Chapter 9

Using siRNA Knockdown in HaCaT Cells to Study Transcriptional Control of Epidermal Proliferation Potential

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Abstract

Compared to primary keratinocytes, HaCaT cells are easier to transfect and yet still maintain at least some features of normal epidermal proliferation and differentiation. This chapter describes methods used in our laboratory to maintain HaCaT cells in an undifferentiated state and to use the siRNA technology to efficiently deplete a gene product of interest from these cells. We also provide protocols on how to couple siRNA knockdown with a clonal assay to examine keratinocyte proliferation potential and a luciferase reporter assay to examine promoter regulation.

Key words: HaCaT, Cell culture, siRNA, Stem cells, Progenitor cells, Proliferation potential, Epidermis, Keratinocytes, Transcription factors, Luciferase reporter.

1. Introduction

Epidermal homeostasis is important to maintain the epidermis for the lifetime of an organism. In a widely-accepted model of epidermal homeostasis, stem cells of the epidermis are relatively quiescent in vivo and hence are able to maintain long-term proliferation potential (1, 2). These undifferentiated, self-renewing cells give rise to daughter transit amplifying (TA) cells which compose the majority of proliferating cells within the basal layer. Unlike stem cells, TA cells can only undergo a limited number of divisions before exiting the cell cycle and undergoing terminal differentiation (1, 3–5). Early experiments from Barrandon and Green described a clonal assay to decipher the difference in proliferation potential between stem cells and TA cells (6). Large colonies are each derived from a cell that has long-term proliferation potential

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and therefore is believed to be a stem cell. Smaller colonies are derived from a cell that has limited proliferation potential and therefore is thought to be a TA cell. This useful assay, if combined with gene manipulations, will allow the dissection of molecular mechanisms controlling epidermal proliferation potential. However, the use of this assay has been largely restricted to primary epidermal keratinocytes which are difficult to manipulate due to low transfection efficiency.

HaCaT cells are a spontaneously immortalized keratinocyte cell line that are easily transfectable. When transplanted onto either nude mice or grown in organotypic cultures, these cells are able to give rise to stratified epithelium that resembles the epidermis to a certain extent (7, 8). This demonstrates that HaCaT cells retain at least some of the functional differentiation properties of normal keratinocytes. Like primary keratinocytes in culture, HaCaT cells represent a heterogeneous cell population. When sorted on the basis of Desmoglein 3 (Dsg3) expression, cells that were $Dsg3^{dim}$ from both HaCaT and primary human keratinocytes possess higher colony forming ability than Dsg^{bri} (9, 10). This shows that HaCaT cells, similar to primary cells, contain cells with different proliferation potentials. These findings demonstrate that HaCaT cells can serve as a valid model to study epidermal keratinocyte proliferation and differentiation.

In this chapter, we first provide a protocol to prepare low-calcium medium in order to maintain HaCaT cells in an undifferentiated state. We then describe how to efficiently deplete a gene product of interest from HaCaT cells using the siRNA technology and how to examine the effect of the depletion on keratinocyte proliferation potential and target promoter activity.

2. Materials

2.1. Cell Culture

1. Chelex 100× Resin (Bio Rad; cat. no. 142-2842).
2. Fetal Bovine Serum, characterized (Hyclone; cat. no. SH30071.03). Store at –20°C away from light (*see* **Note 1**).
3. Millipore Steritop sterile filter bottle tops, 500 mL volume, GP Express Plus membrane, 45 mm neck size, 0.22 μm filter (Fisher; cat. no. SCGP T05 RE).
4. Insulin from bovine pancreas (Sigma Aldrich; cat. no. I5500), 5 mg/mL in 0.1 N HCl, store at –20°C.
5. Apo-transferrin human (Sigma Aldrich; cat. no. T2252), 5 mg/mL in 1× PBS, store at –20°C.

6. T3 (3,3′,5-Triiodo-L-Thyronine) (Sigma Aldrich; cat. no. T6937), 2×10^{-8} M. Weigh out 13.6 mg of T3 and dissolve into 100 mL of 0.02 N NaOH to give a 2×10^{-4} M T3 solution. Then take 0.1 mL of 2×10^{-4} M T3 and add 9.9 mL 1× PBS to make a 2×10^{-6} M T3 solution. Take 1 mL of 2×10^{-6} M T3 and add 99 mL 1× PBS to make a working stock of T3 of 2×10^{-8} M. Store at –20°C (*see* **Note 2**).
7. 100× Cocktail. Add 20 mL of 5 mg/mL insulin, 20 mL of 5 mg/mL transferrin, 20 mL of 2×10^{-8} M T3, and 140 mL of 1× PBS to a 250 mL plastic graduated cylinder. Mix well and aliquot 24 mL each in 50 mL conical tubes. Store at –20°C.
8. Cholera toxin (Fisher; cat. no. ICN15000501), 84 μg/mL in water, store at 4°C. TOXIC (*see* **Note 3**).
9. Hydrocortisone (Calbiochem; cat. no. 386698), 4 mg/mL in 95% ethanol, store at –20°C.
10. DMEM:F12 (3:1) no Ca^{2+} powder media (Gibco/Invitrogen; cat. no. 90-5010EA) (*see* **Note 4**).
11. 6-well sterile TC plate (Fisher; cat. no. 08-772-1B).
12. 12-well sterile TC plate (Fisher; cat. no. 08-772-29).
13. 10 cm sterile TC plate (Fisher; cat. no. 08-772-22).
14. Sterile 15 mL disposable conical tube (Fisher; cat. no. 05-539-5).
15. Sterile 50 mL disposable conical tube (Fisher; cat. no. 06-443-20).
16. 1.7 mL microfuge tubes (Fisher; cat. no. 50400413); autoclave before use.
17. Hemacytometer (Fisher; cat. no. 02-671-5).
18. Hand cell tally (Fisher; cat. no. NC9684786).
19. HaCaT human keratinocyte cell line is available in many tabs including ours (*see* **Note 5**).
20. Penicillin–streptomycin 100× liquid (Invitrogen; cat. no. 15140-122).
21. 1× sterile PBS.
22. 0.25% Trypsin-EDTA (Invitrogen; cat. no. 25200-056).
23. $NaHCO_3$ (Fisher; cat. no. BP-328-500).
24. Stir plate at 4°C.
25. Media bottles of various sizes (including 250 mL, 500 mL, and 1 L) (Fisher; cat. no. 06-414-1B, 06-414-1C, 06-414-1D).
26. 1 N HCl
27. 1 N NaOH

