# Chapter 11

# Use of Reporter Genes to Study the Activity of Promoters in Ovarian Granulosa Cells

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## Abstract

Use of reporter genes provides a convenient way to study the activity and regulation of promoters and examine the rate and control of gene transcription. Many reporter genes and transfection methods can be efficiently used for this purpose. To investigate gene regulation and signaling pathway interactions during ovarian follicle development, we have examined promoter activities of several key follicle-regulating genes in the mouse ovary. In this chapter, we describe use of luciferase and β-galactosidase genes as reporters and a cationic liposome mediated cell transfection method for studying regulation of activin subunit- and estrogen receptor α (ERα)-promoter activities. We have demonstrated that estrogen suppresses activin subunit gene promoter activity while activin increases ERα promoter activity and increases functional ER activity, suggesting a reciprocal regulation between activin and estrogen signaling in the ovary. We also discuss more broadly some key considerations in the use of reporter genes and cell-based transfection assays in endocrine research.

**Key words:** Reporter genes, luciferase, β-galactosidase, promoter activity, estrogen, estrogen receptor, activin.

## 1. Introduction

There are many approaches to investigate gene expression in eukaryotic cells. Ultimately, one is often interested in the levels, modification, localization, or activity of the protein encoded by the gene of interest, as such measurements are perhaps most closely correlated with the biological response. However, in many cases, it is useful to measure levels of the RNA transcript, for example between cell or tissue types, in response to a stimulus, or over time. Thus, many assays for RNA measurement have been developed, including S1 nuclease protection, primer extension, Northern blot hybridization,

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and RT-PCR. However, these assays all measure mRNA transcript levels which are, of course, affected by both the rate of transcription and the rate of mRNA degradation (and potentially by other steps including RNA processing and transport). To investigate directly the rate of gene transcription, an important assay is nuclear run-on transcription, which allows transcription to continue in isolated nuclei and then hybridizes labeled transcripts to known DNA for identification (1, 2). This assay provides a direct way to assess which genes are active in a given cell and examine the effects of environmental conditions on gene transcription (3, 4). Using this assay, gene transcription occurs largely in its native structural and cellular context. However, some paused RNA polymerases may be artificially activated thus leading to incorrect interpretation of the results (5). Isolation of purified nuclei can also be technically difficult for some cells (6). A complementary assay is the measurement using RT-PCR of unspliced nuclear pre-mRNA precursors using primers that span introns (7). While both are very valuable assays and give fairly direct measurements of gene transcription, they are complex assays most often utilized in specialized circumstances.

An alternative way to indirectly measure gene transcription is to study gene promoter activities using reporter genes, where a reporter is placed next to and controlled by the promoter region of a gene of interest and the accumulation of the reporter gene mRNA or protein product is measured as an indicator of the promoter activity. Mutations can also be introduced into the promoter region to identify important regulatory elements, and ultimately, transcription factors controlling its activity.

A reporter gene encodes a product that can be easily and quantitatively measured. Examples of common reporter genes include the gene that encodes jellyfish green fluorescent protein (GFP) which emits green fluorescent light when excited by appropriate wavelengths (8, 9), the luciferase genes that encode firefly or Renella luciferases which catalyze a reaction in which the substrate luciferin is acted upon to produce light (10, 11), the *LacZ* gene that encodes the enzyme β-galactosidase which catalyzes a reaction in which the substrate analog X-gal gives a blue colored product (12) or 1,2-dioxetane substrates produce light (13–16), the bacterial gene that encodes the enzyme chloramphenicol acetyl transferase (CAT) whose enzymetic activity can be measured with thin layer chromatography or immunological assays, and the human growth hormone gene that encodes growth hormone which can be secreted into the culture medium and measured by radioimmunoassay (17). There are many others that can be used in the appropriate context. A reporter gene normally is not endogenously expressed or only expressed at a very low level. Although endogenous β-glactosidase activity can be detected in most mammalian cell lines, through manipulating assay conditions, the endogenous β-glactosidase activity can be minimized.

The luciferase gene and the *LacZ* gene have been widely used as reporter genes to study promoter activities due to the fact that they can be quickly translated into proteins, these proteins are enzymes which allow for amplification of the signal, the enzymatic activity can be measured with simple, highly sensitive, and quantitative assays, and no radioactive materials are involved. In addition, luciferase has a fast turn-over rate, which reduces accumulation in an inducible system and provides a more dynamic view of gene transcription (18). In this chapter, we describe use of these two reporter genes to study promoter activities in endocrine systems.

A promoter-reporter DNA construct is most often produced and propagated using bacterial plasmids. The plasmid DNA can be introduced into mammalian cells through transient or stable transfection (19). The former is widely used as an initial testing step and is described in this chapter. If a promoter does not show strong activity in transiently transfected cells, stable transfection may be considered. There are numerous ways of transfecting cells with DNA, including calcium phosphate precipitation, lipid vesicle encapsulated DNA transfer, and electroporation (20). We have been using a cationic liposome mediated transfection method to study promoter activities in ovarian granulosa cells. This process involves encapsulating DNA with lipid, transfecting cells with the DNA/lipid mix, collecting cell lysates, and performing luciferase or β-galactosidase assays to quantitatively measure promoter activity (**Fig. 11.1**).

