Nuclear Orphan Receptors Regulate Transcription of the Gene for the Human Luteinizing Hormone Receptor*

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An imperfect estrogen receptor half-site response element direct-repeat, located within the TATA-less promoter of the human luteinizing hormone receptor (hLHR), was identified as an inhibitory site for Sp1/Sp3-driven basal transcription. Isolation of proteins recognizing this site by yeast one-hybrid screening of a human placenta cDNA library revealed three nuclear orphan receptors, EAR2, EAR3/COPUT-TFI, and TR4. Electrophoresis mobility shift assays demonstrated that the in vitro translated nuclear orphan receptors specifically bound the direct-repeat motif of the hLHR promoter. Also, endogenous EAR2 and EAR3/COPUT-TFI from JAR cell and human testis and TR4 from testes bound this motif in electrophoresis mobility shift assays. Functional analyses in CV-1 cells showed that EAR2 and EAR3/COPUT-TFI repressed the hLHR promoter activity by up to 70% in a dose-dependent and sequence-specific manner. Conversely, TR4 activated the hLHR promoter activity up to 2.5-fold through binding to the same cis-element. The stimulation was reversed by coexpression of EAR2 or EAR3/COPUT-TFI, indicating their competitive binding for this site. Such recognition of a common cognate site by the proteins with antagonistic functions implies that a net regulation of the hLHR gene may result from the relative availability of repressors and activator in a physiological state. This also may contribute to the differential expression of the hLHR gene in gonadal and non-gonadal tissues.

The luteinizing hormone receptor (LHR)1 is an essential G-protein-coupled receptor located on the plasma membrane of gonadal cells. It mediates gonadotropin signals and triggers intracellular responses that participate in maturation and function of the gonads as well as the regulation of steroidogenesis and gametogenesis (1, 2). The LHR has also been identified in several non-gonadal tissues, including human nonpregnant uterus, placenta, fallopian tubes, uterine vessels, umbilical cord, brain, and lymphocyte, and in rat prostate (for review, see Ref. 6). Therefore, we propose that one or more of these receptors may bind the EREhs domain and participate in the transcriptional regulation of the hLHR gene expression during differentiation and development cascades.

In this report, we have employed a yeast one-hybrid system to isolate regulatory proteins through binding to a target sequence containing the EREhs domain. Three human nuclear orphan receptors, EAR2, EAR3/COPUT-TFI, and TR4, were cloned and shown to specifically bind the EREhs region in which an imperfect direct-repeat motif was identified as a cognate binding site. Functional analysis showed that EAR2 and EAR3/COPUT-TFI repressed the hLHR promoter activity, whereas TR4 activated hLHR gene transcription. Furthermore, we demonstrated that the inhibition of the hLHR gene in JAR cells was caused by endogenous EAR2 and EAR3/COPUT-TFI proteins binding to the direct-repeat motif.

MATERIALS AND METHODS

cDNA Isolation by Yeast One-hybrid Screening—The MATCH-MAKER One-hybrid System (CLONTECH, Palo Alto, CA) was used to isolate the cDNA encoding for the EREhs domain binding proteins. The preparation of the target reporter constructs, integration of these constructs into yeast Saccharomyces cerevisiae strain YM 4271, isolation of plasmid from each candidate clone, as well as the screening procedures were performed following established protocols recommended by the manufacturer. Basically, 4 tandem copies of 31-bp wild-type DNA frag-
ment from the hLHR promoter (−176 to −145, 5′-GTGCAAGGT-
CAAGCCAGAGCATCGACGGG-3′) containing the EREhs were in-
serted into pHis, pHis-1, and pLacZ yeast reporter vectors. The pHis /
phis-1 plasmids were then linearized and integrated into YM271
chromosome to obtain yeast reporter strains YM-wtERE/His and YM-
wtERE/His-1, respectively. After assessment of leaky His expression
on titrated 3-AT (aminotriazole) (Sigma) plates, the YM-wtERE/His-1
was selected as the candidate strain. Then the pLacZ construct was
linearized and integrated into the YM-wtERE/His-1 strain to obtain YM-
w2ERE/His/LacZ/His-1 dual reporter strain. Another yeast reporter
strain, YM-mERE/His-1, was also created in which mutation at the
ERE half-site (m1) was introduced into the target sequence (5′-
GTCG-
CAATTCAGAGCCAGAGCATCGACGGG-3′), and it was used in
binding selectivity assay. The YM-wtERE/His/LacZ strain was then
transformed with the GAL4 activation domain fused human placenta
cDNA library (CLONTECH) and selected on histidine-deficient plates
containing 30 mM 3-AT. Large colonies from the −His plates were
transferred onto Hybond N filters (Amersham Pharmacia Biotech).