2.2. Transfection Reagents

1. OptiMEM I reduced serum medium 1× liquid (Invitrogen; cat. no. 31985-070).

2. Lipofectamine 2000 Transfection Reagent (Invitrogen; cat. no. 11668-019). Store at 4°C (Do *not* freeze).
3. Ambion siRNA (catalog number varies according to siRNA; visit www.ambion.com; *see* **Note 6**).
4. Negative control siRNA #1 (Ambion; cat. no. AM4635).
5. pGL3 basic vector (Promega; cat. no. E1751) (*see* **Note 7**).
6. β-Actin promoter – β-gal plasmid.
7. pEGFP-N1 plasmid (Clontech).
8. Filler DNA (any plasmid that does not code for a protein. This is used to bring the amount of DNA up to 1600 ng for efficient transfection).

2.3. Luciferase Assay

1. 1 M Na_2HPO_4. Add 14.196 g Na_2HPO_4 and bring the volume to 100 mL with milliQ water.
2. D-luciferin (Sigma; cat. no. L-9504). Make a 1 mM stock solution by adding 1.75 mL 0.2 M Na_2HPO_4 to the 10 mg bottle of D-luciferin. Mix well and transfer to a graduated cylinder. Bring the volume to 35 mL with milliQ water (*see* **Note 8**).
3. Tricine (Sigma; cat. no. T0377). Make a 1 M Tricine-Tris buffer, pH 7.8 stock solution by adding 89.5 g Tricine to 450 mL milliQ water. Adjust the pH to 7.8 with Tris (about 30.38 g). Bring the volume to 500 mL.
4. 1 M $MgSO_4$. Add 24.65 g $MgSO_4$ and bring the volume to 100 mL with milliQ water.
5. 1 M $MgCl_2$. Add 20.3 g $MgCl_2$ and bring the volume to 100 mL with milliQ water.
6. 0.5 M EDTA, pH 8.0. Add 18.6 g EDTA to 90 mL milliQ water. Adjust the pH to 8.0. Bring the volume to 100 mL with milliQ water.
7. Three vials ATP (Sigma; cat. no. A1852). Make a 0.1 M ATP solution by adding 544 μL milliQ water to each vial that contains 30 mg ATP.
8. Sodium pyrophosphate (PPi) (Fisher; cat. no. S390-500). Make a 0.5% PPi stock solution by adding 0.5 g PPi and bring the volume to 100 mL with milliQ water.
9. Luciferase assay substrate. Add the following to a 250 mL graduated cylinder: 35 mL 1 mM D-luciferin, 3.5 mL 1 M Tricine-Tris pH 7.8, 938 μL 1 M $MgSO_4$, 210 μL 1 M $MgCl_2$, 140 μL 0.5 M EDTA pH 8.0, 70 μL 1 M DTT, 1.4 mL 0.1 M ATP, and 350 μL 0.5% PPi. Bring the volume to 140 mL with milliQ water. Mix well and make 1 mL aliquots. Store at –80°C (*see* **Note 9**).

10. Reporter Lysis buffer 5× (Promega; cat. no. E397A); store for long term at –20°C (*see* **Note 10**).
11. Luminometer with printer (Fisher; cat. no. 12-200-17).
12. 12 × 75 mm polystyrene test tubes for luminometer (Fisher; cat. no. 22-170-142).
13. Cell scraper.

2.4. CRPG Assay (β-Galactosidase Assay)

1. CRPG (chlorophenol red-β-D-galactopyranoside monosodium salt) (Roche Molecular; cat. no. 10884308001). Make a 40 nM CRPG stock solution by adding 10.29 mL milliQ water to the bottle with 250 mg CRPG. Mix well and aliquot in 1.5 mL Eppendorf tubes. Store at –20°C.
2. 1 M NaH_2PO_4. Add 11.99 g NaH_2PO_4 and bring the volume to 100 mL with milliQ water.
3. 2× CRPG buffer. Add 1.4 mL 1 M Na_2HPO_4, 526 μL 1 M NaH_2PO_4, and 15 mL milliQ water. Adjust the pH to 7.3 and then bring the volume to 19.4 mL with milliQ water. Add 283.2 μL β-mercaptoethanol and 360 μL 1 M $MgCl_2$. Good for 2 weeks when stored at 4°C.
4. 37°C water bath.
5. Disposable polystyrene 1.5 mL cuvettes (Fisher; cat. no. 14-385-942).
6. Spectrophotometer.

