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Rcho-1 Trophoblast Stem Cells

A Model System for Studying Trophoblast Cell Differentiation

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Summary

The biology of trophoblast cell development can be investigated using in vitro model systems. The Rcho-1 trophoblast stem cell line was derived from a rat choriocarcinoma and is an effective tool for elucidating regulatory mechanisms controlling trophoblast cell differentiation. In this chapter, we describe methods used in the maintenance and manipulation of the Rcho-1 trophoblast cell line.

Key Words: Trophoblast differentiation; rat placenta; trophoblast giant cells; Rcho-1 trophoblast stem cells; choriocarcinoma.

1. Introduction

Trophoblast cells possess specialized phenotypes and arise from a common stem cell population directed along a multi-lineage differentiation pathway ***(1)***. Trophoblast stem cells develop from the blastocyst and are maintained by signals emanating from the inner cell mass ***(2,3)***. In the rat, trophoblast stem cells can be directed toward at least five recognizable differentiated trophoblast cell phenotypes: trophoblast giant cells, spongiotrophoblast cells, invasive trophoblast cells, glycogen cells, and syncytial trophoblast (**Fig. 1**) ***(4,5)***. Differentiated trophoblast cell populations can be distinguished on the basis of morphology, location, and patterns of gene expression. These cell types are arranged into two distinct zones of the chorioallantoic placenta—the junctional zone and the labyrinth zone—and contribute to a complex uteroplacental structure prominent during the last week of gestation, the metrial gland (**Fig. 1**). Each differentiated cell lineage specializes in activities supportive of pregnancy, some of which are well established whereas others are the source of both speculation and ongoing investigation. Some specific trophoblast func-

From: *Methods in Molecular Medicine, Vol. 121: Placenta and Trophoblast: Methods and Protocols, Vol. 1*
Edited by: M. J. Soares and J. S. Hunt © Humana Press Inc., Totowa, NJ