All plasmids were propagated in Escherichia coli DH5α strain, YM-
wtERE/His-1, respectively. After assessment of leaky His expression
in vitro transcription start sites are indicated with arrow. The functional
domains are shown in bold italics, and the putative direct-repeat motif resembling a nuclear orphan receptor cognate site is indicated by horizontal arrows placed under the his1 (EREhs) and his2. An adjacent nonconsensus GREhs is underlined.

The DNA sequence of the 176-bp promoter region of the human LHR gene in
the clones was verified by DNA sequencing. All mutants were verified by sequence
analysis.

For construction of expression plasmids, DNA fragments containing
full-length cDNAs of EAR2 (1.21 kilobases), EAR3/COUP-TFI (1.27
kilobases), and TR4 (1.79 kilobases) were amplified by polymerase chain reaction
from yeast clones 9, 5, and 2, respectively. These three
orphan receptors were cloned into pcDNA 3.1 vector. The fidelity of
expression plasmids was used to synthesize the three nuclear receptor proteins in

In Vitro Transcription and Translation of Nuclear Orphan Receptors—To determine
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on the hLHR gene transcription in JAR- and SV40-transformed placental cells (5). Since putative repressor proteins from the nuclear receptor family may bind to this site and binding selectivity could depend not only on the core EREs sequence but also on the adjacent nucleotides, the sequences surrounding the EREs were also considered. The lack of AT-rich nucleotides preceding the EREs indicated that nuclear receptors that bind to the AGGTCA sequence as monomers were not likely candidate ligands. For these reasons, initial studies were centered on an imperfect direct-repeat motif that contains the consensus EREs (termed as hs1) and a putative imperfect half-site (hs2) 3’ adjacent to the EREs. Luciferase-reporter gene analyses were carried out to evaluate the function of these putative binding sites as well as to address their binding specificity. Wild type hLHR promoter (WT) and constructs with mutated hs1 (m1) and hs2 (m2) were transfected into JAR cells. The luciferase activity of the WT hLHR promoter is shown in Fig. 2. Mutation at either the hs1 (m1) or the hs2 (m2) caused a marked increase of promoter activity by 100%, indicating that both hs1 and hs2 participated in the repression of the hLHR gene promoter. In contrast, mutation of the 3’ adjacent GREhs (see Fig. 1) had no effect. These results demonstrated that the inhibition of hLHR transcription was conferred by the imperfect direct-repeat motif. The required functionality of the two half-sites indicated that the repressor protein(s) likely binds to the cognate site as a dimer. Members of the RAR/thyroid receptor subfamily of the nuclear receptor superfamily including many orphan receptors can bind to a direct-repeat sequence as homodimers or heterodimers (for review, see Refs. 6 and 10). Thus, it was reasonable to assume that one or more of these may contribute to the observed inhibition of the hLHR promoter.

Isolation of Nuclear Receptors through Binding to the hLHR Promoter in Yeast—The yeast one-hybrid system was employed to identify regulatory proteins that specifically bind to the hLHR promoter via the direct-repeat motif. A target sequence containing tandem hs1, hs2, and adjacent sequences (designated as HS) was used to screen a human placenta cDNA library. Nine His+ and LacZ+ dual positive clones were selected from 3.5 million yeast transformants and then verified by restored His+/LacZ+ phenotypes in the subsequent several rounds of transformation into wild-type reporter strain (see “Materials and Methods”). Sequence analysis of the nine positive clones followed by a search in the GenBank identified three human nuclear orphan receptors and two human retinoid acid receptors. Two clones with different lengths of 5’- and 3’-flanking regions encoded the full-length EAR2 receptor. Three clones corresponded to the EAR3/COUPTFI orphan receptor; one of these was full-length, and the others were identical clones lacking 11 amino acids at the N terminus. The third gene was identified as the full-length orphan receptor TR4 in two clones. The other two clones that corresponded to RARα and RXRβ receptors were N-terminus-truncated forms lacking 110 amino acids and 38 amino acids, respectively.

