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A Correction: Inhibitory Activity in the Conditioned Medium of Embryonic Chick Bones Is Due to Thymidine (41945)

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Abstract. Earlier studies from this laboratory suggested that embryonic chick bones in organ culture released into the culture medium a specific inhibitor of bone cell proliferation as defined by inhibition of [^{3}H]TdR incorporation into DNA. Dialysis and membrane ultrafiltration experiments suggested that the inhibitory substance (IS) had a molecular weight between 6000 and 14,000. However, subsequent studies on the purification of IS have revealed that the inhibitory activity in bone-conditioned medium is of lower molecular weight and has several properties in common with thymidine (TdR): (1) IS coeluted with [^{3}H]TdR upon gel filtration chromatography on Sephadex G-10. (2) IS bound to charcoal but not to cation or anion exchange resins. (3) Bone-conditioned medium decreased incorporation of [^{3}H]TdR into the free [^{3}H]TdR pool of cells in monolayer culture. (4) Conditioned medium inhibited [^{3}H]TdR incorporation into [^{3}H]thymidine monophosphate in a reaction catalyzed by thymidine kinase. The equivalent concentration of TdR in conditioned medium as estimated by thymidine kinase assay was sufficient to account for the reduction in [^{3}H]TdR incorporation into bone cell DNA. No evidence was found for a specific inhibitor of bone cell proliferation other than TdR. Hence we conclude that the inhibitory effect of IS is due to dilution of [^{3}H]TdR by nonradioactive TdR. Furthermore, media conditioned by several tumor cell lines also contained a low-molecular-weight component which inhibited [^{3}H]TdR incorporation. The results suggest that organ- and cell-conditioned media can contain significant concentrations of TdR which can artifactually inhibit [^{3}H]TdR incorporation in cell proliferation assays. © 1984 Society for Experimental Biology and Medicine.

Previous work in our laboratory suggested that conditioned medium of embryonic chick bones grown in organ culture contained an inhibitor of bone cell proliferation (1). Dialysis and selective membrane filtration of this inhibitory substance (IS) suggested that it had a molecular weight between 6000 and 14,000. The inhibitory effect of conditioned medium was also shown to be specific to bone cells with no effect on chick skin, liver, or muscle cells. Furthermore, the activity of IS was not destroyed after treatment of CM with trypsin, phospholipase, RNAse, neuraminidase, or heat. Based on these results, it was concluded that IS was a small polypeptide which specifically inhibited bone cell proliferation. We now present evidence that the IS in the embryonic chick bone-conditioned medium is thymidine (TdR) and the inhibitory effect observed was due to an artifact in the cell proliferation assay which was based on incorporation of [^{3}H]TdR into DNA.

Materials and Methods. *Conditioned medium.* Femur and tibiae from 8- and 11-day-old chick embryos were removed under aseptic conditions and were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air and serum-free medium (BGJ/b, Fitton Jackson modified, Grand Island Biological Co.) containing antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml). The phosphate concentration was increased to 4.8 mM as described by Howard *et al.* (2). The medium was removed every 24 hr for 2–5 days, clarified at 1500 rpm for 10 min and filtered using a filter of 0.45-μm pore size. This conditioned medium served as the source of IS. Conditioned media collected from Leydig tumor, human lung carcinoma (SKLC6), and breast tumor tissue were also used as sources of inhibitory activity. Aliquots of fresh medium were saved and used as control unconditioned medium. Ultrafiltration membranes were obtained from Amicon and dialysis tubing was obtained from Spectrapor.

Assay for inhibitory activity. Inhibition of DNA synthesis was assessed by the reduction of [^{3}H]TdR incorporation into TCA insoluble

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material in serum free monolayer cultures of chick embryo calvarial cells, as described in our previous report (1). Osteoblast-enriched cell populations were obtained by collagenase digestion (0.1% collagenase, 60 min) of 13- to 14-day-old embryonic chick calvaria as described previously (1). The cells were suspended in serum free BGJ/b medium (which contains no TdR) and plated into 24-well culture dishes at a concentration of 350 cells/mm^2 of surface area in 1 ml and incubated at 37°C in 5% CO_2 and 95% air. After 18 hr of incubation, the factor to be tested (e.g., conditioned media or control media) and 20 μl containing 2 μCi of [^{3}H]TdR (ICN Chemicals, 48 Ci/mmole) were added and incubated for an additional 4 hr. The radiolabeled medium was removed and the cells were washed with phosphate-buffered saline (PBS) and used for the [^{3}H]TdR incorporation assay. Skin, muscle, and heart cells were dispersed from 11-day-old embryonic chick tissues by collagenase digestion (0.05% collagenase, 20 min) and used for inhibition assay as described for calvaria cells.

