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A Rat Primary Hepatocyte Culture Model for Aging Studies

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Abstract

The purpose of this protocol is to establish a primary hepatocyte culture system as a suitable model to examine age-related changes in Phase II detoxication gene expression. Hepatocytes are isolated using a two-step collagenase perfusion technique from young (3 to 6 months) and old (24 to 28 months) rats and placed in primary culture using collagen (Type I)-coated plates as the extracellular matrix. A supplemented William's E Medium is used as the medium. This culture system maintains hepatocyte viability from both young and old rats for ~60 hr, as measured by lactate dehydrogenase activity, while also maintaining their respective phenotypes relative to Phase II detoxification. We thus conclude that a collagen-based cell culture system is suitable to study age-associated deficits in Nrf2/ARE-mediated Phase II gene regulation provided that experiments can be conducted within 60 hr after cell isolation.

Keywords

collagen Type 1; William's Medium E; primary hepatocyte cell culture; aging; Phase II detoxification

INTRODUCTION

There is growing awareness that Phase II detoxification processes become impaired with age. However, work in this area has been hampered because of the lack of experimental models that maintain the physiological aging context but also allow molecular manipulation to study gene expression reliably. These criteria exclude immortalized cell lines, the main experimental system for molecular biology, as transformed cells cannot adequately mimic the cellular aging phenotype of the liver. Freshly isolated hepatocytes taken from animals of appropriate ages may be an excellent means to study age-related molecular and cellular changes that affect detoxification reactions. Isolated rat hepatocytes only survive for a few hours after collagenase dispersion if they are not placed in culture. Thus, appropriate culture conditions must be used in order for differentiated primary hepatocytes to be used as a cellular model for Phase II-dependent reactions. Therefore, we have established a primary hepatocyte culture system that will permit us to address mechanistic questions related to age-associated changes in cell function.

This unit describes procedures for isolating parenchymal hepatocytes from the livers of young and old rats (see Basic Protocol 1), culturing the hepatocytes on collagen-coated

plates (see Basic Protocol 2), and for assessing their viability by using the lactate dehydrogenase (LDH) release assay (see Support Protocol).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All solutions and equipment coming into contact with hepatocytes after the isolation procedure is complete must be sterile and aseptic technique used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless specified otherwise.

BASIC PROTOCOL 1: ISOLATING HEPATOCYTES FROM YOUNG AND OLD RATS

This procedure describes the initial isolation of hepatocytes from rat livers and purification for parenchymal cells. Dissection can be conducted on a laboratory surgical table and need not be conducted in an aseptic environment for short-term culture of the hepatocytes. The highest number of healthy viable hepatocytes is achieved by a well-coordinated dissection when materials and solutions are prepared fresh, and the total time between anesthesia and purification of isolated hepatocytes is minimized. Hepatocyte isolation from a second rat can be started as soon as the dissociation of liver cells from the first rat is underway.

A liver from a young rat typically provides 400 to 500 million hepatocytes, while a liver from an old rat yields upwards of 600 million cells. Depending on the experimental protocol, this yield may be far more hepatocytes than can be conveniently used at once. For example, for viability assessments, gene expression determination, and nuclear extract preparation, hepatocytes are plated at 2 million cells/well of a 6-well plate. Thus, a single rat would provide hepatocytes for hundreds of cultures. Cell suspensions may therefore be cryopreserved and used for other studies (Stevenson et al., 2007).

Materials

Pre-Hanks' 1× solution (see recipe), prewarmed to 37°C and bubbled for 30 min with carbogen gas

Hanks' I solution with BSA and EGTA (see recipe)

Hanks' II solution with $CaCl_2$ (see recipe)

Heparin (0.2% in saline; sterile filtered)

Young (4 to 6 months old) and/or old (24 to 28 months old) Fischer 344 rats

Ethyl ether

Collagenase D (Boeheringer-Manheim)

Krebs' 1× solution, pH 7.4 (see recipe), bubbled for 30 min with carbogen gas

DNase I (Sigma)

