Assays for *in vitro* monitoring of proliferation of human airway smooth muscle (ASM) and human pulmonary arterial vascular smooth muscle (VSM) cells

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Vascular and airway remodeling, which are characterized by airway smooth muscle (ASM) and pulmonary arterial vascular smooth muscle (VSM) proliferation, contribute to the pathology of asthma, pulmonary hypertension, restenosis and atherosclerosis. To evaluate the proliferation of VSM and ASM cells in response to mitogens, we perform a [^{3}H]thymidine incorporation assay. The proliferation protocol takes approximately 48 h and includes stimulating cells synchronized in G_0/G_1 phase of the cell cycle with agonists, labeling cells with [^{3}H]thymidine and examining levels of [^{3}H]thymidine incorporation by scintillation counting. Although using radiolabeled [^{3}H]thymidine incorporation is a limitation, the greatest benefit of the assay is providing reliable and statistically significant data.

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INTRODUCTION

Proliferation is one of the most important cell functions, reflecting cells' ability to respond and adapt to the changing environment in normal tissue homeostasis and in disease states. Asthma and pulmonary arterial hypertension are both characterized by structural changes in the airways or vasculature, e.g., airway or vascular remodeling, respectively. These pathological changes occur, in part, due to increased growth of airway smooth muscle (ASM) or pulmonary arterial vascular smooth muscle (VSM) cells and proliferation stimulated by inflammatory mediators, contractile agonists and growth factors. The *in vitro* proliferation assay of human ASM and VSM cells is a cell-based approach to examining how polypeptide growth factors, contractile agonists, inflammatory mediators and cytokines modulate ASM and VSM proliferation[1–5]. Although the relevant data generated from cell culture models must be viewed with caution, much has been learned about human VSM and ASM cells using *in vitro* assays of cell proliferation.

Cell proliferation can be assessed using three basic approaches: (i) a direct cell count, (ii) a radiographic technique with [^{3}H]thymidine and (iii) immunological 5-bromodeoxyuridine (BrdU) labeling. Each technique has its own strengths and limitations that define the range of its applicability depending on the goals of the study, cell type and available resources. As the focus of this protocol is the [^{3}H]thymidine incorporation assay, the cell count technique and BrdU incorporation assay will be mentioned when appropriate for comparison, which will enable the reader to make a decision as to the suitability of the protocol to the proposed experimental task.

The assessment of proliferation of VSM and ASM cells using the [^{3}H]thymidine incorporation assay can be outlined as follows: cells in their early passage (third or fourth) are grown for 5–7 d to reach near confluence in a 24-well plate and then serum-deprived for 48 h to synchronize them in the G_0/G_1 phase of the cell cycle. To determine whether a specific agonist, e.g., epidermal growth factor (EGF) (**Fig. 1**) or platelet growth factor-BB (PDGF-BB) (**Fig. 2**), promotes cell proliferation, synchronized or quiescent ASM or VSM cells are stimulated with 1 ng ml^{-1} EGF or 10 ng ml^{-1} PDGF-BB for 16 h to initiate cell cycle progression from the G_0/G_1 phase into the S phase. Next, [^{3}H]thymidine is added to cells to label newly synthesized DNA[6]; 24 h later, cells are fixed and [^{3}H]thymidine incorporation is detected by scintillation counting followed by statistical analysis of the data.

Among cell proliferation methods, the [^{3}H]thymidine incorporation assay is most suitable for the assessment of the proliferation rate of human VSM and ASM cells because of the specific growth characteristics of these cells[1–5]. DNA synthesis of VSM and ASM is a rare event *in vivo*; in culture, human VSM and ASM cells maintain not only their *in vivo* characteristics such as expression of smooth muscle α-actin and contractility[7] but also a slow proliferation rate[2,5]. For example, human ASM cells in the presence of a very potent mitogen, 10% FBS, double their numbers in approximately 2.5 d; human pulmonary arterial VSM cells double their number in approximately 4.5–5 d in the presence of 10% FBS. In contrast, EGF or PDGF, well-established smooth muscle mitogens, stimulate only a 20–25% increase in ASM cell numbers. Therefore, the cell count technique, which is very suited to quickly proliferating cells (e.g., cancer cells), is not well suited to assessing VSM or ASM cell proliferation because the effects are less and it is difficult to obtain statistically significant data. In contrast, the [^{3}H]thymidine incorporation assay allows the measurement of the incorporation of radiolabeled [^{3}H]thymidine into DNA during the S phase of the cell cycle as a quantitative measure of new DNA synthesis and yields statistically significant data even when the number of divided cells is relatively low. The [^{3}H]thymidine incorporation assay uses a 24-well plate, with each condition performed in triplicate; this requires the availability of enough cells, e.g., around 3×10^5 cells per sample at the time of the assay. The possible nonradioactive alternative (but less sensitive) approach to this assay is BrdU ELISA

provided by Roche and Exalpha Biologicals, Inc. The BrdU ELISA assay is based on the measurement of cell proliferation by colorimetric quantitation of BrdU incorporated into the newly synthesized DNA of replicating cells. Other colorimetric assays for measurement of cell proliferation, available from Molecular Probes, Roche and Chemicon, are based on the ability of living cells to cleave a pale yellow substrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT), to yield a dark blue formazan product.