2.5. Clonal Assay

1. 4% paraformaldehyde (*see* **Note 11**).
2. Rhodamine B (Sigma Aldrich; cat. no. R6626-100G) and Nile Blue A (Sigma Aldrich; cat. no. N5632-25G). Make a 1% Rhodamine B/Nile Blue solution by adding 1 g Rhodamine B and 1 g Nile Blue and then bring volume to 100 mL with milliQ water.

3. Methods

3.1. Making Low Ca^{2+} Complete Media

3.1.1. Making Ca^{2+}-Free Serum

1. Weigh out 200 g Chelex 100× Resin and place in a 2 L beaker with a stir bar.
2. Mix well with 2 L milliQ H2O.
3. Adjust pH to 7.5 with 12 N HCl. Stir at room temperature for 1 hour.
4. Cover and place at 4°C for 4 hours or overnight to allow the beads to settle.
5. Decant the slurry and add 2 L fresh milliQ H2O. Allow the beads to come to room temperature.

6. Repeat pH adjustment to 7.5. Stir at room temperature for 1 hour.
7. Cover and place at 4°C for 4 hours or overnight.
8. Allow beads to come to room temperature. Check the pH. (If pH is not 7.5, repeat Steps 5.8.)
9. Carefully pipet out as much water as possible. Try not to disturb the beads.
10. Carefully pour in 500 mL characterized Fetal Bovine Serum.
11. Stir gently (no bubbles) at 4°C for 1 hour.
12. Let resin settle in cold room overnight.
13. Pipet out the serum.
14. Filter-sterile through 0.22 μm filter (use a 500 mL filter apparatus).
15. Aliquot 179 mL serum into sterile 250 mL TC bottles. Store at –20°C until needed.

3.1.2. Regenerating Chelex beads

The Chelex beads can be regenerated and used up to three times to chelate Ca^{2+} ions. Regenerate immediately after use.

1. Add 600 mL 1 N HCl to the beads. Stir at room temperature for 1 hour.
2. Cover and place at 4°C for 4 hours or overnight to allow the beads to settle.
3. Decant and add 2 L milliQ water. Stir at room temperature for 1 hour.
4. Cover and place at 4°C for 4 hours or overnight to allow the beads to settle.
5. Decant and add 600 mL 1 N NaOH. Stir at room temperature for 1 hour.
6. Cover and place at 4°C for 4 hours or overnight to allow the beads to settle.
7. Decant and add 2 L milliQ water. Stir at room temperature for 1 hour.
8. The beads are now ready to re-use. Start with step 5 for the "Making Ca^{2+}-free serum" protocol.

3.1.3. Making Ca^{2+}-Free Serum Mix

Do not store the serum mix. Make just enough for the amount needed for the final media preparation.

1. Thaw one bottle of 179 mL Ca^{2+}-free serum.
2. Add the following to the serum: 11.8 mL of 100× cocktail, 11.8 μL of 84 μg/mL cholera toxin, and 179 μL of 4 mg/mL hydrocortisone.
3. Mix well and store at 4°C until the low Ca^{2+} base media is ready.

3.1.4. Making Low Ca^{2+} Base Media (1 L)

1. Place 800 mL milliQ water in a 2 L beaker with a stir bar.
2. Add 1 bag of DMEM:F12 (3:1) no Ca^{2+} powder media. Rinse out the inside of the package with milliQ water to remove all traces of powder.
3. Add 3.07 g of $NaHCO_3$ and 10 mL of 100× pen/strep. Mix well.
4. Adjust the pH to 7.2 (*see* **Note 12**).
5. Bring volume up to 1 L with milliQ water.

3.1.5. Making Low Ca^{2+} Complete Media

1. Add the Ca^{2+}-free serum mix to 1 L of Low Ca^{2+} base media made in the 2 L beaker.
2. Mix gently, avoiding bubbles.
3. Filter media through a sterile 0.22 μm Millipore filter in the TC hood. Place in sterile TC bottles.
4. Store at 4°C away from light.

3.2. Titrating siRNA Knockdown

Test the efficiency of siRNA knockdown by transfecting cells with siRNA and then 72 hours later collecting the cells, making cell lysates, and performing a Western blot. The amount of siRNA used should be titrated first, followed by a time course assay to determine the length of knockdown. For each concentration of siRNA being tested, make sure to include a negative-control siRNA of the same concentration. The negative-control siRNA should be a siRNA that has a scrambled sequence that will not target any gene.

3.2.1. Plating HaCaT Cells (Day 0)

1. Wash plate with 8 mL 1× PBS. Aspirate off.
2. Add 2 mL 0.25% Trypsin-EDTA to the plate and swirl the plate. Place the plate in the 37°C incubator for about 15 minutes until the cells have lifted off the plate.
3. Inactivate the trypsin by adding 8 mL media to the plate. Pipet up and down to create a single cell suspension. Collect in a 15 mL conical tube.
4. Count the cells using a hemacytometer. Calculate the number of cells needed to plate 4×10^5 cells per 10 cm plate (*see* **Note 13**).
5. Pipet the number of cells needed into a new 50 mL conical tube.
6. Centrifuge the cells at 1500 rpm for 5 minutes at room temperature.
7. Aspirate off the old media. Resuspend the cells in the amount of media needed (add 10 mL of media for each plate that is to be plated).
8. Pipet 10 mL of cell suspension to each plate of a 10 cm dish.
9. Place the cells in the 37°C, 5% CO_2 incubator.