- 0.4% (w/v) trypan blue
- 37°C water bath
- Perfusion apparatus including:
  - Peristaltic pump and tubing
  - Glass vial for bubble trap
- Inverted microscope
- Cotton swabs
- Vacuum aspirator
- Carbogen tank and tubing
- 1-ml syringe
- Anesthetizing chamber
- Nose cone
- Dissecting steel pan (sacrifice tray)
- Tape
- Dissection tools including:
  - Surgeon’s scissors
  - Cross-action forceps
  - Iris scissors
  - Blunt forceps
  - Hemostats
  - 18-G and 21-G surgical needles for designing cannulae for young and old rats, respectively
  - 23-G needle for heparin injection
- Kimwipes
- 0.4-mm surgical sutures
- Glass rod
- Gauze pads
- 500-ml Erlenmeyer flask
- 50-ml round-bottom flask
- Rotavapor (Buchi Laborteknik AG)
- 0.5-ml microcentrifuge tube
- Hemacytometer and coverslip

Set up for surgery

1 Place pre-Hanks' 1×, Hanks' I, and Hanks' II solutions in a 37°C water bath. Maintain the Krebs' solution at room temperature.

2 Secure the bubble trap in the clamp and arrange tubing through the pump teeth so that tubing is able to reach the water bath and easily reach the bubble trap and surgical area.

3 Adjust the perfusion flow rate to 10 ml/min for a young rat and 13 ml/min for an old rat. Transfer the tubing to pre-Hanks' 1× solution.

> It is very important to adjust the flow rate, as a too-low flow rate can lead to incomplete perfusion and low hepatocyte yield, while a too-high flow rate can compromise hepatocyte viability.

4 Fill a 1-ml syringe with a 23-G needle with 0.4 ml (young rat) or 0.6-ml (old rat) heparin solution, remove any air bubbles. Bend the needle to a 90° angle for injection of heparin into the iliac vein.

Prepare animal for surgery

5 Charge the anesthetizing chamber and nose cone with ethyl ether for 5 min.

6 Place the animal in the charged anesthesia chamber. Remove the animal after it is immobilized and the eyes stop blinking.

7 Place the animal on the sacrifice tray under anesthesia (nose cone) being sure to allow enough anesthesia to maintain unconsciousness but enough air as not to suffocate the animal.

Perform the surgery

8 Secure the animal's limbs to the tray with tape, ventral side up. Place the nose cone over the animal's nose so that it receives a mixture of anesthesia and air. Ensure that the animal is insensate before proceeding by pressing firmly on the rear foot pads and watching for a reflex response.

9 Use the surgeon's scissors to make a midline incision and lateral incisions on each side.

> Take care not to cut through the diaphragm.

10 Expose the iliac vein by separating the musculature using the forceps to remove the connective tissue. Position the bevel of the 90°-bent needle over the iliac vein and gently slide it in. Slowly inject the heparin (0.4 ml for young, 0.6 ml for old rats). Place a Kimwipe over the injection site to minimize exsanguination while slowly withdrawing the needle.

11 Use the cross-action forceps and the 0.4-mm surgical sutures to place a loose ligature around the vena cava and secure the end of the ligature with a hemostat. Place two more ligatures ~1 cm apart around the portal vein near the base of the liver, also securing loosely with hemostats.

12 Make a small incision between the two portal vein ligatures, being careful not to sever the portal vein completely.

13 Quickly place the cannula into the portal vein incision and secure the cannula by tightening the ligature around the portal vein closest to the liver. Tighten the ligature around the portal vein furthest from the liver to secure the cannula to the vein for additional support.

14 Use the surgeon's scissors to cut upward completely through the sternum/rib cage. Use the iris scissors to cut the diaphragm and the vena cava above the liver, also tightening the vena cava ligature.

15 Supporting the liver by the cannula, use the iris scissors and the forceps to sever the connective tissue between the liver and the stomach and the spleen. Cut through any adipose, connective, and vasculature tissue beneath the perfusion area.

16 While holding up the liver by the cannula, sever the connective tissue between the liver and the dorsal coelomic area (cut along the spine) to completely free the liver.

Use extreme caution to avoid damaging the liver during excision.