If a limited number of cells are available, immunocytochemical analysis of BrdU incorporation could be used[8,9]. If the goal of the study is not only to determine DNA synthesis but also to estimate the fraction of cells in the S phase, then immunochemical quantitation of BrdU incorporation by flow cytometry could be used[10]. The major limitation of the [^{3}H]thymidine incorporation assay is essentially associated with the use of radioactivity; however, this method is one of the best and most reliable experimental approaches providing statistically significant data.

MATERIALS

REAGENTS

- [*methyl* -^{3} H]thymidine (Amersham/Pharmacia, cat. no. TRK418)
  ! CAUTION Radioactive material. Consult the material safety data sheet for proper handling instructions and get training to work with radioactive materials according to your institution's environmental health and radiation safety procedures.
- 0.05% Trypsin-EDTA (GIBCO, cat. no. 25300)
- 96% Ethanol solution (Pharmco Products, Inc., cat. no. 111ACS200)
- Trichloracetic acid (TCA) (Sigma, cat. no. T4885) (see REAGENT SETUP)
- Scintillation fluid: Econo-Safe (Research Products International, cat. no. 111175)

EQUIPMENT

- 24-well plate (Nunc, cat. no. 143982)
- Harvester (Brandel, Gaithersburg, MD)
- Reeves Angel 934AH paper (Brandel, cat. no. FPB-24)
- Scintillation flasks (Fisher Scientific)
- Scintillation counter: Beckman Coulter LS6500 (Beckman)

REAGENT SETUP

20% TCA water solution Keep at 4 °C up to several months. ▲ CRITICAL Use ice-cold TCA solution in the experiment.

PROCEDURE

Cell culture

1| Plate 18,000 cells per well in 24-well plate in F12 media supplemented with 10% FBS and allow cells to grow for 5–7 d in a 37 °C humidified 5% CO_2 incubator.
? TROUBLESHOOTING

2| To synchronize cells in the G_0/G_1 phase of the cell cycle, growth-arrest cells in serum-free media: rinse cells once with warmed PBS solution, add 1 ml serum free F12 media supplemented with 0.1% BSA in each well and incubate cells for 48 h in the CO_2 incubator.
▲ CRITICAL STEP Allow cells to grow for at least 5 d for complete recovery from trypsinization and expression of all surface receptors.

Stimulation of cell proliferation and radiolabeling of cellular DNA with [*methyl*-^{3}H]thymidine ● TIMING Approximately 2 d

3| Add agonists into each well according to experimental conditions and incubate cells in the CO_2 incubator for 18 h.
▲ CRITICAL STEP To reduce experimental error, perform each experimental condition in triplicate.
? TROUBLESHOOTING

4| Add [*methyl*-^{3}H]thymidine into each well to a final concentration of 3 μCi ml^{-1} and incubate cells for 24 h in the CO_2 incubator.
! CAUTION [*methyl*-^{3}H]thymidine is radioactive. Consult material safety data sheet for proper handling instructions and get training to work with radioactive materials according to your institution's environmental health and radiation safety procedures.

Extraction of [*methyl*-^{3}H]thymidine-labeled DNA and assessment of [^{3}H]thymidine incorporation

● TIMING Approximately 1 h 45 min per 24-well plate; add 20 min for every extra plate

TABLE 1 | [^{3}H]thymidine incorporation (e.g., c.p.m.) from one representative experiment. Confluent, growth-arrested ASM cells were pretreated with either 3, 10, 30 and 100 mM LY294002 or 0.5, 5, 50 and 500 nM rapamycin for 30 min and then stimulated with either 10% FBS, 1 ng ml^{-1} EGF, 1 U ml^{-1} thrombin (Thr) or diluent. Data are means ± s.e.m. from six replicates for each condition.