3.2.2. Transfecting siRNA (Day 1)

To titrate the amount of siRNA needed for efficient knockdown, use at least three different concentrations of siRNA. Start by using siRNA at a final concentration of 30 nM, 60 nM, and 90 nM. For most siRNAs, 60 nM will knockdown efficiently; however, this should be empirically determined for different siRNAs. Also, use a negative-control siRNA at each concentration to determine if there are any non-specific effects from the siRNA. **Table 9.1** shows the amounts needed for different size plates when using 60 nM siRNA (*see* **Note 14**).

1. For each transfection sample, prepare siRNA–Lipofectamine 2000 complex in a laminar flow hood as follows:
   a. Dilute siRNA into 1.5 mL OptiMEM I Reduced Serum Medium in a 15 mL conical tube (*see* **Note 15**). Mix gently.
   b. Mix Lipofectamine 2000 gently before use, then dilute 30 μL into 1.5 mL OptiMEM in a 15 mL conical tube. Mix gently and incubate for 5 minutes at room temperature (proceed to step c within 25 minutes).
   c. After the 5-minute incubation, combine the diluted siRNA with the diluted Lipofectamine 2000. Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy).
2. Add the siRNA–Lipofectamine 2000 complexes to each plate containing cells and medium. Mix gently by rocking the plate back and forth (*see* **Note 16**).
3. Place the cells in the 37°C, 5% CO_2 incubator.
4. Change the media 24 hours after transfection (*see* **Note 17**).
5. 72 hours after transfection, harvest the cells and perform a Western blot to determine the concentration at which there is efficient knockdown.
6. Titrate the length of knockdown by repeating the above section, but only use the concentration of siRNA that gives the most efficient knockdown. Then harvest the cells at

Table 9.1
Reagent amounts for siRNA transfection

Culture vessel	Surface area per well	Volume of plating media (mL)	Volume of OptiMEM	Volume of 50 μM siRNA (μL)	Volume of Lipofectamine 2000 (μL)
12 well	~4 cm^2	1	2 × 100 μL	1.44	2
6 well	~10 cm^2	2	2 × 250 μL	3	5
10 cm	~60 cm^2	10	2 × 1.5 mL	15.6	30

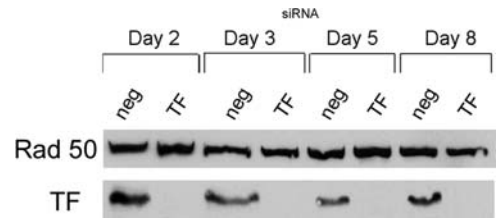

Fig. 9.1. Knockdown in HaCaT cells occurs efficiently and lasts for at least 6 days. Rad50 was used as a nuclear loading control. TF, transcription factor. Neg, negative control.

various days after transfection. **Figure 9.1** shows that this method results in efficient knockdown of a transcription factor (TF) that we study.

3.3. Clonal Assay

A clonal assay can be used to determine the proliferation potential of individual keratinocytes. By transfecting siRNA into HaCaT cells, genes can be knocked down and the effect on the proliferation potential of cells can be monitored. The results from the first 14 days uncover the initial effects from the siRNA. However, replating the cells from day 14 at clonal density allows the monitoring of longer-term proliferation potential. For example, **Fig. 9.2** shows that when a transcription factor that we study was knocked down, there was an initial increase in colony-forming ability. However, when colonies were harvested at the end of the first plating and equal number of knockdown and control cells was re-plated at clonal density and allowed to grow for an additional 14 days in the absence of siRNA, there was now a decrease in colony-forming ability in the knockdown. This result indicates that the expansion seen in the first-generation plating was likely due to an increase in the number of cells that are faster proliferating but have only limited proliferation potential (i.e., TA cells). Concurrently, there was a loss of cells with long-term proliferation potential, as demonstrated by reduced colony-forming ability in the second generation. This illustrates how a colony-forming assay

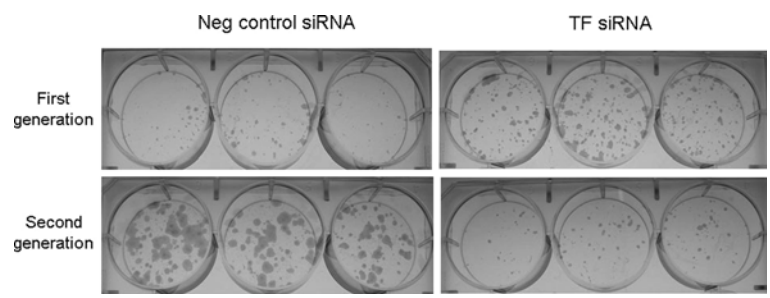

Fig. 9.2. HaCaT cells can be used in a clonal assay to determine the proliferation potential of individual cells.