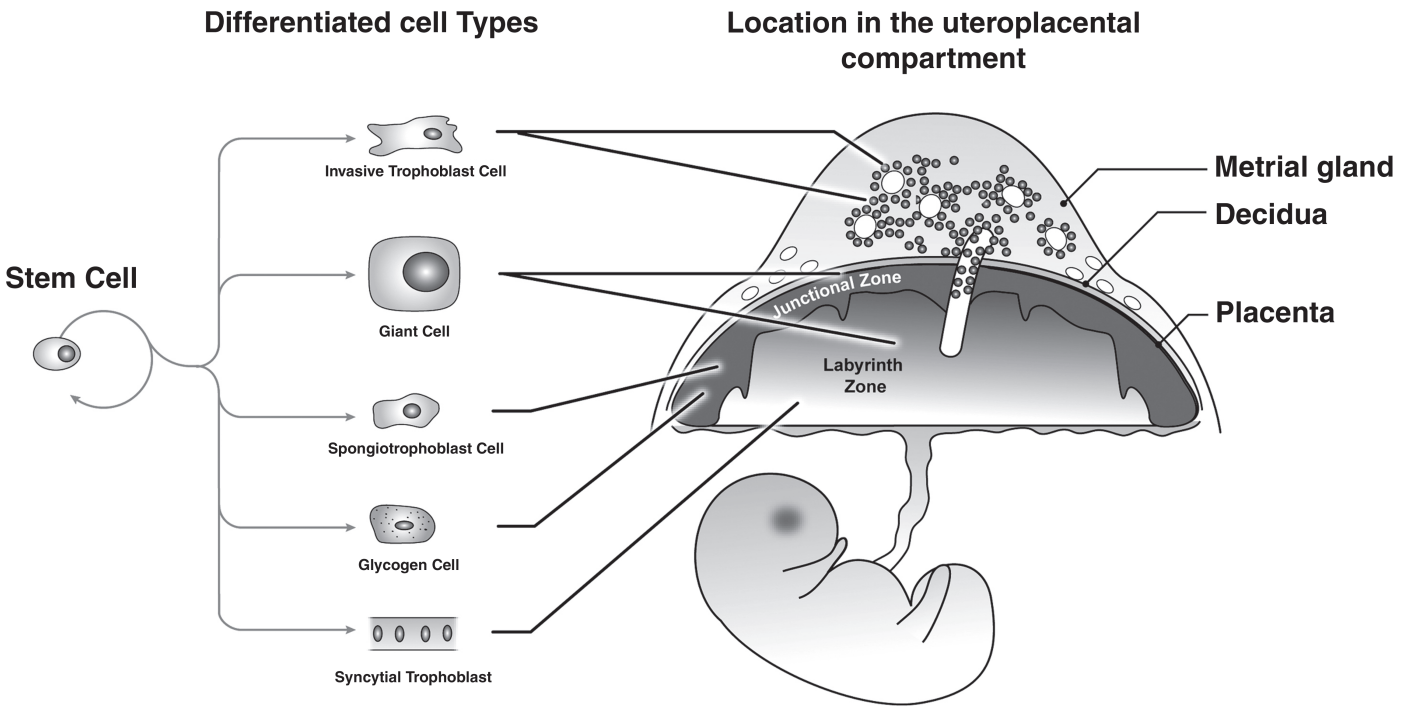

Fig. 1. A schematic representation of rat trophoblast cell lineages and their location within the mature uteroplacental compartment. In the rat, trophoblast stem cells can be directed toward at least five recognizable differentiated trophoblast cell phenotypes: trophoblast giant cells, spongiotrophoblast cells, invasive trophoblast cells, glycogen cells, and syncytial trophoblast. These cell types are arranged into two distinct zones of the chorioallantoic placenta, the junctional zone and the labyrinth zone; and contribute to a complex uteroplacental structure prominent during the last week of gestation: the metrial gland.

tions include remodeling uterine vasculature, hormone/cytokine production, energy storage, and transcellular transport. The normal growth and differentiation of trophoblast cells is crucial for the establishment and maintenance of pregnancy.

Insights about placental development have been derived from the generation of mutant mice by gene targeting ***(6)*** and through the use of cell culture models. The latter efforts have been primarily based on two in vitro systems: blastocyst-derived trophoblast stem cell lines ***(2)*** and trophoblast stem cell lines derived from a rat choriocarcinoma ***(7–9)***. The choriocarcinoma derived cell lines are remarkable in their ability to differentiate into trophoblast phenotypes.

More than two decades ago, Dr. Shinichi Teshima and his colleagues at the National Cancer Institute (Tokyo, Japan) induced a transplantable rat choriocarcinoma with extraordinary features ***(7)***. Initial observations suggested the trophoblast tumor contained trophoblast giant cells and produced lactogenic hormones ***(7,10,11)***. Subsequently, trophoblast stem cell lines were established from the same choriocarcinoma by Dr. Michel Vandeputte's laboratory at the University of Leuven (Leuven, Belgium) ***(8)*** and by our laboratory ***(9)***. The cell line derived by Dr. Vandeputte and colleagues is termed RCHO, while we refer to our trophoblast stem cell line as Rcho-1. These trophoblast stem cell lines are aneuploid, are easy to maintain and expand, and possess the capacity to differentiate in vitro and in vivo into trophoblast giant cells.

RCHO and Rcho-1 trophoblast stem cell lines have become part of the experimental arsenal for studying trophoblast cell biology (**Table 1**). These trophoblast stem cell lines have been used to investigate the regulation of trophoblast cell cycle ***(12–15)***, the regulation of trophoblast cell differentiation ***(8,9,16–32)***, the trophoblast cell phenotype ***(33–47)***, trophoblast cell-specific transcriptional regulation ***(48–67)***, trophoblast cell transport processes ***(68–72)***, trophoblast cell DNA methylation ***(73,74)***, trophoblast cell invasion ***(19,75)***, and trophoblast tumor development ***(76,77)***.

The merit of the RCHO and Rcho-1 trophoblast stem cell models is their plasticity. These cells can be maintained under conditions that facilitate proliferation, or the culture conditions can be changed to promote robust differentiation. Thus, relatively homogenous populations of proliferating and differentiating trophoblast cells can be retrieved from the cultures. The most prominent differentiated phenotype observed in RCHO and Rcho-1 trophoblast stem cell cultures is the trophoblast giant cell ***(7,8)***. This differentiated phenotype is easy to track by monitoring cell morphology (large nucleus) or a variety of functional endpoints. The trophoblast giant cell phenotype is also the most common direction for in vitro differentiation of blastocyst-derived trophoblast stem cells ***(2)***. Differentiation toward other trophoblast cell pheno-

Table 1
Rcho-1 Trophoblast Stem Cell Line Applications for Studying Trophoblast Cell Biology

Trophoblast cellular process	References
Regulation of cell cycle regulation	***12–15***
Regulation of cell differentiation	***8,9,16-32***
Characterization of trophoblast cell phenotypes	***33–47***
Trophoblast cell-specific gene transcription	***48–67***
Cell transport processes	***68–72***
DNA methylation	***73,74***
Cell invasion	***19,75***
Trophoblast tumor development	***76,77***

types is possible, but is not optimal using classic monolayer culture practices (Canham, L. N. and Soares, M. J., unpublished results).