17 Let the liver perfuse with Hanks' I solution for another 2 to 3 min to remove any residual blood.

Digest the liver tissue

18 While the liver is perfusing, add collagenase D to the Hanks' II solution (40 to 45 mg for young and 60 to 65 mg for old rats in 100 ml Hanks' I solution).

19 Perfuse the liver for 1 min in the Hanks' II solution, allowing the liquid to flow onto the tray.

This will remove any residual EGTA remaining from the Hanks' I perfusion.

20 Transfer the liver to the beaker containing Hanks' II solution and collagenase D and submerge liver completely to maintain temperature and so that the perfusate will continually recirculate; allow the perfusion to continue until the liver feels soft and displays a cauliflower-like appearance.

Isolate and purify the hepatocytes

21 Detach the cannula from the tubing.

22 Using a glass rod, gently massage the liver against the side of the beaker containing the Hanks' II solution and collagenase D to begin to liberate hepatocytes.

The liver massage for extracting hepatocytes can be continued until the liver looks like a transparent sac.

23 Filter the cell mixture through gauze into a 500-ml Erlenmeyer flask to remove any larger contaminants (connective tissue or undissociated liver). Rinse the gauze with ~150 ml of Krebs' 1× solution.

24 Allow the cells to settle for 20 to 30 min.

These steps are undertaken to separate the hepatic parenchymal cells from the stellate cells on the basis of differences in their densities. The parenchymal cells settle out, and the stellate cells are largely removed by the aspiration. The hepatic parenchymal cells are primarily involved in detoxification reactions.

25 Aspirate the solution above the settled cells.

Aspirate as much Krebs' buffer as possible without losing cells. This will leave a small layer of buffer atop the cells.

26 Swirl the cells to resuspend. Add 1 to 2 mg DNase I directly into to the cell solution. Swirl again gently until the cell suspension is homogeneous.

27 Add 100 to 120 ml Krebs' 1× solution to the cells, swirling gently to mix. Let the cells settle and remove the supernatant.

28 Repeat the wash cycle one more time for a total of three washes.

29 After the final aspiration, measure the volume of the cell suspension (~20 ml) and then transfer the cell suspension to a 50-ml round-bottom flask.

30 Secure the flask to a Rotavapor, and gently rotate the cells.

Rotate the cells for at least 1 hr, but no more than 4 hr.

Measure cell viability by trypan blue exclusion

31 In a 0.5-ml microcentrifuge tube, add 90 µl Krebs' 1× solution + 90 µl trypan blue +20 µl cells. Mix well.

32 Put 10 µl of the cell suspension on a hemacytometer. Count total cells and blue cells in 4 quadrants.

33 Calculate the number of cells in the preparation.

$$\text{Total counted cells} - \text{number of blue cells} = \text{number of viable cells in the } 10 - \mu\text{l aliquot}.$$
$$\text{Cell number} \times 10,000 \times \text{dilution factor} = \text{cells/ml}.$$
$$\text{Cells/ml} \times \text{number of ml in the isolate} = \text{total yield (total number of live cells)}.$$

Viability is typically ~94%. It should not be <85% to carry out experiments.

BASIC PROTOCOL 2: CULTURING PRIMARY RAT HEPATOCYTES ON COLLAGEN-COATED PLATES

There is no general consensus for the optimal set of culture conditions for primary rat hepatocytes, in part because the appropriate plating substrate, culture medium, additives, and cell density may vary depending on experimental design. This protocol describes plating of hepatocytes on collagen-coated, plastic 6-well culture dishes in William's Medium E containing fetal bovine serum and other supplements. See Background Information for a brief description of the function of each additive in maintaining optimum hepatocyte function. The following should be performed under aseptic conditions.

Materials

Collagen I (rat tail; Sigma)

50 mM HCl Sterile water

Hanks' Balanced Salt Solution (HBSS; Sigma)

Complete William's Medium E (Sigma; see recipe)

Hepatocyte cell suspension (see Basic Protocol 1)

37°C water bath

6-well plastic cell culture dishes (such as Corning or Nunc brands)

Laminar flow hood

Pipet tips, sterile

Prepare collagen-coated plates

1 Suspend collagen in 50 mM HCl for a final concentration of 1 mg/ml.

This solution will be diluted 3 parts collagen to 50 parts water to obtain the final working solution in step 3. Store any extra stock collagen up to 6 months at 4°C.