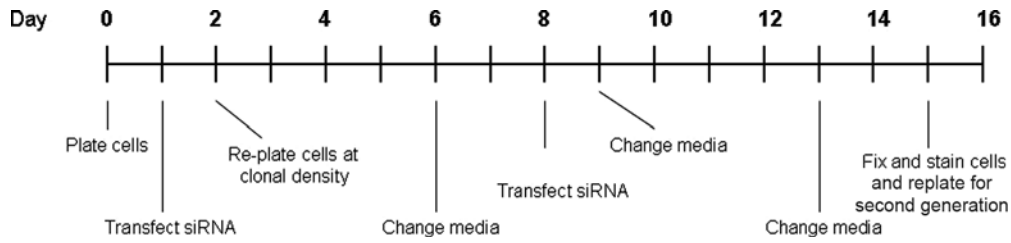

Fig. 9.3. Timeline for clonal assay.

can be used in conjunction with siRNA knockdown to study the molecular control of the keratinocyte proliferation potential (*see* **Fig. 9.3** for a timeline of the first generation assay).

3.3.1. Plating HaCaT Cells (Day 0)

1. Follow steps 1–3 of **section 3.2.1**.
2. Count the cells using a hemacytometer. Calculate the number of cells needed to plate 8×10^4 cells per well (*see* **Note 13**).
3. Pipet the number of cells needed into a new 15 mL conical tube.
4. Centrifuge the cells at 1500 rpm for 5 minutes at room temperature.
5. Aspirate off the old media. Re-suspend the cells in the amount of media needed (add 2 mL of media for each well that is to be plated).
6. Pipet 2 mL of cell suspension to each well of a 6-well plate.
7. Place the cells in the 37°C, 5% CO_2 incubator.

3.3.2. Transfect siRNA (Day 1)

1. Transfect cells using Lipofectamine 2000 according to **Section 3.2.2**. Make sure to adjust the volume for a 6-well plate.
2. Place cells in the 37°C, 5% CO_2 incubator.

3.3.3. Re-plate HaCaT at Clonal Density (Day 2)

1. Wash each well with 2 mL 1× PBS. Aspirate off.
2. Add 400 µL 0.25% Trypsin-EDTA to each well of the plate. Place the plate in the 37°C incubator for about 15 minutes until the cells have lifted off the plate.
3. Inactivate the trypsin by adding 600 µL media to each well. Pipet up and down to create a single cell suspension. Collect in a 1.5 mL microfuge tube.
4. Count the cells using a hemacytometer. Calculate the number of cells needed to plate 3500 cells per 6-well plate (this is for 500 cells/well with enough for one extra well) (*see* **Note 18**).
5. Add 3500 cells into a volume of 14 mL media in a 15 mL conical tube. Do this for each sample.

6. Place 2 mL of the diluted cell suspension to each well of a 6-well plate.
7. Place the cells in the 37°C, 5% CO_2 incubator.

3.3.4. Change Media (Day 6)

1. Aspirate off old media.
2. Add 2 mL of fresh media to each well.
3. Place cells in the 37°C, 5% CO_2 incubator.

3.3.5. Transfect siRNA (Day 8)

1. Transfect cells using Lipofectamine 2000 according to **Section 3.2.2**. Make sure to adjust the volume for a 6-well plate. Prepare a master mix for the samples and make enough for one extra well. For example, for the negative control siRNA you would make a master mix for the transfection that contains enough reagents for 7 wells.
2. Place cells in the 37°C, 5% CO_2 incubator.

3.3.6. Change Media (Day 9 and Day 13)

1. Aspirate off old media.
2. Add 2 mL of fresh media to each well.
3. Place cells in the 37°C, 5% CO_2 incubator.

3.3.7. Fix and Stain HaCaT Cells (Day 15)

1. For half of the wells, for each sample, follow **Section 3.3.3.** to replate cells at Clonal density for second generation. Change media every four days. Fourteen days after replating, follow step 2–13 below.
2. For the remaining wells, (wash) wash each well with 2 mL 1× PBS. Aspirate off.
3. Add 2 mL 4% paraformaldehyde to each well to fix the cells. Incubate at room temperature for 15 minutes.
4. Aspirate off.
5. Wash each well with 2 mL 1× PBS. Aspirate off.
6. Repeat step 4 two more times.
7. Add 2 mL ice-cold 90% methanol to each well to permeabilize the cells. Incubate at room temperature for 5 minutes.
8. Wash each well with 2 mL 1× PBS. Aspirate off.
9. Repeat step 7 two more times.
10. Stain the cells by adding 2 mL of the staining solution (1% Rhodamine B/1% Nile Blue). Incubate at room temperature for 30 minutes.
11. Wash plate with DI water several times until there is no background staining.
12. Pour off water and allow plates to air dry.
13. Take pictures of the plates and quantify the number and size of the colonies.