Cancer cells, such as those represented by the RCHO and Rcho-1 trophoblast stem cell lines, are caricatures of normal development and represent potentially important models for dissecting molecular mechanisms controlling differentiation ***(78)***. The key is in identifying and appreciating which regulatory pathways are characteristic of normal development and which are associated with the transformed phenotype. Thus, it is imperative to perform complementary experimentation using primary cultures of trophoblast cells and in vivo models.

In this chapter, we describe methods developed in our laboratory for using the Rcho-1 trophoblast stem cell model to study various aspects of trophoblast cell biology.

2. Materials

1. Culture media:
   a. Standard Growth Medium: RPMI-1640 culture medium (Mediatech Cellgro, Herdon, VA) containing 50 μ*M* 2-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA), 1 m*M* sodium pyruvate (Sigma Chemical Co., St. Louis, MO), 100 μg/mL penicillin, and 100 U/mL streptomycin (Mediatech Cellgro), and 20% heat-inactivated fetal bovine serum (FBS, Altanta Biologicals, Norcross, GA).
   b. Standard Differentiation Medium-Type I: NCTC-135 culture medium (Sigma) containing 50 μ*M* 2-mercaptoethanol (Bio-Rad), 1 m*M* sodium pyruvate (Sigma), 100 μg/mL penicillin and 100 units/mL streptomycin (Mediatech Cellgro), and 1–10% heat-inactivated donor horse serum (HS; Atlanta Biologicals).

   c. Standard Differentiation Medium-Type II: RPMI-1640 culture medium (Mediatech Cellgro) containing 50 μ*M* 2-mercaptoethanol (Bio-Rad), 1 m*M* sodium pyruvate (Sigma), 100 μg/mL penicillin and 100 U/mL streptomycin (Mediatech Cellgro), and 1% heat-inactivated donor HS (Atlanta Biologicals).
2. Hank's balanced salt solution (HBSS; Sigma).
3. Cell Dissociation Medium: Trypsin-ethylenediamine tetraacetic acid (EDTA) Solution (0.25% Trypsin/0.1% EDTA in HBSS) (Mediatech Cellgro).
4. Cell Freezing and Storage Medium: Standard Growth Medium containing 10% dimethylsulfoxide (Sigma) and an additional 25% FBS (Atlanta Biologicals).
5. Cryovials (2-mL, Nalge Company, Rochester, NY).
6. StrataCooler® Cryopreservation Module (Stratagene, La Jolla, CA).
7. Phosphate-buffered saline (PBS).
8. Crystal Violet Solution: 5% formalin, 50% ethanol, 150 m*M* NaCl, and 0.5% crystal violet (Sigma).
9. TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA).
10. 1% Formaldehyde-agarose gels. Formaldehyde (Fisher Scientific, Pittsburgh, PA); agarose (Sigma).
11. Nylon membranes (Nytran Super Charge, Schleicher & Schuell Biosciences, Inc., Keene, NH).
12. Crosslinker (Model XL-1000, Spectronics Corporation, Westbury, NY).
13. [α-P^{32}]dCTP (Perkin Elmer, Boston, MA).
14. cDNAs and polyclonal antibodies for monitoring proliferating and differentiating trophoblast cells (**Tables 2** and **3**).
15. Androstenedione and progesterone radioimmunoassay kits (Diagnostic Products Corporation , Los Angeles, CA).
16. Extracellular matrix-coated BioCoat® Matrigel™ Invasion chambers (BD Biosciences, Bedford, MA).
17. Diff-Quick stain for cells (Allegiance Scientific Products, McGaw Park, IL).
18. Lipofectamine reagent and OPTI-MEM Reduced Serum culture medium (Invitrogen Life Technologies).
19. Geneticin (Sigma) is prepared as a 40X stock solution (10 mg/mL) in HBSS (Sigma) and stored at 4°C.
20. Holtzman Sprague-Dawley rats are obtained from Harlan Sprague-Dawley (Indianapolis, IN).

3. Methods

3.1. Routine Maintenance and Expansion of Rcho-1 Trophoblast Stem Cells

1. Rcho-1 trophoblast cells are routinely maintained in 75-cm^2 flasks in Growth Medium, in an atmosphere of 5% CO_2/95% air at 37°C in a humidified incubator. Cells are grown under subconfluent conditions. Initially, cells are plated at 1–2 × 10^6 cells per flask and fed at two day intervals (*see* **Notes 1–3**).