Using reporter genes to study the activity of promoters is convenient, sensitive, and reliable. It is however important to understand that this assay is an indirect measurement, and it assumes that the amount of reporter gene product is a reasonable indicator of transcription rate and therefore promoter activity. This assumption is justified if reporter mRNA turnover or translational rate is not affected by the treatment conditions being employed. Using reporter genes to study the activity of promoters may also introduce bias due to the incorporation of foreign elements from the vector or elimination of intrinsic elements from the endogenous gene within the test genes (21). Therefore, a particular promoter-reporter system needs to be carefully evaluated and validated before use. Indeed, this approach is perhaps best applied once other methods like nuclear run-on have been used to verify that what is being studied is actually a transcriptional effect. Reporter gene assays then allow this transcriptional effect to be studied in greater detail. Finally, transfection efficiency, cell viability, and sample recovery play a critical role in interpreting results; therefore, internal controls need to be included in each assay. Although not described in detail here, for internal control purposes, dual-reporters are commonly employed, where one plasmid containing an experimental reporter gene is co-transfected with another plasmid containing a distinct control reporter gene whose activity can be measured using a different assay. The experimental reporter gene is normally driven by a promoter of interest and the control

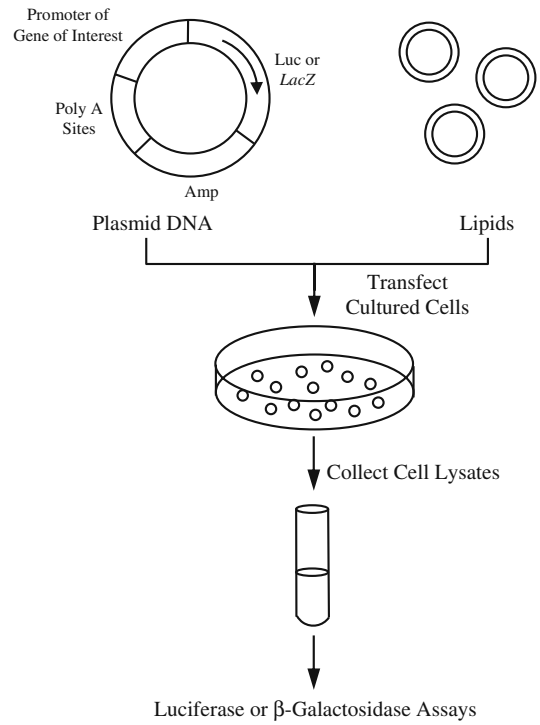


Fig. 11.1. Flow chart showing the use of reporter genes and cationic liposome-mediated cell transfection to study promoter activity. Plasmid DNA is mixed with a liposome suspension. Cells are transfected via incubating with the DNA/lipid mix. Cell lysates are then collected and luciferase or β-galactosidase assays are performed to measure promoter activity. Luc: luciferase reporter gene that encodes firefly luciferase. *LacZ*: *LacZ* reporter gene that encodes β-galactosidase. Amp: gene for ampicillin resistance in *E. coli*. *Arrow* within the Luc gene shown in the circular plasmid indicates the direction of transcription.

reporter is normally driven by a constitutive viral promoter whose activity is not affected by the experimental conditions (19, 22). Although the dual-reporters can be a combination of any two distinct reporter genes, the dual-luciferase reporter system, where firefly luciferase is used as an experimental reporter and Renilla luciferase is used as a control reporter, allows detection of both reporters using the same assay but with different substrates (22).

We are interested in investigating interactions between the signaling pathways involving the steroid hormone estrogen and the TGF-β superfamily member activin. We have demonstrated that estrogen suppresses activin subunit expression and decreases activin signaling in the early mouse ovary (23), while activin stimulates estrogen receptor α and estrogen receptor β (ERα and ERβ) expression (24). To further examine if this reciprocal regulation is at the level of gene transcription and to confirm an effect of activin on functional ER activity, we used reporter genes to study the promoter activities of the activin subunits and ERα as well as the activation of

estrogen response element (ERE). We have shown that estrogen suppresses activin subunit gene promoter activities while activin increases ERα promoter activity and increases functional ER activity (23, 24). Select examples from this work related to the use of reporter genes will be used for illustrative purposes in presenting the specific methods used in these cell-based transfection assays.

## 2. Materials

### 2.1. Cell Culture

1. CD-1 mice at 21–23 day of age for primary granulosa cell culture.
2. GRMO2 cells: from a mouse granulosa cell line provided by N.V. Innogenetics, Ghent, Belgium (25, 26).
3. Medium for primary cell culture (4F medium): phenol red-free D-MEM/F-12 medium (Invitrogen Corporation, Grand Island, NY) supplemented with 2 μg/ml insulin, 5 nM sodium selenite, 5 μg/ml transferrin, 0.04 μg/ml hydrocortisone, and 50 μg/ml sodium pyruvate. To obtain 4F medium containing serum, add 10% charcoal/dextran treated fetal bovine serum (Hyclone, Logan, UT) (see **Note 1**).
4. Pre-incubation medium: 0.5 M sucrose and 10 mM EGTA in 4F medium (without serum).
5. Medium for GRMO2 cell culture (HDTIS medium): phenol red-free D-MEM/F-12 medium (Invitrogen Corporation, Grand Island, NY) supplemented with 5 μg/ml insulin, 5 nM sodium selenite, 10 μg/ml transferrin, 50 μg/ml sodium pyruvate, and 2% charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, UT) (*see* **Note 1**).
6. 12-well (# 92012, Techno Plastic Products Ltd., Zurich, Switzerland) or 24-well tissue culture plates (#662-160, Greiner Bio-One, Monroe, NC).
7. Cell strainer (#352340, 40 μm, BD Falcon, Bedford, MA).