2 Incubate the collagen-HCl suspension in a 37°C water bath until the collagen is completely dissolved.

3 Dilute the collagen solution in sterile water for a concentration of 60 μg/ml.

4 Place 6-well culture plates in a laminar flow hood.

5 Pipet 1 ml of collagen solution into each well, replace lids, and place in the 37°C incubator for 4 hr.

Many other substrates are used for hepatocyte culture, including fibronectin, laminin, and combinations of these with collagen. Several companies also sell precoated dishes (e.g., Biocoat dishes from BD Biosciences). Other substrates used as matrices in hepatocyte cell culture include proprietary formulations like Matrigel (BD

Biosciences) and AlgiMatrix (Invitrogen) provide a three-dimensional matrix.

6 Rinse each well three times, each time with 2 ml HBSS to neutralize the acid. Aspirate the last rinse.

If dishes are to be used immediately, proceed to cover the wells with medium. However, dishes may be used for ≥2 weeks after coating, if they are stored dry at 4°C.

Culture hepatocytes

7 Dispense 2 ml of complete William's Medium E into each well and leave in the incubator for 30 min to equilibrate with the substrate/matrix.

8 Determine the desired number of cells per well and calculate the volume of hepatocyte suspension needed to achieve this number. In the laminar flow hood, using sterile pipet tips, pipet the appropriate volume of cell suspension into each well containing complete William's Medium E.

The hepatocyte cell density is crucial for toxicological studies. For a high-density plating, 2 million cells per well of a 6-well plate was found to be an appropriate number.

The hepatocytes will settle to the bottom of the flask before they are plated. If necessary, gently resuspend the cells before drawing them into the pipet.

9 Replace the medium after 4 hr with 2 ml/well fresh William's Medium E.

This step is especially important for high-density hepatocyte cultures to remove debris produced during collagenase digestion and cell dissociation. Perform all subsequent experiments only after an overnight incubation.

10 Replace the medium every 24 hr during the entire cell culture period.

SUPPORT PROTOCOL: DETERMINATION OF HEPATOCYTE VIABILITY BY LACTATE DEHYDROGENASE (LDH) RELEASE

Cytotoxicity in primary culture of rat hepatocytes can be measured by two methods: cytosolic enzyme leakage and detachment of damaged cells from the monolayer culture. Leakage of LDH, an intracellular enzyme, is typically used for hepatocyte viability measurements. The lactate dehydrogenase method is simple, accurate, and yields reproducible results. When a cell is damaged, LDH is released into the culture medium. Thus, by measuring the intracellular LDH activities of cells that remain intact and attached to the collagen substrate, hepatocyte survival in culture can be determined. The protocol presented here is a modification of the method developed by Moldeus and coworkers (Moldeus et al., 1978). In this assay, LDH activity is measured by following the consumption of NADH during the conversion of pyruvate to lactate.

Materials

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

10% Triton X-100

Potassium phosphate buffer (0.1 M, pH 7.4)

NADH, 20 mM

20 mM sodium pyruvate

1-ml microcentrifuge tube

UV spectrophotometer

1. Wash each well twice, each time with 2 ml PBS.
2. Add 10 µl of 10% Triton X-100 in 1 ml potassium phosphate buffer to each well to lyse hepatocytes.
3. Scrape cells and transfer to a 1-ml microcentrifuge tube.
4. Incubate on ice for 30 min.
5. Prewarm the spectrophotometer to 37°C and turn on UV bulb to allow beam to stabilize.
6. Add 50 µl of cell lysate to 2.85 ml of reaction buffer (0.1 M phosphate buffer, pH 7.4, 5 mM NADH).
7. Initiate the reaction by adding 100 µl of 20 mM sodium pyruvate solution.
8. Measure the rate of decreasing absorbance at 340 nm.

   A unit of LDH activity is defined as an optical density change of 0.001 per minute.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**. All reagents and chemicals in the recipes are from Sigma unless otherwise specified.