No.	Conditions	[^{3}H]-Thymidine count, c.p.m.	
		Mean	s.e.m.
1	Control	909.2	141.8
2	10% FBS	72,937.0	6,079.1
3	EGF 1 ng ml^{-1}	44,923.3	1,736.9
4	LY294002 3 μM	519.4	70.0
5	LY294002 10 μM	372.2	48.0
6	LY294002 30 μM	354.2	32.7
7	LY294002 100 μM	141.5	13.1
8	EGF/ LY294002 3 μM	27,238.6	4,364.0
9	EGF/ LY294002 10 μM	9,380.9	808.0
10	EGF/ LY294002 30 μM	3,114.2	256.7
11	EGF/ LY294002 100 μM	543.9	70.8
12	Rapamycin 0.5 nM	401.7	42.9
13	Rapamycin 5 nM	247.7	30.2
14	Rapamycin 50 nM	512.8	132.9
15	Rapamycin 500 nM	1,216.7	722.7
16	EGF/ Rapamycin 0.5 nM	27,479.0	2,290.9
17	EGF/ Rapamycin 5 nM	18,016.9	1,071.8
18	EGF/ Rapamycin 50 nM	16,601.6	1,349.9
19	EGF/ Rapamycin 500 nM	14,675.2	411.1
20	Thr	12,113.0	1,723.0
21	Thr/ LY294002 3 μM	7,269.9	486.2
22	Thr/ LY294002 10 μM	2,282.2	120.6
23	Thr/ LY294002 30 μM	936.5	88.8
24	Thr/ LY294002 100 μM	402.2	65.0
25	Thr/ Rapamycin 0.5 nM	5,138.5	642.4
26	Thr/ Rapamycin 5 nM	2,682.8	179.8
27	Thr/ Rapamycin 50 nM	2,733.2	225.7
28	Thr/ Rapamycin 500 nM	2,174.4	301.0

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5| Aspirate media from each well of the 24-well plate.

6| Rinse the cells carefully with 1 ml PBS per well three times.

7| Aspirate PBS and add 0.5 ml 0.05% trypsin-EDTA into each well and incubate cells for 15 min in the CO_2 incubator at 37 °C.

8| Add 0.5 ml ice-cold 20% TCA solution into each well. Incubate for 20 min at 4 °C.
▲ **CRITICAL STEP** For better DNA precipitation, use ice-cold PBS and 20% TCA.
■ **PAUSE POINT** DNA precipitates in 20% TCA solution can be stored in the 24-well plate for several days at 4 °C.
? TROUBLESHOOTING

9| Place the sheet of Reeves Angel 934AH filter paper on the harvester platform, wet filter paper with PBS and clamp down a platform.

10| Remove the cover from the 24-well plate with DNA precipitates and place the plate on the harvester's plate holder.

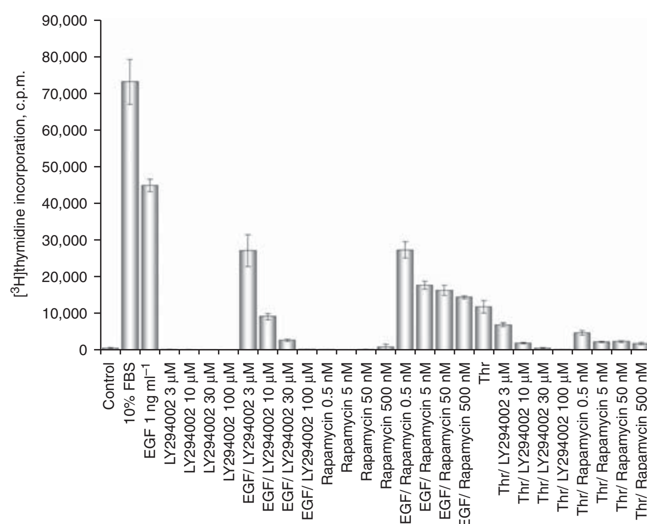

Figure 1 | Typical graph of [^{3}H]thymidine incorporation experiment.

11| The next three steps are performed automatically using the harvester.

12| Aspirate precipitate from each well of the 24-well plate into the Reeves Angel 934AH filter paper sheet.

13| To collect all precipitate, rinse each well with PBS and aspirate PBS to the same paper filter. Repeat three more times.

14| Wash filter paper with 96% ethanol to remove PBS.
! CAUTION 96% ethanol is flammable and harmful if inhaled (see material safety data sheet at http://www.pharmco-prod.com/pages/msds.html).

15| Carefully pick paper filters up and place each filter in a separate scintillation flask containing 8 ml scintillation fluid.

16| To measure [*methyl*-^{3}H]thymidine incorporation, perform a count of the vials in c.p.m. using a scintillation counter.
? TROUBLESHOOTING

Statistical analysis ● TIMING 20–30 min

17| Compare quantitative measurements between experimental conditions using ANOVA and present experimental data as mean values ± S.E. in c.p.m. or as a percentage of the value for the control ([*methyl*-^{3}H]thymidine incorporation without stimulation) taken as 100%.