### 2.2. Lipid and Transfection

1. Plasmid DNA: DNA construct that contains a promoter fragment of a gene of interest linked to a luciferase reporter gene or a β-galactosidase reporter gene.
2. Liposomes: Dissolve 100 mg of DDAB (Dimethyldioctadecylammonium bromide) (Sigma # D-2779. Powder stored at room temperature) in 1 ml chloroform. Add 100 μl of PtdEtn (L-a-phosphatidylethanolamine, dioleoyl[C18:1, cis9]) (Sigma #P-0510. Supplied in 10 mg/ml chloroform, stored at −20°C) and 4 μl of DDAB solution in a microcentrifuge tube. Dry in a speed vac and store at −20°C (*see* **Note 2**) (27).

3. Liposome suspension: Resuspend the liposome pellet in 101 µl of 100% ethanol. Take 1 ml of sterile distilled water in a 15 ml polystyrene tube. Pipette 50 µl of the 101 µl resuspended pellet into the water while vortexing the tube at medium speed (27). Store at 4°C and can be used for up to 4–5 months.
4. Polystyrene tubes (#2058 Falcon 5 ml tubes and #2057 Falcon 14 ml tubes) (*see* **Note 3**).
5. Phenol red-free Opti-MEM: #11058-021, Invitrogen Corporation, Grand Island, NY (*see* **Note 4** for preparation of regular phenol red containing Opti-MEM).

### 2.3. Transfection Efficiency Evaluation

1. Poly-L-Lysine: #P4832, SIGMA, St. Louis, MO. Store at 2–8°C.
2. Microscope cover slips (12 mm, circle): #12-545-80, Fisher Scientific, Pittsburgh, MA.
3. Fixative: prepare a 1% (w/v) paraformaldehyde solution in PBS fresh before use. To dissolve paraformaldehyde in PBS, heat at low to medium settings (avoid boiling) and then cool to room temperature.
4. Pmax green fluorescent protein (pmax GFP) control DNA is obtained from Amaxa (Cologne, Germany) (*see* **Note 5**).
5. DAPI mounting medium (Vector Laboratories, Inc.).
6. Fluorescent microscope (Leica DM 5000B).

### 2.4. Luciferase Assay and β-galactosidase Assay

1. Luciferase assay cell lysis buffer: 25 mM HEPES (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol (DTT), and 0.1% Triton X-100 in $ddH_2O$.
2. Luciferase assay reaction buffer: 25 mM HEPES, pH 7.8; 15 mM $MgSO_4$, 4 mM EGTA, 2.5 mM ATP, 1 mM DTT and 1 µg/ml BSA in $ddH_2O$ (*see* **Note 6**).
3. Luciferin: aliquot 10 mM luciferin stock solution (sodium salt) (Analytical Bioluminescence Lab, San Diego, CA) into 500 µl aliquots and store at −20°C.
4. β-galactosidase assay kit: Galacto-Light Plus Systems kit (Applied Biosystems, Bedford, MA).
5. Luminometer (Analytical Luminescence Laboratory Monolight 2010).
6. Luminometer cuvettes (#556862, 12 × 75 mm, BD Biosciences Pharmingen).
7. Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Richmond, CA).
8. Teflon cell scrapers (Fisher).

## 3. Methods

Use of reporter genes provides a convenient way to study the function and regulation of promoters. Herein we describe a cationic liposome mediated transfection method to study regulation of activin subunit- and ERα-promoter activities as well as activation of a synthetic ERE in primary cultured granulosa cells and in a mouse granulosa cell line, GRMO2 cells. Using this transfection method, DNA plasmids are encapsulated by cationic liposomes. The cationic liposomes can be attracted to negatively charged cell surface where they can fuse with plasma membranes and thus lead to uptake and expression of the DNA in transfected cells (20).

For most mammalian cells, transfection efficiency is generally poor. However, this can be optimized by varying DNA concentration, transfection reagent, and transfection time. The optimal procedure or reagent for transfection varies between cell lines or primary cell types. The protocol described in this chapter has been optimized for transfecting mouse granulosa cells and it has also been successfully used with many other cell types, including 293/293T cells, 3T3 cells, and JEG3 cells. To optimize the transfection method and to facilitate the interpretation of experimental results, a protocol for evaluating transfection efficiency is also detailed below. For experimental control purposes, each transfection assay should include a mock transfection negative control (no plasmid), an empty vector negative control (omit promoter), and a constitutively active viral promoter positive control.

To further analyze promoter function and identify important regulatory elements within a promoter and ultimately, the transcription factors that occupy these sites, 5' deletion mapping and site-directed mutagenesis can be utilized. The flow chart shown in **Fig. 11.2** outlines the basic steps to generate 5' deletion mutations of a promoter at different lengths and plasmids containing these promoter fragments. The plasmids can then be transfected into cells to study changes in promoter activity. To make site-directed mutations, we generally use a QuickChange site-directed mutagenesis kit (Stratagene). A detailed protocol can be found in the manufacturer's instruction manual, and this technique is therefore not included in this chapter.

Using luciferase or *LacZ*as reporter genes, we have studied the effect of estrogen on activin βA and βB promoter activities, the effect of activin on ERα promoter activity and the effect of activin on ERE activation, and shown a negative regulatory feedback system between these two important signaling pathways.