Determination of incorporation of [^{3}H]TdR into free TdR pool. From the specific activity (48 Ci/m*M*) it was calculated that 10.58 ng of [^{3}H]TdR (2 μCi) was added per well during the inhibition assay. For the determination of free [^{3}H]TdR pool, calvaria cells were plated as described previously and incubated with 2 μCi of [^{3}H]TdR along with 100 ng of nonradioactive TdR or 100 μl of conditioned/control medium. After 30, 60, 180, and 240 min of incubation, the radiolabeled medium was removed and the cells were washed five times with PBS containing 50 μM nonradioactive TdR. Five hundred microliters of ice cold 12.5% TCA was added to the wells, and the cells were scraped off with a rubber policeman. The contents, an additional 500-μl rinse, and 50 μl of carrier protein (BSA 10 mg/ml), were transferred to 10 × 75-mm culture tubes and were centrifuged. An aliquot of the TCA supernatant was used for estimation of free [^{3}H]TdR (in the PBS-rinsed intracellular pool), and the TCA precipitate was used to measure the amount of [^{3}H]TdR incorporated into DNA.

Thymidine kinase assay. TdR concentration in the conditioned medium was determined by a competition assay in which the nonradioactive TdR competes with [^{3}H]TdR as a substrate for thymidine kinase (TK). This assay system, based on the method of Morgan *et al.* (3), used TdR and ATP (regenerated from creatine phosphate by creatine kinase) as substrates and a cell lysate from G2 osteosarcoma cells as enzyme source. Briefly, G2 osteosarcoma cells grown to confluency in 120-mm culture dishes were scraped off in 20 m*M* Tris buffer, pH 8.0, with 10 m*M* DTT and 1.6 × 10^{-5} *M* TdR (TK extraction buffer). The mixture was sonicated for 10 sec and centrifuged at 10,000 rpm for 10 min in a microfuge. Thirty-microliter aliquots of the supernatant (source for TK) were transferred to 10 × 75-mm culture tubes in ice to which 25 μl of TK reaction mixture (600 m*M* Tris, pH 7.8, 20 m*M* $MgCl_2$, 20 m*M* ATP, 100 m*M* NaF, 30 m*M* creatine phosphate, 12.0 units/ml of creatine kinase (Sigma), and 10 μCi/ml of [^{3}H]TdR) and 45 μl of antagonist (known concentration of nonradioactive TdR or conditioned medium) were added. The tubes were transferred to a 37°C water bath for incubation. At 10, 20, and 30 min, a 30-μl aliquot was withdrawn and mixed with 9 μl of 25 m*M* EDTA to stop the reaction. Ten microliters of this aliquot was applied to DE-81 filter disks (Whatman). After drying, disks were washed three times with 1 m*M* ammonium formate (10 ml/disk), once with water and once with absolute ethanol. [^{3}H]Thymidine monophosphate (TMP) bound to the DE-81 ion exchange filter paper was quantitated by scintillation counting.

Results. In the initial experiments we used inhibitory factor preparations from 11-day-old chick embryo long bones since they are easy to isolate. However, since the original study was based at least in part on preparations from younger embryos, we also examined conditioned medium collected from long bones of 8-day-old chick embryos grown in culture. The inhibitory factor activities were essentially the same in both 8- and 11-day bone-conditioned media. Subsequent to the initial observation of inhibitor activity in bone-conditioned medium, we initiated studies to purify the IS. Conditioned medium collected from 11-day-old chick embryo long bones grown *in vitro* was fractionated in an Amicon concentrator with a PM10 mem-