Table 2
Genes Expressed in Proliferating Rcho-1 Trophoblast Stem Cells

Gene	Functional group	GenBank accession no.	References
Cdx2	Transcription	AJ278466	unpublished[a]
Eomes	Transcription	AY457971	unpublished[a]
Id-1	Transcription	L23148	***17*** and unpublished[a,b]
Mash2	Transcription	X53724	***17*** and unpublished[a]
SOCS 3	Signal transduction	AF075383	***32*** and unpublished[a]
Cyclin D3	Cell cycle	D16309	***14*** and unpublished[b]

Abbreviations: Eomes, Eomesodermin; Id-1, Inhibitor of DNA binding 1; Mash, mammalian achaete schute; SOCS3, suppressor of cytokine signaling 3.

[a]Sahgal, N., Canham, L. N., and Soares, M. J., unpublished results.

[b]Canham, L. N., Sahgal, N., and Soares, M. J., unpublished results.

Table 3
Trophoblast Giant Cell-Associated Genes Expressed in Differentiating Rcho-1 Trophoblast Cells[a]

Gene	GenBank accession no.	Antibodies: source (cat. no.)	References
PRL family			
PL-I	D21103	Chemicon International, Temecula, CA (AB1288)	***9,13,26,38,44***
PL-II	M13749	Chemicon (AB1289)	***9,13,26,38,44***
PLP-A	NM_017036	Chemicon (AB1290)	***9,13,44***
PLP-Fα	NM_022530	None currently available	***42,44***
PLP-M	NM_053791	None currently available	***44***
Steroidogenic regulators			
P450scc	J05156	Chemicon (AB1244, AB1294)	***35,36***
3β-HSD	L17138	None currently available	Unpublished[b]
P450c17	NM_012753	See references	***37***
Others			
PSG36	M32474	None currently available	Unpublished[b]
HAND1	NM_021592	Santa Cruz Biotechnology, Santa Cruz, CA (sc-9413)	***17*** and unpublished[c]

Abbreviations: PRL, prolactin; PL, placental lactogen, PLP, prolactin-like protein; P450scc, side chain cleavage; P450c17, 17α hydroxylase; 3βHSD, 3β hydroxysteroid dehydrogenase; PSG, pregnancy specific glycoprotein.

[a]This list of genes reflects the trophoblast giant cell phenotype of the differentiating Rcho-1 trophoblast stem cells.

[b]Canham, L. N., Sahgal, N., and Soares, M. J., unpublished results.

[c]Sahgal, N., Canham, L. N., and Soares, M. J., unpublished results.

2. After 48 h of culture, 5 mL of Growth Medium is added to each flask.
3. Following an additional 24 h (72 h from the time of initial plating), the culture medium is removed, cells are washed with HBSS, and then briefly (1–2 min) exposed to 3–4 mL of Cell Dissociation Medium, followed by vigorous agitation of the culture flask.
4. Following dissociation of the cells from the culture flask, an equal volume of Standard Growth Medium is added to inactivate the trypsin-EDTA.
5. Cells are collected by centrifugation, resuspended in Standard Growth Medium, and re-plated at a splitting ratio of 1 to 3.
6. Under normal conditions the cells are usually passaged at 3-d intervals.

3.2. Cloning by Limiting Dilution (see *Note 4*)

Limiting dilution strategies can be used to obtain clones of Rcho-1 trophoblast stem cells. Cells are harvested and counted with the aid of a hemacytometer. Cells are distributed into 96-well plates at an estimated concentration of one-half of a cell per well. The number of cells per well should be verified. Under standard growth conditions, colonies of cells can be observed within a week of culture in approx 40–50 wells of the 96-well plate. Colony outgrowths are then harvested and expanded.

3.3. Freezing, Storage, and Retrieval (see *Note 5*)

Rcho-1 trophoblast stem cells can be routinely frozen, stored frozen in liquid nitrogen, and retrieved for the establishment of new cultures.

1. Cells are harvested and counted with the aid of a hemacytometer.
2. Cells are equilibrated in Cell Freezing and Storage Medium at a concentration of $1–2 \times 10^6$ cells/mL.
3. One milliliter aliquots of the cell suspension are then transferred into 2-mL cryovials.
4. Cryovials are positioned within a StrataCooler® Cryopreservation Chamber precooled to 4°C.
5. The Cryopreservation Chamber is transferred to –80°C.
6. After 3 d to 3 wk at –80°C, frozen vials are moved to a liquid nitrogen storage container, where they can be stored indefinitely.
7. Upon retrieval, frozen aliquots should be rapidly thawed at 37°C, washed once in Standard Growth Medium, and reseeded into culture plates.