3.4. Luciferase and CRPG β-Galactosidase Assays

A luciferase assay is a reporter assay that allows one to determine if the transcription factor being studied regulates a specific promoter. By combining siRNA transfection with reporter plasmid transfection, one can assess the effect of gene depletion on reporter promoter activity. A CRPG β-galactosidase assay is performed to normalize the data for possible differences in transfection efficiencies. The CRPG assay can also be used to determine if experimental conditions may have non-specific effects on promoter activity. For example, if knockdown of a gene under study results in consistent dosage-dependent increase in both luciferase expression driven by the specific promoter and β-galactosidase assay driven by the control β-actin promoter, this could indicate that the siRNA being used is causing a non-specific activation of promoters. In this case, additional control promoters such as CMV should be tested. In addition to having samples that are transfected with the negative-control siRNA, there should also be samples that are transfected with an empty pGL3 basic vector. This control allows one to determine if the siRNA is having any promoter-independent effect on the luciferase read-out. A comparison between luciferase read-outs from pGL3 vector and pGL3 with target promoter will also ensure that the promoter is indeed active under the experimental conditions. **Table 9.2** provides examples of experimental conditions, with the proper controls included.

Table 9.2
Example of Luciferase assay experimental design

Sample #	siRNA	Promoter plasmid
1	30 nM neg	Empty pGL3
2	30 nM gene being studied	Empty pGL3
3	30 nM neg	Target promoter pGL3
4	30 nM gene being studied	Target promoter pGL3
5	60 nM neg	Empty pGL3
6	60 nM gene being studied	Empty pGL3
7	60 nM neg	Target promoter pGL3
8	60 nM gene being studied	Target promoter pGL3

Each condition being studied should be done in triplicate. In the example above you would need 24 wells (two 12-well plates). However, when preparing master mixes, make enough for one extra well (*see* **Fig. 9.4** for a timeline for this assay).

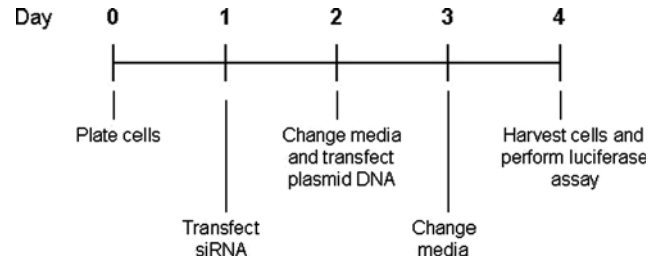

Fig. 9.4. Timeline for luciferase and CRPG β-galactosidase assay.

3.4.1. Plating HaCaT cells (Day 0)

1. Follow steps 1–3 of **Section 3.2.1.**
2. Count the cells using a hemacytometer. Calculate the number of cells needed to plate 7×10^4 cells per well (*see* **Note 13**).
3. Pipet the number of cells needed into a new 15 mL conical tube.
4. Centrifuge the cells at 1500 rpm for 5 minutes at room temperature.
5. Aspirate off the old media. Re-suspend the cells in the amount of media needed (add 1 mL of media for each well that is to be plated).
6. Pipet 1 mL of cell suspension to each well of a 12-well plate.
7. Place the cells in the 37°C, 5% CO_2 incubator.

3.4.2. Transfecting siRNA (Afternoon, Day 1)

1. Transfect cells using Lipofectamine 2000 according to **Section 3.2.2**. Make sure to adjust the volume for a 12-well plate and the samples are prepared in triplicate. Prepare a master mix for the samples and make enough for one extra well.
2. Change the media 24 hours after transfection.

3.4.3. Transfecting Plasmid DNA (Early Evening, Day 2)

The cells should be at least 80% confluent at the time of transfection to minimize the amount of cell death caused by Lipofectamine toxicity. The following plasmids will be transfected into HaCaT cells for the luciferase assay in triplicate for each sample (*see* **Note 19**):

Plasmid name	Amount
Reporter plasmid (i.e., promoter of interest-pGL3 basic)	800 ng
β-actin promoter – β-gal plasmid (for normalization)	300 ng
Filler DNA (empty vector plasmid)	500 ng
Total DNA	1600 ng

1. Calculate the amount of each plasmid needed to transfect the samples in triplicate, but add enough plasmid for one extra well. For example, if you have four samples, you would

prepare them each in triplicate. This means that you should have a master mix that contains enough DNA for 12 wells. However, to account for pipetting errors, you should prepare the DNA for 13 wells.

2. Calculate the amount to OptiMEM I Reduced Serum Medium needed to dilute both the DNA and the Lipofectamine 2000. For each you will need 100 µL per well. Using the example above, you would need 13 wells, therefore you would dilute the DNA in OptiMEM to make the final volume 1300 µL. You would also dilute the Lipofectamine 2000 in OptiMEM to make a final volume of 1300 µL.
3. Calculate the amount of Lipofectamine 2000 needed. You will need 4 µL per well. Using the example above for 13 wells, you would need to add 52 µL to 1248 µL OptiMEM media.
4. For each transfection sample, prepare DNA-Lipofectamine 2000 complex in a laminar flow hood as follows:
   a. Dilute the plasmids into OptiMEM I Reduced Serum Medium in a 15 mL conical tube. Mix gently.
   b. Mix Lipofectamine 2000 gently before use, then dilute into OptiMEM in a 15 mL conical tube. Mix gently and incubate for 5 minutes at room temperature (proceed to step c within 25 minutes).
   c. After the 5-minute incubation, combine the diluted DNA with the diluted Lipofectamine 2000. Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy).
5. Add the DNA–Lipofectamine 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
6. Place the cells in the 37°C, 5% CO_2 incubator.
7. Change the media 16 hours after transfection.