These results suggested that multiple proteins recognized the same HS region, and this was confirmed in binding selectivity assays. A mutant yeast reporter strain in which the direct-repeat motif was disrupted through mutation of the hs1 site (m1) was established. The cDNAs corresponding to the five receptors were transformed into this mutant strain. The complete lack of growth (data not shown) of the transformants in histidine-deficient plates confirmed that we had cloned EAR2, EAR3/COUPTFI, TR4, and RARα and RXRβ through their specific binding to the direct-repeat motif of the hLHR gene promoter.

Characterization of the Binding Specificity of the Nuclear Receptors for the hLHR Promoter in Vitro—The nuclear orphan receptors EAR2, EAR3/COUPTFI, and TR4 belong to the same RAR/TR/orphan receptor subfamily within the nuclear receptor superfamily. These three receptors have been reported to recognize a cis-element that is composed of a direct-repeat of the AGGTCA sequence, with elasticity regarding interspacing and nucleotide differences from the consensus core sequences (11). The initial identification of an imperfect direct-repeat motif as an inhibitory domain of hLHR gene promoter indicated that the hLHR could be transcriptionally regulated by these orphan receptors. EMSAs were therefore carried out to characterize their binding to the hLHR promoter. The receptors were first in vitro translated, and [35S]methionine was incorporated for detection of nascent protein synthesis. Expression of EAR2, EAR3/COUPTFI, and TR4 cDNAs yielded proteins with the expected molecular mass of 42.9, 46.1, and 65 kDa, respectively, whereas no product was detected in mock translation control (not shown).

EMSAs were performed using an oligonucleotide probe corresponding to the HS region of the hLHR promoter (−176 to −145). Incubation of the probe with in vitro translated EAR3/COUPTFI resulted in formation of a single DNA-protein complex, whereas no band was observed when unprogrammed lysate (NP) was used (Fig. 3A, lanes 1 and 2). The complex was eliminated upon the addition of a 100-fold excess of unlabeled wild-type competitor (lane 3), demonstrating its specific binding to the HS region. The identity of the complex was verified by the evidence that it was completely supershifted by an EAR3/COUPTFI antibody, but it was not affected by normal rabbit IgG (lanes 7 and 8). Furthermore, the EAR3/COUPTFI complex was not competed by mutated oligomers m1 or m2 (lanes 4 and 5), indicating that both half-sites (hs1 and hs2) were required for the binding. The results were compatible with the notion that EAR3/COUPTFI is able to recognize a broad range of direct-repeat motifs to which it binds as a stable homodimer (18). In addition, the GREhs, shown not to participate in the repression of hLHR gene (Fig. 2), when mutated, competed for the EAR3/COUPTFI binding as the wild-type DNA (Fig. 3A, lane 6). This further confirmed that EAR3/COUPTFI specifically bound to the imperfect direct-repeat motif of the hLHR promoter.

Similar results were obtained when in vitro translated EAR2 and TR4 were investigated in EMSAs (Fig. 3, B and C). Both
EAR2 and TR4 formed specific complexes with the HS probe (lanes 10 and 18), and these were absent in the controls lanes (lanes 9 and 17). The complexes were abolished by the wild-type competitor but not by oligomers with mutation at either hs1 or hs2 (lanes 11–13 and 19–21). The mutant GREhs inhibited the binding as the wild-type oligomer (lanes 14 and 22). The supershifted bands caused by an EAR2 antibody (lane 15) and a TR4 antibody (lane 23) verified the binding attributed to the expressed receptors. No supershift was observed with normal rabbit IgG (lanes 8, 16, and 24). Thus, the EMSAs illustrate that the nuclear orphan receptors EAR2, EAR3/COUP-TFI, and TR4 specifically bound the hLHR promoter region through the direct-repeat sequence.