3.4. Method to Monitor Trophoblast Cell Proliferation/Survival (13) (see *Note 6*)

1. Cells are harvested and counted with the aid of a hemacytometer.
2. A total of 500 cells per well are transferred in Standard Growth Medium to a 24-well plate.
3. Following cell attachment overnight, the culture medium is replaced and treatments added. Medium is changed as required over the treatment period. Standard Growth Medium is used as a positive control for maximal growth.

4. After a maximum of seven days, the wells are rinsed with PBS, and stained with Crystal Violet Solution (300 µL/well) for 10 min with agitation.
5. Cell cultures are then washed repeatedly in tap water, and allowed to dry.
6. Crystal violet dye is then eluted with ethylene glycol.
7. Cell density can be quantified by measuring absorbance of each eluate at 600 nm. In this assay, cell number is directly correlated with absorbance of the cellular eluates.

3.5. Induction of Trophoblast Cell Differentiation (see *Notes 7 and 8*)

Trophoblast giant cell differentiation is induced by growing Rcho-1 trophoblast stem cells to confluence in Standard Growth Medium and then replacing the medium with differentiating conditions. High cell density and the absence of mitogens (removal of FBS) facilitate trophoblast giant cell differentiation.

1. Cells are harvested and counted with the aid of a hemacytometer.
2. A total of $1–2 \times 10^6$ cells in Standard Growth Medium are plated in a 75 cm^2 flask.
3. The cells are fed after 48 h with Standard Growth Medium.
4. After another 24 h, one of two protocols can be used to promote differentiation.
5. Protocol I involves replacing the culture medium with Differentiation Medium Type I. Cultures are re-fed daily and the appearance of giant cells is evident within 2–4 d (**Fig. 2**). Differentiation is progressive and differentiated cells maintained in culture for up to 3 wk.
6. Protocol II involves replacing the culture medium with Differentiation Medium Type II. Cultures are re-fed daily for 6 to 8 d and then the cells are returned to Standard Growth Medium with daily changes for another 6 to 8 d. Trophoblast giant cells are evident as in Protocol I; however, become more robust in size during the reintroduction of Standard Growth Medium (**Fig. 2**).

3.6. Methods to Evaluate Trophoblast Cell Differentiation (see *Note 9*)

Trophoblast differentiation can be assessed by monitoring changes in cell morphology/endoreduplication, changes in gene expression, the production of steroid and polypeptide hormones, and invasiveness.

3.6.2. Morphology/Endoreduplication

Differentiated trophoblast giant cells are easy to recognize and distinguish from undifferentiated trophoblast stem cells. They are large cells with an enlarged nucleus and prominent nucleoli. These cells arise by endoreduplication and their DNA content is polyploid. Nuclear size is proportional to DNA content. Differentiated trophoblast giant cells can be easily quantified by monitoring nuclear size by image analysis ***(9)*** or by monitoring cellular DNA content by flow cytometry ***(2)***.