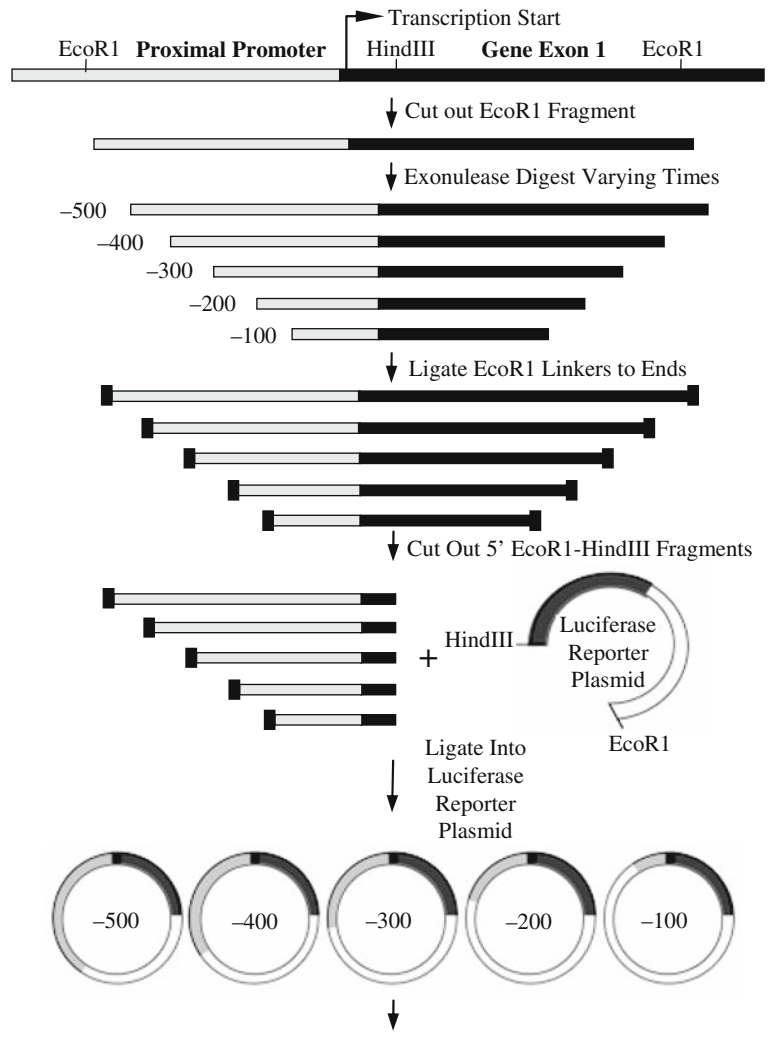


Fig. 11.2. Flow chart showing the basic steps to generate 5' deletion mutations. The plasmids containing the promoter fragments can then be transfected into cells to study the activity of the different promoter fragments. Although not shown here, rather than non-specific exonucleases, termini are often defined by unique restriction enzyme recognition sites.

### *3.1. Preparation of Primary Cultured Granulosa Cells and GRMO2 Cells for Transfection*

Granulosa cells are collected through follicle puncture as reported previously by our laboratory in rats (28–32) and in mouse (24). Preparation of mouse cells is described below.

1. CD-1 mice are sacrificed on postnatal days 21–23. Ovaries from 5–10 animals are rapidly dissected out and collected into a 10 cm petridish containing cold 4F medium.
2. Dissect out fat and oviduct under a dissection microscope. Transfer ovaries to a clean 10 cm petridish and wash the ovaries twice with cold 4F medium.

3. Transfer the ovaries into a 15 ml Corning tube containing 10 ml pre-incubation medium and incubate at 37°C for 30 min, or until the ovaries sink (*see* **Note 7**). The purpose of this step is to disrupt gap junctions and improve cell integrity and yield (33).
4. After the ovaries have gravitated to the bottom of the tube, wash ovaries 2–3 times with cold fresh medium and transfer the ovaries along with 5 ml medium to a 10 cm petridish.
5. Use a 25 gauge needle to hold an ovary steady and use another needle to puncture follicles. Release of granulosa cells from follicles can be clearly observed under a dissection microscope. Continue puncturing until no more granulosa cells are extruded. Discard the punctured ovary and move on to a fresh one.
6. After all the ovaries are punctured and discarded, collect the extruded granulosa cells from the petridish into a 15 ml Corning tube under a cell culture hood. Centrifuge the cells at 150*g* for 5 min. Aspirate the medium and resuspend cells in 5 ml of 4F medium. Centrifuge again and aspirate the medium.
7. Repeat this wash once with 4F medium and once with 4F medium containing serum. Resuspend the final cell pellet in 1–2 ml of 4F medium containing serum. Filter out oocytes and small follicles with a 40 μm cell strainer.
8. Count viable cells using Trypan-blue exclusion and plate approximately $5 \times 10^5$ cells per well in 12-well plates or $2 \times 10^5$ cells per well in 24-well plates in 4F medium containing serum.
9. Cells are cultured in a humidified incubator at 37°C and 5% $CO_2$, and they normally attach to the bottom of the plates within 1 or 2 days.
10. If GRMO2 cells are to be used, cells are thawed and cultured in 12-well plates in a humidified incubator at 37°C and 5% $CO_2$ in HDTIS medium.
11. Start transfection when the cells are about 50–70% confluent.

### 3.2. Transfection

1. After 3- days of culture in the estrogen-deprived condition and when the cells reach the desired confluency, primary cultured granulosa cells or GRMO2 cells are transiently transfected with DNA constructs using cationic liposomes in a phenol red free Opti-MEM medium. The DNA constructs used here are either an activin βA subunit promoter-luciferase reporter construct (34), an activin βB subunit promoter-luciferase reporter construct (28), a 2x estrogen response element (ERE)—luciferase reporter construct (kindly provided by Dr. Larry Jameson from Northwestern University (35)), or a mouse ERα-promoter-β-galactosidase construct (kindly provided by Dr. Alessandro Weisz from Seconda Università degli Studi di Napoli, Italy (36)).