In contrast, EMSAs failed to detect RARα or RXRβ binding to the hLHR HS probe. Furthermore, functional analyses in CV-1 cells did not show retinoid acid or 9-cis-retinoid acid-mediated effect on hLHR transcription when the promoter was cotransfected with full-length RARα or RXRβ or both together (data not shown). These results are consistent with the notion that the RAR and RXR retinoid receptors, unlike EAR2, EAR3/COUP-TFI, and TR4, require a restricted spacer for high-affinity binding (10, 12–14). It is therefore evident that RARα and RXRβ were detected in yeast by the highly sensitive system employed in this study through binding to tandem copies of the HS sequence containing the direct-repeat motif with zero spacing (DR0). However, such binding is not present or is too weak (not detected in the EMSA) to mediate a change in the hLHR promoter activity.

Transcriptional Repression of the hLHR Gene by EAR2 and EAR3/COUP-TFI—The specific recognition of the hLHR promoter by the nuclear orphan receptors made it necessary to examine their potential functions in the regulation of the hLHR gene transcription. For these studies, cotransfection assays of the nuclear receptors with the hLHR promoter/luciferase reporter constructs (wild type or mutated hs1 or hs2) were carried out in CV-1 cells. In Fig. 4A, it is shown that EAR2 repressed the hLHR wild-type promoter activity but had no effect on the basic promoter-less construct. Increasing the dose of EAR2 lowered the promoter activity by up to 70%. In contrast, the hs1 mutation (m1) abolished the inhibitory effect of EAR2, indicating that this site was essential for EAR2 to exert its action on the hLHR gene. Furthermore, coexpression of EAR2 did not repress the hs2-mutated construct (m2) but inhibited the activity of the GREhs mutant promoter (m3) to the same extent as the wild-type promoter (Fig. 4B). The results demonstrated that the negative regulation of the hLHR gene by EAR2 depended on the presence of an intact direct-repeat motif but not on the adjacent GREhs. These findings were consistent with those derived from the EMSA binding analysis (Fig. 3). Taken together, it was shown that EAR2 was able to repress the hLHR promoter activity in a dose-dependent and sequence-specific manner.

Similar results were obtained with EAR3/COUP-TFI, which repressed the hLHR promoter activity by up to 55% in a dose-dependent manner. The inhibition was also sequence-specific since the repression was eliminated by mutation of either hs1 or hs2 site but was not affected by the GREhs mutation (Fig. 4, C and D). In the absence of EAR2 or EAR3/COUP-TFI in CV-1 cells, the wild-type hLHR promoter was released from the inhibition, and therefore, the luciferase activity of the hs1- or hs2-mutated construct was not different from the wild-type promoter. The effect of the orphan receptor TR4 on the hLHR promoter activity was next examined in the cotransfection experiments in CV-1 cells. Unexpectedly, cotransfection of TR4 with the wild-type hLHR 176-bp promoter/luciferase construct activated the transcriptional activity up to 2.5-fold in a dose-
dependent manner (Fig. 5A). The activation was abolished by mutation of either half-site (hs1 or hs2), but it was present in cells transfected with the GREhs mutant construct (Fig. 5, A and B). The results show that TR4 is a transcriptional activator of the hLHR gene, and it exerts its function through the imperfect direct-repeat element. Occupancy of the same cognate site by EAR2, EAR3/COUPTFI, and TR4 with apparent opposite functions suggested that they may antagonize each other in the regulation of the hLHR gene. This possibility was tested by coexpression of TR4 with equal amounts of expression plasmid for EAR2 or EAR3/COUPTFI in the presence of the wild-type hLHR promoter. Upon the addition of EAR2 or EAR3/COUPTFI, the TR4-mediated induction was completely abolished, and it was replaced by a repression comparable with the repression exhibited by EAR2 or EAR3/COUP-TFI alone (Fig. 5C). This suggested that the three proteins competitively bound to the same site. Taken together, these results demonstrate that the hLHR gene was subject to transcriptional repression and activation by nuclear orphan receptors.