Complete William's Medium E (in William's E base medium)

5% FBS

1:100 (v/v) antibiotic/antimycotic mix containing 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 25 µg/ml amphotericin B (ATCC)

2 mM L-glutamine

100 ng/ml insulin

1 µM dexamethasone

Store up to 2 months at 4°C

Hanks' stock solution, 10×

80 g NaCl

4 g KCl

0.6 g Na_2HPO_4

0.6 g KH_2PO_4

Add H_2O to 1 liter

Store up to 2 months at 4°C

Hanks' I solution, pH 7.4

100 ml Pre-Hanks' 1× solution (see recipe)

2 g bovine serum albumin

0.5 ml of 120 mM EGTA

Adjust pH to 7.4 using 1 N HCl

Prepare fresh

Hanks' II solution, pH 7.4

100 ml Pre-Hanks' 1× solution (see recipe)

1 ml 0.4 M $CaCl_2$

Adjust pH to 7.4 using 1 N HCl

Prepare fresh

Krebs' solution, 1×, pH 7.4

250 ml Krebs' 2× stock solution (see recipe)

1.5 g HEPES

0.18 g glucose

0.5 g glutamate

Add H_20 to 500 ml

Adjust pH to 7.4 using 1 N HCl

Prepare fresh

Krebs' stock solution, 2×

13.8 g NaCl

0.72 g KCl

0.59 g $MgSO_4 \cdot 7H_2O$

4 g $NaHCO_3$

0.26 g KH_2PO_4

Add H_2O to 1 liter

Store up to 2 months at 4°C

Pre-Hanks' solution, 1×

50 ml 10× Hanks' stock solution (see recipe)

3 g HEPES

1 g $NaHCO_3$

Add H_2O to 500 ml

Prepare fresh

COMMENTARY

Background Information

A progressive age-associated decline in stress response mechanisms has been noted in many animal species, which leads to heightened risk for both morbidity and mortality (Harman, 1996; Beckman and Ames, 1998; Balaban et al., 2005; Humphries et al., 2006). However, the molecular mechanisms leading to this overt aging phenotype have yet to be elucidated. In part, this is because of a lack of in vitro models that can be molecularly manipulated to discern the precise lesion(s) involved in the aging cellular environment. The present protocol describes a primary hepatocyte culture system that fills this gap. Using this protocol, hepatocytes from young and old rats can be kept in primary culture for at least 60 hr. Thus, this model provides a sufficient experimental window to carry out cellular and molecular studies related to detoxification mechanisms during aging.

Methods for culturing rat hepatocytes have been established for a number of years, but to our knowledge no definitive studies have described conditions showing this model is suitable for toxicological research related to aging. Holbrook and co-workers successfully used cultured rat hepatocytes to monitor age-associated changes to cell signaling cascades. They found that a similar culture system, as described herein, was suitable for discerning age-related differences in mitogen-activated protein kinase (MAPK) signaling mechanisms (Liu et al., 1996) and endoplasmic reticulum stress response (Li and Holbrook, 2004). In addition to this, studies from other groups utilized the collagen matrix in combination with various media to demonstrate that the aging phenotype was maintained in culture in a caloric restriction model as well as under growth factor stimulation (Kitano et al., 1998; Lambert and Merry, 2000). Thus, these previous studies combined with the present method suggest that culturing hepatocytes from old rats is an appropriate model to examine a variety of age-dependent changes that affect cell signaling and stress response mechanisms.

Critical Parameters and Troubleshooting

Acclimatization of animal colonies—It is important that animals be acclimatized in the animal facilities for at least 1 week prior to the experimental procedures. Since a major application of the isolation and cell culture of the hepatocytes is to assess age-associated

differences in detoxication capacity, it is critical that the transportation-induced stress does not affect these parameters.

Cell isolation and culture—Cell damage and death begin as soon as the animal is anesthetized. Therefore, speed of surgery, as well as timely dissociation and purification of cells, is of utmost importance. To minimize cell damage, avoid contact with the perfusing liver either by hand or pointed surgical instruments. Instead, support the perfusing liver by the cannula and use a cotton swab to reposition the liver if necessary. After isolation, resuspend hepatocytes in buffer by gently swirling the flask. Do not pipet too vigorously or through a narrow-bore pipet. Viability of hepatocytes before plating is the single-most important predictor of suitability for culture. Discard a hepatocyte preparation that has <85% viability as the survival in culture will not be optimum. This is especially true for hepatocytes isolated from old rats.