2. Transfection reagent is prepared as listed in **Table 11.1**. Typically, we use 0.25 μg DNA per well for 24-well plates and 0.5 μg DNA per well for 12-well plates. First, the DNA mix and lipid mix are prepared separately by combining appropriate amount of DNA or lipid with an equal amount of OptiMEM. Second, these two mixes are combined and incubated at room temperature for at least 20 min. Finally, the combined mixes are diluted with a larger volume of OptiMEM (see **Table 11.1**). This final mix is used to transfect cells.

**Table 11.1**
**Preparation of transfection reagent**

| | DNA Mix | | Lipid Mix | | | |
|---|---|---|---|---|---|---|
| Plate Size | DNA per well (ug) | OptiMEM (ul) | Lipid per well (ul) | OptiMEM (ul) | Diluent OptiMEM (ul) | Final Volume (ml) |
| 24 well | 0.25–1 | 25 | 5 | 25 | 200 | ~ 0.25 |
| 12 well | 0.5–2 | 50 | 10 | 50 | 400 | ~ 0.5 |
| 6 well | 2–5 | 100 | 15 | 100 | 800 | ~ 1 |
| 6 cm | 5–10 | 200 | 25 | 200 | 1600 | ~ 2 |
| 10 cm | 10–20 | 400 | 45 | 400 | 3200 | ~ 4 |

3. If co-transfection of a promoter-reporter gene construct together with a particular gene-expression construct is desired, add 0.025–0.05 μg of the gene-expression construct to the above DNA mix. Total DNA contents need to be normalized in all samples with appropriate vectors.
4. Aspirate medium from cells and wash once with PBS. Add the transfection mix to each well and incubate in a humidified $CO_2$ incubator for 6 h (*see* **Note 8**).
5. After 6 h transfection, the transfection mix is aspirated and cells are maintained in fresh culture medium for 14–16 h (overnight) to recover. Transfection efficiency evaluation or luciferase/β-galactosidase assays can then be performed. In our studies, after recovery, fresh medium containing various hormonal treatments (see result figures later) is given to the cells for an additional 24 h to examine effects of those treatments on the promoter activities.

### 3.3. Transfection Efficiency Evaluation

1. Lysine coat coverslips: heat coverslips in a loosely covered glass beaker in 1 M HCl at 50–60°C for 4–16 h; cool and wash coverslips extensively in $dH_2O$ and then $ddH_2O$; rinse coverslips in 100% ethanol and leave to dry; coat coverslips in bulk in 10–15 ml 1 mg/ml poly-L-lysine with gentle shaking for 30 min in a 10 cm petridish; wash the coverslips in $dH_2O$ and then $ddH_2O$ at least 5 changes in each; rinse coverslips in 100% ethanol and let dry before use (*see* **Note 9**).
2. Plate cells in 24-well plates with a lysine-coated cover slip on the bottom of each well. Transfect cells with pmaxGFP control DNA (0.5 μg/μl, use 0.5 μl for each well) for 6 h following transfection protocol described above.
3. After overnight recovery, aspirate medium and rinse cells briefly with PBS. Add 0.5 ml of 1% paraformaldehyde to each well and fix cells at 4°C for 30 min. Aspirate the fixative and rinse cells with PBS once. Add several drops of PBS to each well so the cover slips are easy to pick up with a forceps.
4. Each slide can accommodate three 12 mm diameter circle coverslips. Add three drops of DAPI mounting medium on a slide and make sure that they are separated enough, then put one coverslip on each drop, cell side facing down. Use a Kimwipe to absorb excess DAPI mounting medium and seal with nail polish.
5. Let nail polish dry and take pictures from randomly selected 10–20 fields using a fluorescent microscope. Count the number of transfected cells (GFP, green) and the total number of cells (DAPI nuclear stained, blue) in each field, calculate transfection efficiency by dividing the number of transfected cells by the total number of cells, and then average the results from all fields. Examples in **Fig. 11.3** show that in a particular field, 6 out of 16 primary cultured granulosa cells were transfected and 9 out of 53 GRMO2 cells were transfected. The final transfection efficiency was 26% for primary cultured granulosa cells and 6% for GRMO2 cells after averaging results from 15 different fields.

### 3.4. Luciferase Assay and β-Galactosidase Assay

1. For luciferase assay, prepare cell lysis buffer as described in Materials.
2. Aspirate medium from wells and wash cells 1–2 times with cold PBS. Add 150 μl of lysis buffer to each well (12-well or 24-well culture plates). Incubate on ice for 20–30 min with gentle shaking.

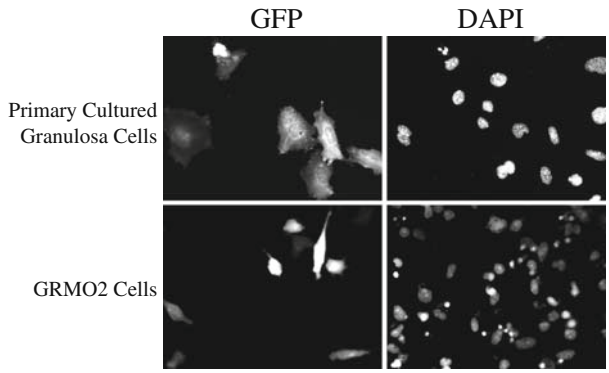


Fig. 11.3. Examples for transfection efficiency evaluation in primary cultured mouse granulosa cells and in GRMO2 cells. Using the cationic liposome-mediated cell transfection method described in this chapter, cells were transfected with 250 ng per well pmax GFP control DNA in 24-well plates. Cells were then fixed and pictures were taken under a fluorescent microscope. GFP: shows cells that were transfected and gave green fluorescent light. DAPI: shows the total number of cells that were nuclear stained with DAPI. In the particular fields shown here, 6 out of 16 primary cultured granulosa cells were transfected and 9 out of 53 GRMO2 cells were transfected. The final transfection efficiency was 26% for primary cultured granulosa cells and 6% for GRMO2 cells after averaging results from 15 different fields.

