an unexplored complexity of steroid hormone actions, opening new lines of research to delineate the ER/AR transcriptome, as well as potentially new signalling pathways.

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Critical review

Table 1  Alternative spliced variants of ERs in the testis of vertebrates

<table>
<thead>
<tr>
<th>Splice variant</th>
<th>Splicing mechanism</th>
<th>Feature</th>
<th>Species</th>
<th>References</th>
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<td>Exon deletion</td>
<td>Deletion of exon 2 with introduction of a premature termination codon</td>
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<td></td>
<td></td>
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<tr>
<td>ERβcx</td>
<td>Alternative 3′ splice site</td>
<td>Receptor lacking part of classical ERβ LBD, which was replaced by a unique 26 amino acid sequence in ERβcx</td>
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<td>35, 36</td>
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<td>ERβ2</td>
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<td>Deletion of exon 8</td>
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<td>32</td>
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<td></td>
<td>Receptor lacking part of LBD</td>
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<td></td>
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<tr>
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<td>Insertion of a specific ERβ4 exon 8</td>
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<td></td>
<td>Receptor lacking part of LBD</td>
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<tr>
<td>ERα isoform S</td>
<td>Alternative utilisation of 5′-untranslated exons</td>
<td>Unidentified 5′-sequence followed by ERα exons 4–8 Receptor without DBD</td>
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<td>33</td>
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<td>Deletion of exon 4</td>
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<td>Receptor without DBD</td>
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<td>Nucleotide insertion</td>
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<td>40, 50, 52</td>
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<td>Receptor with insertion of 18 amino acids in LBD</td>
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<td>Deletion of exon 3</td>
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<td>Receptor with partial deletion of DBD</td>
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<td>ERβ263</td>
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<td>Receptor with partial deletion of DBD</td>
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<tr>
<td>ERαΔ1</td>
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<td>Deletion of exon 1</td>
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<td>53</td>
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<td></td>
<td>N-terminal truncated receptor only with part of NTD</td>
<td></td>
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</tbody>
</table>

h, human (Homo sapiens); r, rat (Rattus norvegicus); sb, seabream (Sparus auratus)

Conclusion

The set of events required to carry out sex steroid hormone effects are staggeringly complex, with both nuclear- and membrane-mediated effects clearly regulating hormonal action. However, the general mechanism involving classical SSRHs is widely recognised in nature. The process of alternative splicing seems to play a major role in the functional diversity and complexity of organisms, ultimately facilitating cell specific functions. The identification of structural variants of both ERs and ARs in healthy testis, as well as in a broad range of vertebrate species, highlights the newly revealed complexity.

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of SSHRs signalling mechanisms in this tissue. Therefore, to develop therapeutic options for male infertility targeting ERs and ARs, it is critical to detail specific localisation in distinct cell types of the testis, determine the relative abundance to wild-type receptors and decipher the cellular functions of these variants.

**Abbreviations list**

AR, androgen receptors; ER, oestrogen receptor; GPCR, G-protein coupled receptor; HREs, hormone response elements; HSPs, heat shock proteins; SSHRs, sex steroid hormone receptors.

**Acknowledgements**

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Critical review