brane (10,000 mol wt cutoff) to remove high-molecular-weight components. The retentate and filtrate were tested for activity as defined by inhibition of [^{3}H]TdR incorporation by chick embryo calvarial cells during a 4-hr exposure to effector and [^{3}H]TdR. The retentate showed no activity while the filtrate contained all the inhibitory factors (data not shown) indicating an apparent molecular weight of less than 10,000 as previously reported. Conditioned medium (10 ml) was also dialyzed for 24, 48, and 120 hr against distilled water (1000 ml) by using a Spectrapor membrane tubing with a molecular weight cutoff of approximately 3500. Inhibitor activity was reduced in the retentate after 24 hr and by 120 hr, the retentate showed no inhibitory activity (Table I). When an aliquot (100 ml) of the 120-hr dialysate was lyophilized and reconstituted to the original volume (1 ml), the inhibitor activity was completely recovered in the dialysate. When the 120-hr retentate was concentrated fivefold by lyophilization and tested for inhibitory activity, no inhibitory activity could be demonstrated (98 ± 22% of control). The results of extensive dialysis thus suggest that IS was slowly dialyzable with an apparent molecular weight of less than 3500.

TABLE I. INHIBITORY EFFECTS OF EMBRYONIC CHICK LONG-BONE-CONDITIONED MEDIUM ON CALVARIA CELL [^{3}H]TdR INCORPORATION INTO DNA AS A FUNCTION OF DIALYSIS TIME

Hr of dialysis	% Inhibition[a]
Retentate	
0	82.4 ± 1.7[b]
24	26.0 ± 16.0[c]
48	12 ± 12.1
120	0 ± 14.3
Dialysate (120 hr)	83.3 ± 2.9[b]

Note. Conditioned medium was dialyzed against distilled water at 4°C using Spectrapor dialysis tubing (mol wt 3500 cutoff). The retentate and dialysate (concentrated to original volume by lyophilization) were used for testing the inhibitory activity using the chick calvaria cell [^{3}H]TdR incorporation assay.

[a] Means ± SD ($N = 6$).

[b] Significantly different from control, $P < 0.001$, by Student's t test.

[c] Significantly different from control, $P < 0.01$, by Student's t test.

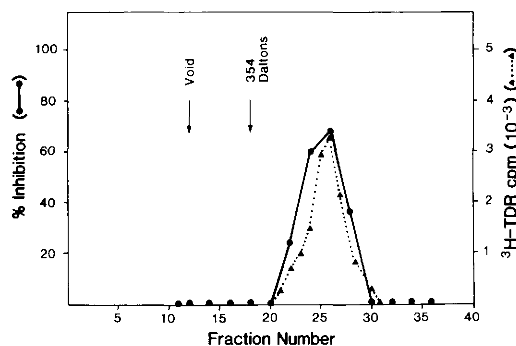

FIG. 1. Fractionation of inhibitory activity (inhibition of incorporation of [^{3}H]TdR into DNA) by gel filtration of conditioned medium concentrate on Sephadex G-10. The active fractions coeluted with [^{3}H]TdR.

To determine the molecular weight of IS more accurately, conditioned medium concentrated by lyophilization (10×) was initially separated by chromatography on a Sephadex G-25 column using PBS as an eluant. No inhibitory activity was detected in the void volume (106 ± 19% of control/DNA synthesis). However, inhibitory activity coeluted with phenol red (mol wt 354) indicating an apparent molecular weight of less than 500 (data not shown). When separated on a Sephadex G-10 column standardized with [^{3}H]TdR and ^{125}I (Fig. 1), the inhibitory activity eluted in the same fractions as [^{3}H]TdR (mol wt 244).

To determine if inhibited [^{3}H]TdR uptake could be responsible for the inhibition of [^{3}H]TdR incorporation into DNA, the effects of conditioned medium and of nonradioactive TdR on the uptake of [^{3}H]TdR into the TCA soluble cell pool as well as into TCA insoluble pool (DNA) were determined. Nonradioactive TdR (100 ng/ml) reduced the incorporation of [^{3}H]TdR (10.58 ng/ml) into the soluble cell TdR pool as well as into DNA (Table II). Similarly, the conditioned medium also reduced the uptake of [^{3}H]TdR into the free TdR pool and into DNA (Table II). These results suggest that IS in the conditioned medium may inhibit [^{3}H]TdR uptake by cells by competing with the radioisotope for the transport system, in a manner similar to nonradioactive TdR.