3. Tilt the plates, scrap the bottom of each well with cell scrapers, and collect lysates into chilled eppendorf tubes. Store the collected lysates on ice until ready to use or store at −80°C for later use (*see* **Note 10**).
4. Dilute 10 mM luciferin to 1 mM in $ddH_2O$ and prepare luciferase assay reaction buffer as described in Materials. Aliquot 400 µl of reaction buffer into luminometer cuvettes.
5. Set the luminometer so that it injects 100 µl of substrate (luciferin) and collects data for 10 s (see **Note 11**).
6. Centrifuge cell lysates at maximal speed using a table top centrifuge before use to remove cell debris. Add 100 µl of cell lysates to 400 µl of reaction buffer, immediately inject 100 µl of 1 mM luciferin using the automatic injector, and measure emitted luminescence using the luminometer for 10 s.
7. For β-galactosidase assay, a Galacto-Light Plus Systems kit is used following the manufacturer's instructions.
8. Use the remaining cell lysates to measure protein concentration in each sample with the Bio-Rad protein assay reagent: add 5–10 µl of cell lysate to 100 µl diluted Bio-Rad assay reagent in a 96-well plate and read absorbance at 595 nm wave length with a 96-well plate reader.

9. Normalize relative light units obtained from luciferase assay or β-galactosidase assay with the protein content in each sample (*see* **Note 12**). The normalization allows one to control possible variations in the number of cells, cell growth rate and recovery of samples. Example results using a luciferase reporter gene to study effect of estrogen on activin βA and βB subunit promoter activities are shown in **Fig. 11.4**. Example results using *LacZ* reporter gene to study effect of activin on ERα promoter activity are shown in **Fig. 11.5**. Example results using luciferase reporter gene to study effect of activin on ERE activation are shown in **Fig. 11.6**.

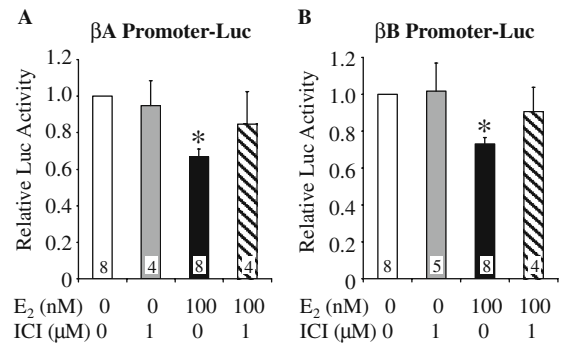


Fig. 11.4. Effects of $E_2$ and ICI 182, 780 on activinβA- and βB-promoter activities in transfected GRMO2 granulosa cells. All the treatments were given for 24 h. The numbers at the bottom of each bar indicate replicate experiments. Each measurement was done in triplicate. *P $<$ 0.05 (Reproduced from **ref.*22*** with permission from *The Endocrine Society*).

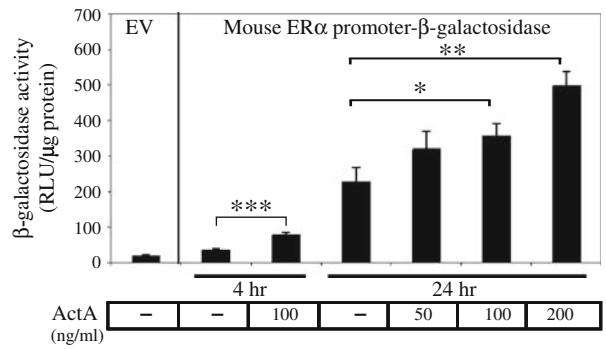


Fig. 11.5. Activin A increases ERα promoter activity. GRMO2 cells were deprived of estrogen for 3 days before being transfected with a mouse ERα promoter-β-galactosidase construct or empty vector (EV) overnight and then treated with activin A (ActA) at indicated doses for 4 or 24 h. Results are the average of three independent experiments each performed in triplicate. *P $<$ 0.05, **P $<$ 0.01, ***P $<$ 0.001 (Reproduced from **ref.*23*** with permission from the *Journal of Biological Chemistry*).