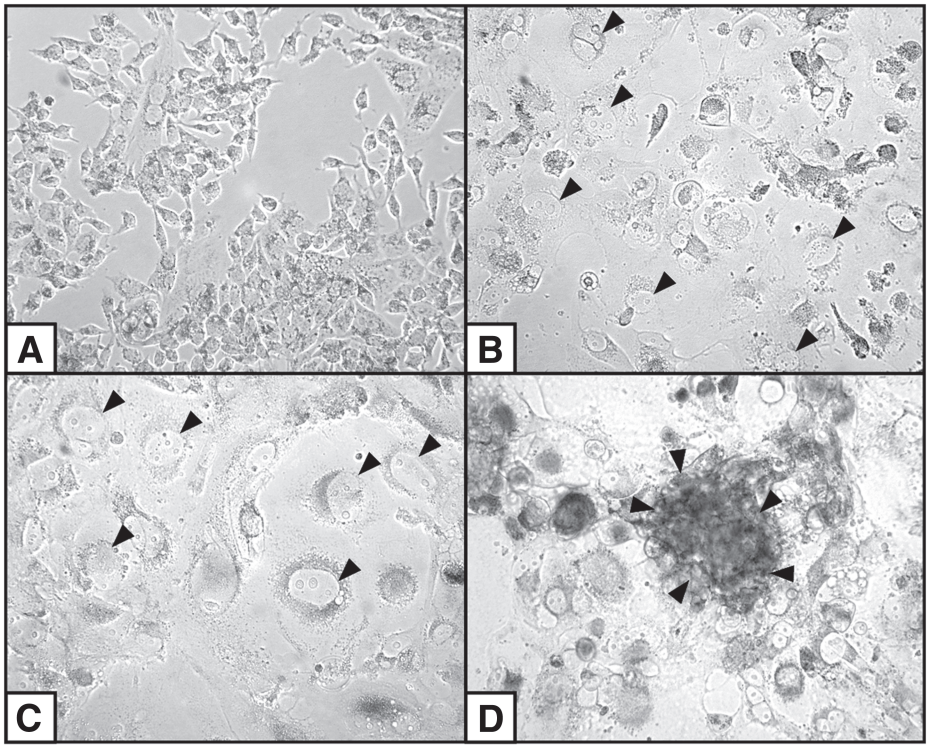

Fig. 2. Morphology of Rcho-1 trophoblast cells at different stages of differentiation. **(A)** Proliferative phase, containing primarily trophoblast stem cells; **(B)** cells induced to differentiate using Protocol I (withdrawal of the mitogen); **(C)** cells induced to differentiate using Protocol II (withdrawal of mitogens + reintroduction of fetal bovine serum [FBS]); **(D)** development of new trophoblast stem cell colonies following reintroduction of FBS.

3.6.2. Gene Expression

The differentiation status of the Rcho-1 trophoblast stem cells can be routinely monitored by Northern blotting.

1. Total RNA is extracted from cells using TRIzol reagent, resolved in 1% formaldehyde-agarose gels, transferred to nylon membranes, and crosslinked.
2. Blots are probed with α-P^{32}-labeled cDNAs (**Tables 2** and **3**).
3. cDNA for a housekeeping gene is used to evaluate the integrity and equal loading of RNA samples (*see* **Note 10**).

3.6.3. Hormone Production

Steroid and peptide hormones accumulate in conditioned medium accompanying the differentiation of trophoblast giant cells. Progesterone and androstenedione are the two major steroid products. They can be measured with commercially available radioimmunoassays ***(35–37)***. Production of members of the prolactin family of polypeptide hormones (placental lactogen-I, placental lactogen-II, and prolactin-like protein-A) are monitored by Western blotting ***(34)***.

3.6.4. Invasion (see ***Note 11***)

The invasive phenotype of trophoblast cells can be assessed by determining the directional movement of cells through an extracellular matrix ***(75)***.

1. Rcho-1 trophoblast stem cells are seeded at 5×10^4 per 3 mL in Standard Growth Medium on the upper chamber of an extracellular matrix-coated BioCoat Matrigel Invasion chamber.
2. Cells are incubated at 37°C in a water-jacketed incubator set at 5% CO_2.
3. The cultures are continued for various durations.
4. Chambers are then removed and the matrix and cells on the upper surface are scraped and the membrane fixed and stained with Diff-Quick.
5. Chamber membranes are then excised and placed on slides, overlayed in immersion oil, and cells that invaded and attached to the under surface of the chamber can be counted using a microscope ocular grid.

3.7. DNA Transfection of Rcho-1 Trophoblast Stem Cells

DNA can be transferred into Rcho-1 trophoblast stem cells using liposome-mediated procedures. Below is a description of our routine transfection protocol.

1. In a six-well plate, seed 2×10^4 cells per well in 2 mL of Standard Growth Medium.
2. After 2–3 d, the cells are then incubated with a DNA/Lipofectamine mixture (Lipofectamine reagent 10 µL, DNA construct 2 µg, Opti-MEM culture medium 200 µL) at 37°C for 7 h.
3. Following the incubation the DNA/lipofectamine mixture is removed and the medium is changed to either Standard Growth Medium or Standard Differentiation Medium.
4. The activity of proteins encoded by the transfected DNA can be monitored 48–60 h following transfection.
5. Stable DNA transfected Rcho-1 trophoblast stem cell sublines can be generated through the introduction of DNA plasmids containing cassettes for selectable genes such as those encoding for neomycin resistance. Effective selection for neomycin resistance generally requires exposure to geneticin at a concentration of 250 µg/mL for 2 to 3 wk.

3.8. Transplantation and In Vivo Maintenance of Rcho-1 Trophoblast Stem Cells (see *Note 12*)

The kidney capsule serves as an effective growth environment for Rcho-1 trophoblast stem cells.

1. Cells are harvested from cultures and counted with the aid of a hemacytometer.
2. Cells ($1–5 \times 10^6$) are transferred beneath the kidney capsule of 4-wk-old female rats (we routinely use Holtzman Sprague-Dawley rats) in a volume of 25–40 µL using a 27-gauge needle and 1-mL syringe.
3. The cells grow rapidly and must be harvested after 10–12 d.
4. Harvested transplants can also be minced and transferred beneath the kidney capsule of additional recipient animals.
5. Rcho-1 trophoblast stem cells transplanted beneath the kidney capsule have the potential to exhibit both endocrine and invasive phenotypes.