TdR has an absorption maxima at 265 nm and does not bind to either DEAE or

TABLE II. INHIBITORY EFFECT OF CONDITIONED MEDIUM AND NONRADIOACTIVE TdR ON THE INCORPORATION OF [^{3}H]TdR INTO FREE [^{3}H]TdR POOL AND INTO DNA IN EMBRYONIC CHICK CALVARIAL CELLS

Factor	% Inhibition[a]	
	Free [^{3}H]TdR pool	DNA
Conditioned medium (100 μl/ml)	42 ± 13.9[b]	62 ± 4.6[b]
TdR (100 ng/ml)	60 ± 8.5[b]	73 ± 3.4[b]

[a] The amount of [^{3}H]TdR in the free TdR pool was constant from 30 to 240 min in both control and experimental cultures. Hence the percentage inhibition was calculated from the average ratio of experimental/control for the four time points (six wells per time point). Means ± SD (N = 24).

[b] Significantly different from control, $P < 0.001$, by Student's t test.

CM cellulose at pH 7.2 but does bind to charcoal. DEAE and CM cellulose treatment of inhibitory factor preparations did not affect the inhibition of [^{3}H]TdR incorporation while charcoal treatment of inhibitory factor preparations eliminated the inhibitory effect (Table III). These results further suggest that the inhibitory factor could be TdR. It would decrease the spec act of [^{3}H]TdR in the DNA synthesis assay and thereby cause an apparent artifactoral inhibition of DNA synthesis.

Further evidence for the similarity between IS and TdR was obtained from the effects of conditioned medium on phosphorylation of [^{3}H]TdR by TK. We reasoned that nonradioactive TdR in bone-conditioned medium would compete with [^{3}H]TdR, the TK substrate, and decrease the production of [^{3}H]TMP. Figure 2 shows the inhibition of [^{3}H]TMP production by addition of known amounts of nonradioactive TdR and by 8-day-old chick embryo long-bone-conditioned medium. Basal level of [^{3}H]TdR and nonradioactive TdR in the reaction mixture were 0.25 μCi and 4.8 μ*M*, respectively. Addition of 45 μl of conditioned medium (final volume 100 μl) caused 46% inhibition of [^{3}H]TdR conversion into [^{3}H]TMP. Using the standard curve in Fig. 2, we calculated that the conditioned medium contained 9.3 μ*M* TdR (101.2 ng/45 μl conditioned medium). Fifty microliters of the same conditioned medium when tested on calvaria cells caused 67% inhibition of [^{3}H]TdR uptake into DNA (Table IV) compared to 73% seen with 100 ng/ml or 8.2 μ*M* of nonradioactive TdR (Table II). Thus the measured amount of TdR in the conditioned medium estimated by the TK assay is sufficient to account for the reduction in [^{3}H]TdR incorporation.

Inhibitory activity of conditioned medium was also tested on chick skin, muscle and heart cells, normal human fibroblasts, and BALB/c 3T3 cells. The results indicated that the inhibitory factor was not specific to bone-derived cells (Table IV).

Conditioned media collected from different tumor cultures grown *in vitro* were also tested

TABLE III. EFFECT OF TREATMENT OF NONRADIOACTIVE TdR OR CONDITIONED MEDIUM WITH CHARCOAL, DEAE CELLULOSE, OR CM CELLULOSE ON INCORPORATION OF [^{3}H]TdR INTO DNA OR ABSORPTION AT 265 nm

Treatment	Inhibitory factor[a] (% inhibition[c])	Nonradioactive TdR[b]	
		% Inhibition[c]	265-nm Absorption
None	48 ± 7[d]	84 ± 3[d]	2.65
Charcoal	−5 ± 11	−2 ± 9	0.00
DEAE cellulose	43 ± 9[d]	84 ± 2[d]	2.58
CM cellulose	47 ± 7[d]	85 ± 3[d]	2.54

Note. One gram of ion-exchange resin (DEAE and CM cellulose) and charcoal were equilibrated to pH 7.4 with 30 m*M* Tris acetate in separate tubes. After centrifugation to remove the buffer, the beads were incubated with control/conditioned media (1 ml) and TdR (1 ml, 100 μg/ml) for 30 min with mixing. The tubes were then centrifuged and the supernatant used for inhibition assay.

[a] 50 μl added/ml medium in the incorporation assay.

[b] Nonradioactive TdR concentration was 100 ng/ml in the incorporation assay and 100 μg/ml for the absorption at 265 nm.

[c] Means ± SD (N = 6).

[d] Significantly different from control, $P < 0.001$, by Student's t test.