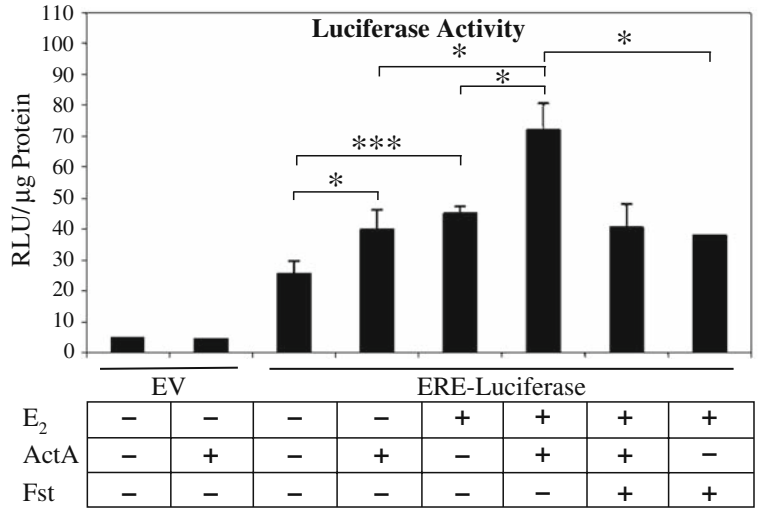


Fig. 11.6. Activin A increases ERE response to $E_2$. Primary granulosa cells were deprived of estrogen for 3 days before being transfected with an ERE-Luciferase construct or empty vector (EV) overnight. Transfected cells were then treated with activin A (ActA, 100 ng/ml), follistatin (Fst, 400 ng/ml), or a combination of these two compounds for 4–8 h followed by addition of $E_2$ (100 nM) or ethanol for another 24 hrs $n = 5$. *$P < 0.05$, ***$P < 0.001$ (Reproduced from **ref.*23*** with permission from the *Journal of Biological Chemistry*).

## 4. Notes

1. Estrogen-free culture conditions are used to minimize any interference from this steroid as this study measures estrogen effects as well as ER levels. If the estrogenic properties of phenol red containing culture medium or of serum are not a concern, use regular D-MEM/F-12 medium and regular fetal bovine serum.
2. Alternatively, a commercially available product such as Lipofectamine (Invitrogen) can be used and transfection can be performed following its manufacturer's instructions, although the homemade liposome preparation described in this protocol is very cost efficient.
3. Polystyrene tubes are the clear ones, not the opaque ones. The opaque ones are polypropylene tubes and lipids tend to adhere to the walls of this kind of tube.
4. Prepare regular Opti-MEM if the estrogenic effects of phenol red are not a concern: dissolve 13.6 g of Opti-MEM I powder (# 22600-050, Invitrogen Corporation, Grand Island, NY) in 1L of $ddH_2O$, add 2.4 g $NaHCO_3$, pH to 7.3, filter, and store at 4°C.

5. Although we use the pmax GFP control DNA from Amaxa, any other GFP-encoding plasmid will work.
6. Leave the ATP aliquot on ice and add it to the reaction solution just before use to minimize degradation at room temperature.
7. Incubation for 30 min is normally enough. Prolonged incubation (>45 min) can cause significant loss of cells due to cell death.
8. Transfection time may vary between 6 and 16 h depending on use of cell types and transfection reagents. Prolonged transfection times may cause cell toxiticity.
9. Treatment with HCl helps cells and polyaminoacids stick to glass. After acid treatment, coverslips can be stored for 1 year without polyaminoacid coating. After polyaminoacid coating, coverslips can also be stored for 1 year and rinsed with 100% ethanol before use. Polyaminoacid can be saved at −20°C and reused for 3–4 times.
10. We have observed that freezing the lysates at −80°C for at least 1 hr before use gives more consistent results between experiments.
11. The amount of reaction buffer and substrate used can be scaled down if desired or if using a different type of luminometer or luminometer cuvettes.
12. Relative light units can also be normalized with an internal luciferase control if using a dual-luciferase reporter assay system, or with another reporter gene expressed from a control constitutive promoter.

## References


1. Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M., Darnell, J.E. Jr. (1981) Transcriptional control in the production of liver-specific mRNAs. *Cell* **23**:731–739.
2. Greenberg, M.E., Ziff, E.B. (1984) Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature* **311**:433–438.
3. Weaver, R.F. (1999) Molecular Biology. 1st ed: McGraw-Hill Companies, Inc.
4. Hu, Z.W., Hoffman, B.B. (2000) Nuclear run-on assays for measurement of adrenergic receptor transcription rate. *Methods in Molecular Biology* **126**:169–180.
5. Eick, D., Kohlhuber, F., Wolf, D.A., Strobl, L.J. (1994) Activation of pausing RNA polymerases by nuclear run-on experiments. *Analytical Biochemistry* **218**:347–351.
6. Sandoval, J., Rodriguez, J.L., Tur, G., et al. (2004) RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription. *Nucleic Acids Research* **32**:e88.
7. Lipson, K.E., Baserga, R. (1989) Transcriptional activity of the human thymidine kinase gene determined by a method using the polymerase chain reaction and an intron-specific probe. *Proceedings of the National Academy of Sciences of the United States of America* **86**:9774–9777.
8. Prendergast, F.G., Mann, K.G. (1978) Chemical and physical properties of aequorin and the green fluorescent protein isolated from Aequorea forskalea. *Biochemistry* **17**: 3448–3453.