4. Notes

1. We routinely use RPMI-1640 culture medium as a base growth medium. Rcho-1 trophoblast stem cells grow vigorously in RPMI-1640 culture medium but sometimes at the cost of poor pH regulation. We compensate for the lack of pH control by changing the culture medium more frequently (daily) and/or by supplementing the cultures with HEPES (10–20 m*M*). High humidity is essential for optimal Rcho-1 trophoblast stem cell growth. A serum-free system has not been defined for propagating the Rcho-1 trophoblast stem cells. At this juncture the inclusion of FBS is essential. We routinely use high concentrations (20%) of FBS, which the cells appear to prefer. The high FBS concentration may also minimize some of the variabilities associated with different lots of serum.
2. Cell density is a key for the appropriate maintenance and expansion of the Rcho-1 trophoblast stem cell line. The most common problem in working with Rcho-1 trophoblast stem cells is the desire to grow them to confluence. Confluence and proliferation are not compatible. As the cells become more dense, they begin to spontaneously differentiate or die. The differentiating cells have a more flattened appearance and will ultimately develop into trophoblast giant cells, whereas the dead cells lift from the surface of the culture plate. In order to prevent spontaneous cell death or differentiation, the Rcho-1 trophoblast stem cells must be passaged as recommended.
3. Rcho-1 trophoblast stem cell cultures are heterogeneous. Both proliferative and differentiated cells can be observed in expanding cultures. Manipulating various aspects of the culture procedure can influence the cellular composition of the cell line. Cell composition can influence growth rates and features of differentiation. Maintaining the cells at higher densities or any type of significant stress (humidity, pH, CO_2 deprivation, and so on) can lead to differentiation (giant cell formation) or cell death, both of which result in an irreversible termination of the culture. Harvesting the Rcho-1 trophoblast cells following brief treatment with

trypsin-EDTA results in isolation of a population of cells enriched in stem cells. This procedure also results in the enrichment of differentiated cells (trophoblast giant cells) that are more adherent and not removed by brief exposure to the trypsin-EDTA solution. Harvesting the differentiated cells generally requires more vigorous dissociation methods such as scraping with a rubber policeman. Unfortunately, the yield of intact trophoblast giant cells by this technique is not optimal. Consistency in cell culture practices is extremely important in working with the Rcho-1 trophoblast stem cell line. Variations in culture densities, passaging methods, and splitting ratios significantly influence the phenotype of the cell line.

4. Rcho-1 trophoblast stem cells grow well at low density, especially in the presence of culture medium containing 20% FBS, and clonal lines can be easily derived. The main concern in isolating clonal lines from Rcho-1 trophoblast stem cells is obtaining a single cell suspension and preventing cell aggregation during their dispersal into multi-well plates.
5. Freezing, storage, and retrieval of Rcho-1 trophoblast stem cells require considerable care. In recent years, we have increased the concentration of FBS in the freezing medium, which seems to improve cell viability at retrieval. We are also careful to rapidly thaw the cells at 37°C and remove the freezing medium by centrifugation before culture. If performed well, the cultures are revived within 24 h and ready to passage in another 48 h. Nonetheless, retrieval of cultures from frozen cell aliquots has been our biggest problem in distributing the Rcho-1 trophoblast stem cells to other laboratories. Because of these problems, we routinely distribute the cells as live cultures.
6. We have described a simple dye-based colorimetric technique for monitoring cell proliferation. There are many other strategies that can be used (cell counts, flow cytometry, and so on). However, it is important to appreciate that a key component of differentiation in Rcho-1 trophoblast stem cells is endoreduplication, e.g., DNA synthesis, without karyokinesis and cytokinesis. Thus, strategies for monitoring Rcho-1 stem cell proliferation that involve monitoring the incorporation of a nucleotide or nucleotide analog will not discriminate between DNA synthesis associated with proliferation and differentiation.
7. One of the experimental advantages of the Rcho-1 trophoblast stem cell line is its capacity to differentiate. We have developed a couple of protocols for enriching differentiated trophoblast cells. These involve achieving high cell density and removal of mitogenic factors. We have the most experience in shifting the cells to an NCTC 135 basal medium containing HS. Morphological and biochemical indices of trophoblast giant cell differentiation are evident within a few days. However, we have noted that the size of the trophoblast giant cells that appear in these cultures is generally much smaller than those appearing spontaneously in the expanding cells cultured in FBS. Consequently, we have recently implemented a second protocol for differentiation. The new strategy involves cell expansion, followed by mitogen withdrawal, and then re-introduction of Standard Growth Medium. Within a few days large trophoblast giant cells appear

throughout the cultures (**Fig. 2**). As these cultures are maintained in Standard Growth Medium, colonies of stem cells will also begin to appear. Cells in these colonies are tightly packed and rise above the surface of the plate. If needed, the stem cell colonies can be removed by brief trypsinization without detachment of the differentiated trophoblast giant cells. In both protocols, mitogen withdrawal is the key. In the absence of FBS, some cells differentiate, others die, and some stem cells apparently become dormant. The enhanced trophoblast giant cell formation following re-introduction of Standard Growth Medium suggests that endoreduplication is stimulated by factors present in FBS.