Be sure that the medium is replaced 4 hr after plating. It is also critical that experiments be initiated only after an overnight culture. After isolation, levels of stress-induced transcription factors are especially high, thus confounding differences in the aging phenotype. After the initial 4 hour medium replacement, replace medium every 24 hr thereafter for the maintenance of cultures.

Anticipated Results

Viability of hepatocytes upon isolation and in primary culture—The percent viability of the final hepatocyte preparation will typically be ≥85% with a greater percentage of viable hepatocytes from younger rats than from older rats. Thus, there may be a greater sensitivity to collagenase disruption of aged liver and subsequent cellular damage. In this unit, cellular release of lactate dehydrogenase (LDH) was used as means of assessing hepatocyte viability over the culture period. Experiments have shown that almost no cells from old rats remained viable after 60 hr in culture, whereas nearly 50% of cells from young rats were alive at this time point. Hence, hepatocytes from old rats can be cultured under similar conditions acceptable for maintaining viability for cells from young rats. However, experimentation on isolated hepatocytes from old rats should be conducted within 60 hr after isolation.

Time Considerations

Prepare reagents and set up for isolation and culture ideally a day before the surgery. The first step in this process should be the preparation of collagen solution and coating of the plates, so that they have sufficient time to set and be used the next day. If a series of isolations is planned for the whole week, coat all the plates at one time and store them appropriately. Collagen-coated plates are preferably made fresh and used between 3 and 24 hr later. However, we have also achieved good results with plates that were made up to 1 week in advance and stored in sterile wrapping at 4°C. Bring the temperature of the plates to 37°C prior to use. Apply the same considerations for the preparation of the media.

Start the surgery and isolation procedure early in the day to leave enough time for plating and the first medium change. This also gives a sufficient overnight incubation time for the

hepatocytes before the start of experiments. Plate the hepatocytes within 3 hr of isolation to prevent a loss of initial viability prior to plating.

Literature Cited

Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. Cell. 2005; 120:483–495. [PubMed: 15734681]

Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev. 1998; 78:547–581. [PubMed: 9562038]

Harman D. Aging and disease: Extending functional life span. Ann N Y Acad Sci. 1996; 786:321–336. [PubMed: 8687032]

Humphries KM, Szweda PA, Szweda LI. Aging: A shift from redox regulation to oxidative damage. Free Radic Res. 2006; 40:1239–1243. [PubMed: 17090412]

Kitano S, Fawcett TW, Yo Y, Roth GS. Molecular mechanisms of impaired stimulation of DNA synthesis in cultured hepatocytes of aged rats. Am J Physiol. 1998; 275:C146–C154. [PubMed: 9688845]

Lambert AJ, Merry BJ. Use of primary cultures of rat hepatocytes for the study of ageing and caloric restriction. Exp Gerontol. 2000; 35:583–594. [PubMed: 10978680]

Li J, Holbrook NJ. Elevated gadd153/chop expression and enhanced c-Jun N-terminal protein kinase activation sensitizes aged cells to ER stress. Exp Gerontol. 2004; 39:735–744. [PubMed: 15130668]

Liu Y, Guyton KZ, Gorospe M, Xu Q, Kokkonen GC, Mock YD, Roth GS, Holbrook NJ. Age-related decline in mitogen-activated protein kinase activity in epidermal growth factor-stimulated rat hepatocytes. J Biol Chem. 1996; 271:3604–3607. [PubMed: 8631968]

Moldeus P, Hogberg J, Orrenius S. Isolation and use of liver cells. Meth Enzymol. 1978; 52:60–71. [PubMed: 672656]

Stevenson DJ, Morgan C, McLellan LI, Helen Grant M. Reduced glutathione levels and expression of the enzymes of glutathione synthesis in cryopreserved hepatocyte monolayer cultures. Toxicol In Vitro. 2007; 21:527–532. [PubMed: 17196364]