9. Tsien, R.Y. (1998) The green fluorescent protein. *Annual Review of Biochemistry* **67**: 509–544.
10. Ow, D.W., JR DEW, Helinski, D.R., Howell, S.H., Wood, K.V., Deluca, M. (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* **234**:856–859.
11. de Wet JR, Wood, K.V., DeLuca, M., Helinski, D.R., Subramani, S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Molecular and Cellular Biology* 7:725–737.
12. Joung, J.K., Ramm, E.I., Pabo, C.O. (2000) A bacterial two-hybrid selection system for studying protein-DNA and protein-protein interactions. *Proceedings of the National Academy of Sciences of the United States of America* **97**:7382–7387.
13. Jain, V.K., Magrath, I.T. (1991) A chemiluminescent assay for quantitation of beta-galactosidase in the femtogram range: application to quantitation of beta-dgalactosidase in lacZ-transfected cells. *Analytical Biochemistry* **199**:119–124.
14. Bronstein, I., Fortin, J., Stanley, P.E., Stewart, G.S., Kricka, L.J. (1994) Chemiluminescent and bioluminescent reporter gene assays. *Analytical Biochemistry* **219**:169–181.
15. Bronstein, I., Fortin, J.J., Voyta, J.C., et al. (1994) Chemiluminescent reporter gene assays: sensitive detection of the GUS and SEAP gene products. *BioTechniques* **17**: 172–174, 6–7.
16. Bronstein, I., Martin, C.S., Fortin, J.J., Olesen, C.E., Voyta, J.C. (1996) Chemiluminescence: sensitive detection technology for reporter gene assays. *Clinical Chemistry* **42**: 1542–1546.
17. Kingston, R.E., Sheen, J., Moore, D. (2001) Isotopic assays for reporter gene activity. Current protocols in molecular biology, edited by Frederick M Ausubel et al. Chapter 9:Unit9 7A.
18. Thompson, J.F., Hayes, L.S., Lloyd, D.B. (1991) Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene* **103**:171–177.
19. Carey, M., Smale, S.T. (1999) Functional Assays for Promoter Analysis. In: Transcriptional Regulation in Eukaryotes: Concepts, Strategies, and Techniques: Cold Spring Harbor Laboratory Press, pp. 137–92.
20. Felgner, P.L., Gadek, T.R., Holm, M., et al. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proceedings of the National Academy of Sciences of the United States of America* **84**: 7413–7417.
21. Zhang, K., Kurachi, S., Kurachi, K. (2003) Limitation in use of heterologous reporter genes for gene promoter analysis. Silencer activity associated with the cloramphenicol acetyltransferase reporter gene. *Journal of Biological Chemistry* **278**:4826–4830.
22. Sherf, B.A. NSL, Hannah, R.R. and Wood, K.V. Dual-Luciferase, T.M. (1996) Reporter Assay: An Advanced Co-Reporter Technology Integrating Firefly and Renilla Luciferase Assays. *Promega Notes Magazine* **57**: 02.
23. Kipp, J.L., Kilen, S.M., Bristol-Gould, S., Woodruff, T.K., Mayo, K.E. (2007) Neonatal exposure to estrogens suppresses activin expression and signaling in the mouse ovary. *Endocrinology* **148**: 1968–1976.
24. Kipp, J.L., Kilen, S.M., Woodruff, T.K., Mayo, K.E. (2007) Activin regulates estrogen receptor gene expression in the mouse ovary. *Journal of Biological Chemistry* **282**:36755–36765.
25. Briers, T.W., van de Voorde, A., Vanderstichele, H. (1993) Characterization of immortalized mouse granulosa cell lines. *In vitro Cellular & Developmental Biology* **29A**:847–854.
26. Vanderstichele, H., Delaey, B., de Winter, J., et al. (1994) Secretion of steroids, growth factors, and cytokines by immortalized mouse granulosa cell lines. *Biology of Reproduction* **50**:1190–1202.
27. Campbell, M.J. (1995) Lipofection reagents prepared by a simple ethanol injection technique. *BioTechniques* **18**:1027–1032.
28. Dykema, J.C., Mayo, K.E. (1994) Two messenger ribonucleic acids encoding the common beta B-chain of inhibin and activin have distinct 5′-initiation sites and are differentially regulated in rat granulosa cells. *Endocrinology* **135**:702–711.
29. Mukherjee, A., Park-Sarge, O.K., Mayo, K.E. (1996) Gonadotropins induce rapid phosphorylation of the 3′,5′-cyclic adenosine monophosphate response element binding protein in ovarian granulosa cells. *Endocrinology* **137**:3234–3245.
30. Park-Sarge, O.K., Mayo, K.E. (1994) Regulation of the progesterone receptor gene by gonadotropins and cyclic adenosine 3′,5′-monophosphate in rat granulosa cells. *Endocrinology* **134**:709–718.
31. Pei, L., Dodson, R., Schoderbek, W.E., Maurer, R.A., Mayo, K.E. (1991) Regulation of the alpha inhibin gene by cyclic adenosine 3′,5′-monophosphate after transfection into

rat granulosa cells. *Molecular Endocrinology* **5**:521–534.

32. Burkart, A.D., Mukherjee, A., Mayo, K.E. (2006) Mechanism of repression of the inhibin alpha-subunit gene by inducible 3′,5′-cyclic adenosine monophosphate early repressor. *Molecular Endocrinology* **20**: 584–597.
33. Campbell, K.L. (1979) Ovarian granulosa cells isolated with EGTA and hypertonic sucrose: cellular integrity and function. *Biology of Reproduction* **21**:773–786.
34. Ardekani, A.M., Romanelli, J.C., Mayo, K.E. (1998) Structure of the rat inhibin and activin betaA-subunit gene and regulation in an ovarian granulosa cell line. *Endocrinology* **139**:3271–3279.
35. Gehm, B.D., McAndrews, J.M., Chien, P.Y., Jameson, J.L. (1997) Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proceedings of the National Academy of Sciences of the United States of America* **94**:14138–14143.
36. Cicatiello, L., Cobellis, G., Addeo, R., et al. (1995) In vivo functional analysis of the mouse estrogen receptor gene promoter: a transgenic mouse model to study tissue-specific and developmental regulation of estrogen receptor gene transcription. *Molecular Endocrinology* **9**: 1077–1090.