Transcriptional Repression of the hLHR Gene in JAR Cells Resulted from Endogenous Nuclear Orphan Receptors—The results obtained from cotransfection assays in CV-1 cells demonstrate that nuclear orphan receptors EAR2 and EAR3/COUPTFI potently repressed hLHR gene promoter activity in a sequence-specific manner. The subsequent studies were directed to determine whether the inhibition of hLHR gene in JAR cells was caused by the corresponding endogenous nuclear orphan receptors. EMSAs performed (Fig. 6A) by incubation of JAR cell nuclear extracts with the HS probe revealed the formation of three major DNA-protein complexes (a, b, and c as indicated by arrows, lane 1). The complexes a and b were competed by the 100-fold unlabeled wild-type competitor (WT, lane 2) but remained unchanged in the presence hs1 (m1)- or hs2 (m2)-mutated oligonucleotides (lanes 3 and 4). However, complex c was significantly but not completely abolished by the wild-type oligomer. It was also not fully retained upon the addition of mutated competitors when compared with the complexes a and b. This indicated that the complex c was composed of more than one protein, some of which bound the HS probe nonspecifically. In addition, the GREhs mutant oligomers competed binding of the complexes (lane 5) as the wild-type DNA. The results illustrated two major complexes (a and b), and part of the complex (c) bound strictly to the direct-repeat motif of the hLHR promoter. These findings were similar to the binding specificity results from the EMSAs using in vitro translated orphan receptors. Supershift assays using antibodies directed to the three receptors were carried out to elucidate the identities of the proteins binding to this site. The complex b was supershifted by the EAR2 antibody, and the complex a was supershifted by the EAR3/COUPTFI antibody. The TR4 antibody had no influence on the observed complexes as the normal rabbit IgG (lanes 8 and 10), indicating TR4, did not participate in the regulation of the hLHR gene in JAR cells. This is consistent with the observed marked repression of the hLHR gene through the direct-repeat motif in these cells. A human SF-1 (steroidogenic factor 1) antibody against its DNA binding domain was also included in the supershift assays as a negative control (lane 9). SF-1 recognizes the AGGTCA as its cognate site to which it binds as a monomer, and the binding also strictly depends on the presence of AT nucleotides just preced-
A wild-type hLHR promoter-luciferase construct (WT) was cotransfected into CV-1 cells with increasing doses (0.2, 0.4, and 0.6 μg) of TR4 cDNA. The basic promoter-less vector was used as negative control. B, the WT or hs1 (m1)-, hs2 (m2)-, or GREhs (m3)-mutated hLHR promoter/luciferase construct were cotransfected into CV-1 cells with 0.6 μg of TR4 cDNA. C, the wild-type hLHR promoter/luciferase construct was cotransfected into CV-1 cells with 0.6 μg of TR4 cDNA in the absence or presence of coexpression of equal concentrations of EAR2 cDNA (0.6 μg) or EAR3/COUP-TFI cDNA (0.6 μg). The relative luciferase activities were indicated as the percentage of the wild-type promoter activity (100%) in the absence of nuclear receptors. Results were normalized with β-galactosidase activity and expressed as the mean ± S.E. of three independent experiments in triplicate wells for each cotransfection.

**DISCUSSION**

An imperfect EREhs direct-repeat motif, compatible with the recognition site bound by members of RAR/TR/orphan nuclear receptor subfamily, was identified in this study as an inhibitory element for hLHR gene transcription in JAR cells. This domain specifically bound the nuclear proteins in JAR cells and human gonadal tissues in the EMSA analyses. The isolation of three nuclear orphan receptors, EAR2, EAR3/COUP-TFI, and TR4, from a one-hybrid screening of a human placenta library in yeast indicated that multiple proteins could be involved in the hLHR promoter regulation through this element. EAR2 is a subtype of EAR3/COUP-TFI, and TR4 shares 70% and 50% sequence identity with EAR3/COUP-TFI at DNA binding domain and putative ligand binding domain, respectively (15). The conserved DNA binding domain of these orphan receptors confers their ability to recognize an identical/similar cognate element that is composed of a direct-repeat (DR) of AGGTCA core sequence with a spacer of variable length. The binding of these orphan receptors to the imperfect DR0 motif of the hLHR promoter was shown to be strictly mediated through the two half-sites (hs1 and hs2).

Members of the orphan receptor subfamily have been found to play an important role in gonad and brain development (16–18). Therefore, it is of major significance that they are
recognized as participants in the transcriptional regulation of the hLHR gene. Functional analysis demonstrated that EAR2 and EAR3/COUP-TFI repressed the hLHR promoter activity in a dose-dependent and sequence-specific manner. EAR2 and, particularly, EAR3/COUP-TFI are generally recognized as repressor proteins regulating an array of different target genes (for review, see Ref. 19). The mechanism of their repression includes active inhibition of basal or activated transcription, quenching a transactivator-regulated transcription, and transrepression (20). The inhibition of the hLHR gene appears to result from the active silencing function of EAR2 and EAR3/COUP-TFI rather than competition with, quenching, or titrating out a hormonal partner (14, 21, 22).