3.4.4. Luciferase Assay (Afternoon, Day 4)

1. Aspirate the media and add 0.5 mL 1× PBS to each well.
2. Scrape each well and collect each sample in a 1.7 mL microfuge tube.
3. Centrifuge for 1 minute at full speed at room temperature to pellet the cells.
4. Prepare 1× lysis buffer by diluting the 5× reporter lysis buffer down to 1× with milliQ water.
5. Aspirate the supernatant and re-suspend cells in 70 µL 1× lysis buffer.
6. Freeze-thaw the samples one time to lyse the cells by placing the samples at –80°C until samples are fully frozen (about 1 hour) and then thawing at room temperature (*see* **Note 20**).

7. While the freeze-thaws are occurring, thaw the luciferase substrate to room temperature.
8. Vortex the samples for 10 seconds.
9. Centrifuge at full speed at room temperature for 30 seconds. Make sure the hinge of the tube faces outward. The pellet will be on the same side as the hinge.
10. Transfer the supernatant for each sample into new 1.7 mL microfuge tubes.
11. Add 50 μL of cell lysate to a luminometer tube.
12. Add 150 μL of luciferase substrate to the first sample.
13. Read sample immediately in luminometer (*see* **Note 21**).
14. Repeat steps 11–13 for each sample.
15. Print the results from the luminometer readings.

3.4.5. CRPG β-Galactosidase Assay (Afternoon, Day 4)

1. For each sample, add 100 μL 2× CRPG and 55 μL milliQ water to a 1.7 mL microfuge tube. Prepare an extra tube for a blank that contains no cell lysate.
2. Then add 5 μL cell lysate to each tube.
3. Finally add 40 μL 40 mM CRPG and close the caps tightly.
4. Briefly vortex the sample.
5. Pulse spin the samples to get the sample to the bottom of the tube.
6. Incubate the samples at 37°C until color develops.
7. After the desired color is obtained (orange, but not brown or red), stop the reaction by adding 600 μL milliQ water.
8. Transfer the samples to the disposable cuvettes.
9. Read the O.D. of the samples in the spectrophotometer at 562 nm (*see* **Note 22**).
10. Print the results from the spectrophotometer readings.
11. To normalize the luciferase samples, divide the luciferase reading by the O.D. (β-gal) reading to give the "Relative Light Unit" (RLU). Do this for each sample. Then average the RLU for the triplicate samples and also determine the standard deviation. Prepare a graph of the average RLU for each sample.

4. Notes

1. Serum lots must be screened because they show variability in supporting keratinocytes growth.
2. When making T3 solutions, make sure to use only plastic. T3 will stick to glass and therefore glass items should be avoided.

3. Cholera toxin is extremely toxic. Make sure to take precautions when handling it.
4. This is a special formula media and therefore must be ordered in large quantities (100 L). Make sure to specify that the media needs to be calcium-free when ordering. The media comes with L-glutamine, but without calcium chloride, HEPES buffer, and sodium bicarbonate.
5. HaCaT cells should be maintained on a 10-cm plate in culture by preventing them from being confluent for longer than a day. Allowing them to be confluent for too long can cause the cells to undergo terminal differentiation. HaCaT cells can be grown in standard DMEM/F12 10% FBS medium. However, by growing in low-calcium medium as described in this protocol, the cells maintain a more basal-like phenotype and are more resistant to undergoing unwanted terminal differentiation. The cells grown in low-calcium medium can be switched to high-calcium medium (2.8 mM calcium) and will undergo normal terminal differentiation, with K1 protein expression occurring 4 days after calcium addition (*see* **Fig. 9.5**).

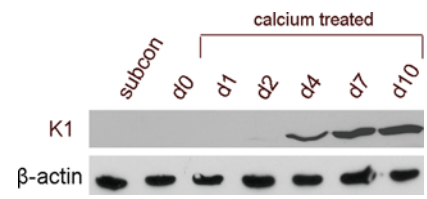

Fig. 9.5. K1 expression is upregulated 4 days after treatment with 2.8 mM calcium in HaCaT cells grown in low-calcium media complete. Subcon, sub-confluent.

6. Check Ambion's web site for availability of siRNA against your gene of choice. If there is not a validated siRNA for your gene, then you should order multiple siRNAs against the gene that you study. When initially screening siRNAs, order only 5 nmol standard purity and re-suspend the siRNA in the nuclease-free water supplied with the siRNA. Make sure to re-suspend the siRNA in the tissue culture hood to avoid contamination and then aliquot into sterile microfuge tubes in small amounts. Avoid repeated freeze-thaws of the siRNA to avoid degradation. Once the siRNAs are screened for efficient knockdown and low toxicity, then order larger scale of the one that works best.
7. Make sure that the DNA preparation is clean and free of contamination by taking O.D. readings at 260 nm and 280 nm using a spectrophotometer. The 260:280 ratio should be greater than 1.8 for good-quality DNA.