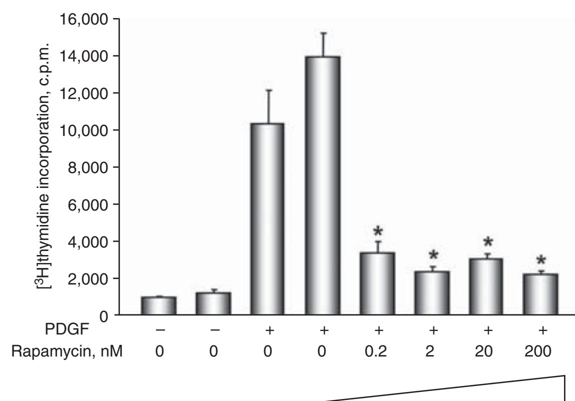

Figure 2 | Representative figure demonstrating that rapamycin modulates DNA synthesis in PDGF-stimulated human VSM cells assessed using [^{3}H]thymidine incorporation. Confluent, growth-arrested VSM cells were pretreated with 0.2, 2, 20 or 200 nM rapamycin for 30 min and then stimulated with either 10 ng ml^{-1} PDGF or diluent. Data represent means ± s.e.m. from six replicates for each condition (*$P < 0.01$ for rapamycin versus PDGF by one-way ANOVA [Bonferonni–Dunn test]).

18| Assess differences in the experimental conditions using the Bonferroni–Dunn post hoc test with a level of significance $P \leq 0.05$.

● TIMING

Steps 1–4, growth of cell cultures to near confluence, growth arrest, stimulation of cell proliferation with agonists and radiolabeling of cellular DNA with [*methyl*-^{3}H]thymidine: approximately 9 d.
Steps 5–16, extraction of [*methyl*-^{3}H]thymidine-labeled DNA and assessment of radioactive nucleotide incorporation: approximately 1 h 45 min per 24-well plate; add 20 min for every extra plate.
Steps 17–18, statistical analysis: 20–30 min.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

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TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	High variability in c.p.m. within each condition	Unequal number of cells per well were plated	Gently resuspend cells during plating into each well
3	Low [^{3}H]thymidine incorporation, e.g., c.p.m.	Insufficient quantity of cells	Perform visual analysis of cells before assay
8	Low [^{3}H]thymidine incorporation, e.g., c.p.m.	Not all DNA precipitate was transferred from a well onto the filter	Use ice-cold PBS and TSA
16	Low [3H]thymidine incorporation, e.g., c.p.m.	Scintillation counter does not work properly	Schedule maintenance of the counter

ANTICIPATED RESULTS

The incorporation of [^{3}H]thymidine allows statistically significant data to be obtained on ASM or VSM cell proliferation induced by mitogens. **Table 1** and **Figure 1** show representative data from one experiment using the [^{3}H]thymidine incorporation assay; **Figures 2** and **3** show typical presentations of [^{3}H]thymidine incorporation data. As shown in **Table 1**, usually the basal level of [^{3}H]thymidine incorporation in serum-deprived ASM cells (Control, lane 1) is below 1,000 c.p.m.; FBS markedly stimulates thymidine incorporation, and the count reaches 72,937 c.p.m. (Lane 2); the EGF- and thrombin-induced [^{3}H]thymidine counts are 44,923 and 12,113 c.p.m., respectively (Lanes 3 and 20). These data show marked changes in DNA synthesis: 73-fold for FBS, 45-fold for EGF and 12-fold for thrombin. If we used the cell count technique, say, it would be difficult to obtain statistically significant data from one experiment, especially to measure a concentration-dependent inhibition of ASM and VSM DNA synthesis induced by PI3K inhibitor LY294002 or mTOR inhibitor rapamycin. Thus, the [^{3}H]thymidine incorporation assay represents a valuable method for the quantitative measurement of DNA synthesis induced by ASM and VSM cell mitogens.

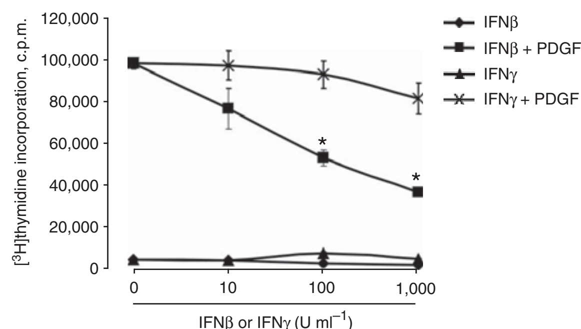

Figure 3 | Representative figure demonstrating different effects of interferon β (IFNβ) and IFNγ on DNA synthesis in smooth-muscle-like LAM-derived (LAMD) cells assessed by [^{3}H]thymidine incorporation. Pre-confluent, serum-deprived LAMD cells were treated with 10, 100, or 1000 U ml^{-1} IFNβ or IFNγ in the presence or absence of 10 ng ml^{-1} PDGF. Data represent means ± s.e.m. from six replicates for each condition (*$P < 0.01$ for IFNβ + PDGF versus PDGF by one-way ANOVA [Bonferonni–Dunn test]).

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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