8. Under our culture conditions, Rcho-1 trophoblast stem cell differentiation is most prominently directed toward the trophoblast giant cell lineage. Giant cell formation proceeds over time and may be accelerated by re-introduction of FBS containing medium. Evidence for differentiation along other trophoblast cell lineages (**Fig. 1**; spongiotrophoblast cells, glycogen cells, syncytial trophoblast, and the specialized invasive trophoblast cells of the metrial gland) is apparent but generally modest to minimal. This restricted differentiation to trophoblast giant cells is likely, at least in part, a reflection of culture conditions rather than developmental capabilities of the Rcho-1 trophoblast stem cells. We may be able to learn from differentiation strategies developed for studying embryonic stem cells ***(79)***. Other cell lineages can be detected by monitoring the expression of genes or gene products specific for spongiotrophoblast cells, syncytial trophoblast, and the specialized invasive trophoblast cells of the metrial gland (**Table 4**). Glycogen cells are generally identified by their accumulation of glycogen. Exposure of differentiating cells to dimethylsulfoxide can inhibit trophoblast giant cell differentiation and reactivate part of the trophoblast stem cell phenotype (Sahgal, N., Canham, L., and Soares, M. J., unpublished results).
9. Balzarini and colleagues use alkaline phosphatase enzyme activity as a measure of differentiation of RCHO trophoblast stem cells ***(22,25)***. The assay is simple and can readily be adapted to a multi-well format. We have not utilized the assay mainly because alkaline phosphatase is known to be expressed in many cell types and thus does not reflect a specific measure of trophoblast cells.
10. We have utilized an assortment of different housekeeping genes to monitor RNA integrity and loading efficiency. These have included β-actin, glyceraldehyde-3'-phosphate dehydrogenase (G3PDH), β-tubulin, and 28S ribosomal RNA. Some of these, including G3PDH and β-tubulin are sometimes problematic in that their expression is affected by cell differentiation or the treatments employed.
11. Aspects of the invasive phenotype can also be monitored by determining the expression of gelatinase B and/or α1 integrin and through the analysis of gelatinase B activity in conditioned medium by substrate gel electrophoresis (zymography; *see* **ref. *75***).
12. Rcho-1 trophoblast stem cells can be maintained in vivo by transplantation into various host tissues. We have routinely used the kidney capsule but these cells have also been successfully transplanted to other sites, including the liver, cerebral ventricles, lungs, testes, and uteri of rats ***(7,10,11,85–92)***. In vivo transplan-

Table 4
Other Trophoblast Cell Lineage-Specific Gene Markers

Trophoblast Cell lineage	Gene name	GenBank accession no.	References
Spongiotrophoblast	PLP-B	M31155	***80,81***
	PLP-Fβ	AY741310	Unpublished[a]
	SSP	NM_172073	***82***
Syncytial trophoblast	GCM-1	NM_017186	Unpublished[b]
Invasive trophoblast	PLP-L	NM_138527	***5,83***
	PLP-N	NM_153738	***84***

Abbreviations: PLP, prolactin-like protein; SSP, spongiotrophoblast-specific protein; GCM-1, Glial cell missing-1.

[a]Ho-Chen, J., Bustamante, J. J., and Soares, M. J., unpublished results.

[b]Sahgal, N., Canham, L. N., and Soares, M. J., unpublished results.

tation of the Rcho-1 trophoblast cells has been effectively used to elevate circulating levels of lactogenic hormones. The predominant lactogen expressed by the transplants appears to be PL-I. Lactogenic and luteotrophic actions on the mammary glands and ovary, respectively, represent effective indicators of systemic action of the products of the transplants. Please be aware that Rcho-1 trophoblast cells are potentially capable of producing other peptide and steroid hormones; thus the physiological consequences of trophoblast stem cell transplantation may be complex.

Acknowledgments

We would like to thank past and current members of our laboratory for their efforts in developing and characterizing the methods described in this chapter. This work was supported by a National Institutes of Health (NIH) KO8 award to NS (HD42171) and grants from the NIH (HD20676, HD39878, HD48861) and the Hall Family Foundation.

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