8. Protect from light. D-luciferin is light sensitive.
9. Protect from light. Do not allow the aliquots to undergo multiple freeze-thaws. An alternative to the homemade luciferase substrate is to buy a luciferase assay kit from Promega (cat. no. E4530) which also includes the 5× reporter lysis buffer. However, making the luciferase substrate is much more cost efficient.
10. Dilute the lysis buffer to 1× with water right before use. Only make enough for what you need right away. For example, if you have 24 wells, then you would need 1680 μL 1× lysis buffer. You want to make extra to account for pipetting error, so you could make 1800 μL (add 360 μL of 5× reporter lysis buffer to 1440 μL milliQ water). Do not store the 1× lysis buffer.
11. Paraformaldehyde is very toxic. Take precautions when handling it.
12. Media should be red-orange in color. If the media is pink, then de-gas the media.
13. Always calculate the amount of cells needed for one extra plate or well. This minimizes variability in the number of cells plated due to pipetting errors. Make sure that after you centrifuge the cells and aspirate the supernatant that you re-suspend the cells in enough medium for the extra plate. For example, if you need four 10-cm plates, then calculate the number of cells needed for five plates. Once the cells have been pelleted, re-suspend the cells in 50 mL low Ca^{2+} complete media and then add 10 mL of the cell suspension to each of the four 10-cm plates.
14. For example, if you are transfecting a 10-cm plate with 30 nM siRNA, then use only 7.8 μL siRNA. If using 90 nM siRNA, then use 23.4 μL siRNA.
15. Use only polypropylene tubes for the transfection mixes because Lipofectamine 2000 sticks to polystyrene.
16. The transfection mixes can be added directly to the plates with the E-low Ca^{2+} complete media. The serum and antibiotics in the media do not have an effect on transfection efficiency in HaCaT cells.
17. Aspirate off the old media and add fresh media.
18. Whenever handling small volumes, the pipetting error is likely to be high. Therefore, it is better to make dilutions first to reduce the variability between samples. For example, if you have a total of 25×10^4 cells, then you can make a dilution for each sample to achieve a concentration of 1×10^4 cells/mL (add 40 μL of cell suspension to 960 μL low Ca^{2+} complete media). Then to get 3500 cells, you would add 350 μL of the

diluted cell suspension to 14 mL media. Then 2 mL of the final dilution would be added to each well of a 6-well plate to give 500 cells per well.

19. The amount of reporter plasmid (promoter of interest-pGL3 basic) must be determined empirically. To titrate the reporter plasmid, follow the protocol for the luciferase and CRPG assays, except that you do not need to transfect in siRNA and you would add different amounts of the reporter plasmid. For example, one triplicate set of wells would contain no reporter plasmid (this is the background level of luciferase activity). You would then have a triplicate set of wells that contains the following amounts of reporter plasmid: 100 ng, 200 ng, 400 ng, 800 ng, 1000 ng. You would perform luciferase and CRPG assays. You should have a dose-dependent increase in basal promoter activity with increasing amounts of reporter plasmid. If you start to see the promoter activity plateau or decrease with higher amounts of reporter plasmid, then squelching has occurred and concentrations from this point on should not be used in future experiments. Pick a concentration of reporter plasmid that is in the linear range.
20. Do not leave the samples at room temperature for extended periods of time as this will allow for protein degradation. Once thawed, place the samples on ice.
21. Once the substrate is added to the cell lysate, the sample needs to be read immediately. The reaction occurs quickly and the luciferase activity will decrease over time.
22. The O.D. readings should be between 0.1 and 1.0 to be within the linear range of the spectrophotometer. If your samples are reading less than 0.1, allow the samples to incubate at room temperature until you see the color become more orange-red. Then repeat the readings in the spectrophotometer. If the readings are above 1.0, then dilute all your samples by adding about 100 μL water to each sample. Repeat the readings. If the samples are still at an O.D. above 1.0, then repeat the addition of water to all your samples until the readings are below 1.0.

References

1. Potten, C. S., and Morris, R. J. (1988) Epithelial stem cells in vivo. *J. Cell Sci. Suppl.* **10,** 45–62.
2. Lavker, R. M., and Sun, T. T. (2000) Epidermal stem cells: properties, markers, and location. *Proc. Natl. Acad. Sci. USA* **97,** 13473–13475.
3. Jones, P. H., and Watt, F. M. (1993) Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell* **73,** 713–724.
4. Jones, P. H., Harper, S., and Watt, F. M. (1995) Stem cell patterning and fate in human epidermis. *Cell* **80,** 83–93.
5. Watt, F. M. (2001) Stem cell fate and patterning in mammalian epidermis. *Curr. Opin. Genet. Dev.* **11,** 410–417.

6. Barrandon, Y., and Green, H. (1987) Three clonal types of keratinocyte with different capacities for multiplication. *Proc. Natl. Acad. Sci. USA* **84,** 2302–2306.
7. Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106,** 761–771.
8. Schoop, V. M., Mirancea, N., and Fusenig, N. E. (1999) Epidermal organization and differentiation of HaCaT keratinocytes in organotypic coculture with human dermal fibroblasts. *J. Invest. Dermatol.* **112,** 343–353.
9. Wan, H., Yuan, M., Simpson, C., Allen, K., Gavins, F. N., Ikram, M. S., et al. (2007) Stem/progenitor cell-like properties of desmoglein 3dim cells in primary and immortalized keratinocyte lines. *Stem Cells* **25,** 1286–1297.
10. Wan, H., Stone, M. G., Simpson, C., Reynolds, L. E., Marshall, J. F., Hart, I. R., et al. (2003) Desmosomal proteins, including desmoglein 3, serve as novel negative markers for epidermal stem cell-containing population of keratinocytes. *J. Cell Sci.* **116,** 4239–4248.