EMSA with JAR cell nuclear extracts revealed that EAR2 and EAR3/COUP-TFI antibodies caused supershift of two DNA-protein complexes of distinct migrating properties, indicating that recruitment of other cofactors via protein-protein interactions may participate in the regulation of hLHR gene transcription. EAR2 and EAR3/COUP-TFI were reported to interact directly with TFIIH, a component of basal transcriptional machinery (23). Their repressive functions were found enhanced by interaction with two common nuclear receptor corepressors, NcoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor (24)). Furthermore, EAR3/COUP-TFI has been shown to interact directly with Sp1 protein, independent of the EAR3/COUP-TFI binding site, to enhance the activation of target gene transcription (25, 26). It still remains to be determined whether EAR3/COUP-TFI-mediated repression of the hLHR promoter activity requires a direct interaction between EAR3/COUP-TFI and Sp1 protein. However, if such interaction occurs, it would involve EAR3/COUP-TFI binding to its cognate site, since our cotransfection studies with the h1- or h2-mutated constructs did not reveal the additional increase on the promoter activity over what was observed by exclusion of the inhibitory effect of the EAR3/COUP-TFI (Fig. 4B). The repression of the TATA-less hLHR gene by EAR2 and EAR3/COUP-TFI is probably achieved by direct or corepressor-bridged interaction with one or more components of the basal transcriptional machinery or, alternatively, by direct interaction with Sp1 to perturb the Sp1-driven hLHR gene transcription.

In contrast to the actions of EAR2 and EAR3/COUP-TFI, the nuclear orphan receptor TR4 was demonstrated to be a transcriptional activator of the hLHR promoter through the same direct-repeat motif (DR0). To our knowledge, this is first demonstration that TR4 regulates target gene transcription through a DR0 direct-repeat cis-element. It has been reported that TR4 up-regulated the rat α-myosin heavy chain and S14 genes through a DR4 motif, whereas it inhibited the SV40 gene through a DR2 and squelched EAR2/RXR-mediated stimulation of several genes through the DR1/DR5 element (14, 27, 28). In addition, it has been recently proposed that TR4 may adopt different conformations once bound to a DR3 vitamin D response element (DR3VDR) or a DR4 thyroid hormone response element (DR4T3RE). Such change would allow TR4 to recruit different coregulators and, hence, to function as a repressor through DR3VDR or as an activator through DR4T3RE (21). It is proposed that the induction of the hLHR gene by TR4 through the DR0 element will be dependent on the context of its full promoter region, taking into account the possible participation of coactivators. Coactivator proteins as transcriptional mediator/intermediary factor 2, steroid receptor coactivator 1 (SRC-1), and human receptor-interacting protein 140 (RIP140) have been found to interact with several nuclear receptors (for review, see Ref. 29). However, it remains to be determined whether such interaction is present in TATA-less genes and involved in the TR4-mediated induction of the hLHR gene. In JAR cells, the hLHR promoter appeared to be regulated solely by EAR2 and EAR3/COUP-TFI, since no DNA-TR4 complexes were observed in the EMSA. However, such complexes, including TR4, are readily observed in gonadal nuclear proteins, indicating that dual regulation of the hLHR gene by orphan receptors with opposite functions could occur in these tissues.

Cotransfection of TR4 with EAR2 or EAR3/COUP-TFI converted the marked activation of the hLHR promoter induced by TR4 into an inhibitory effect conferred by EAR2 or EAR3/COUP-TFI through their competitive occupancy of the same binding site. Therefore, binding of the hLHR promoter by these nuclear orphan receptors with apparent opposite functions implies that the net outcome of the hLHR gene transcription could be determined by the relative availability of the repressors (EAR2, EAR3/COUP-TFI) and the activator (TR4) at a physiological or pathological stage. Such a mechanism provides a mode for regulation of the Sp1-driven hLHR gene expression in various states of differentiation and development.

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