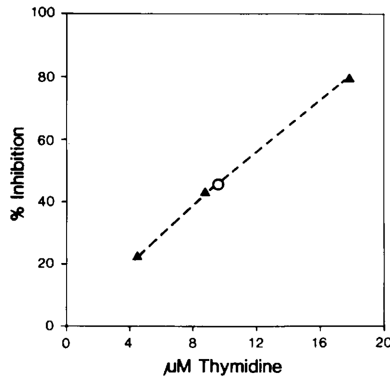

FIG. 2. Standard curve for TdR inhibition of the incorporation of [^{3}H]TdR into [^{3}H]TMP by thymidine kinase. The concentration of TdR in the conditioned media was calculated to be 9.3 μM (O).

TABLE V. CONDITIONED MEDIUM EFFECT ON CHICK CALVARIA CELL [^{3}H]TdR INCORPORATION INTO DNA

Source of conditioned medium	Vol (µl)	% Inhibition[a]
Chick long bone	100	77.9 ± 1.9[b]
	50	66.9 ± 1.6[b]
	10	22.1 ± 11.0[c]
Leydig tumor	100	32.0 ± 21.2[c]
	50	0.9 ± 16.9
Breast tumor	100	63.7 ± 8.9[b]
	50	43.5 ± 11.9[b]
SKLC6 (human lung carcinoma)	100	46.1 ± 7.5[b]
	50	12.5 ± 6.8[d]

[a] Means ± SD ($N = 6$).
[b] Significantly different from control, $P < 0.001$, by Student's t test.
[c] Significantly different from control, $P < 0.02$, by Student's t test.

for inhibitory activity using the chick calvaria cell assay. It was found that all three tumor CM contained inhibitory activity (Table V). In addition, we found that fetal calf serum also contained a low-molecular-weight (<3500) inhibitor (Table VI).

Discussion. When this study was initiated, the objective was to purify and characterize IS. However, during the course of these studies, IS was found to be of low molecular weight. Indeed, the molecular weight of IS as judged by gel-filtration chromatography was similar to that of TdR. Characterization of IS as TdR was further confirmed by other studies. Similar to TdR, IS bound to charcoal but not to ion-exchange resins. Early on in these studies we felt that there probably was insufficient TdR in the conditioned medium to account for the observed inhibition. By means of TK assay, we found that the conditioned medium contained more than 9 μM TdR, a concentration adequate to sufficiently dilute the radioactive TdR so as to account for the decreased uptake of [^{3}H]TdR into DNA in our chick calvarial cell assay. TdR has been shown to inhibit DNA synthesis at high concentrations (above 2 mM) because of its feedback effects on the synthesis of other nucleotide precursors (4). However, the amount of TdR in the conditioned medium is in the micromolar concentration range and hence the cytotoxic effect of TdR is ruled out.

TABLE IV. INHIBITORY EFFECT OF CHICK LONG-BONE-CONDITIONED MEDIUM ON [^{3}H]TdR INCORPORATION INTO DNA IN CELLS OF DIFFERENT TISSUES

Cells	% Inhibition[a]: Conditioned medium (50 µl/ml)	% Inhibition[a]: TdR (100 ng/ml)
Chick calvaria	66.9 ± 1.6[b]	73.0 ± 3.4[b]
Chick skin	58.6 ± 15.8[b]	64.4 ± 7.9[b]
Chick muscle	64.0 ± 8.6[b]	76.5 ± 3.0[b]
Chick heart	71.1 ± 8.3[b]	75.0 ± 3.9[b]
Normal human fibroblasts	50.0 ± 9.5[b]	—
Mouse 3T3	47.5 ± 5.3[b]	—

[a] Means ± SD ($N = 6$).
[b] Significantly different from control, $P < 0.001$, by Student's t test.

The most likely source of the TdR is dying bone marrow cells. The bone marrow cell population (3–4 × 10^6/bone, 10- to 11-day-old embryo), which is not viable under the conditions used, contains 10–15 µg DNA or 1.3–2.0 µg TdR. This together with DNA from bone cells and the free TdR metabolite pool in the cells (four bones/ml, three to five medium changes) could account for the estimated 2 µg/ml free TdR in the conditioned medium.

We found no evidence for the presence of a specific inhibitor (mol wt > 6000) besides TdR in the conditioned medium. If there was an IS with molecular weight greater than

TABLE VI. EFFECTS OF DIALYZED (48 hr) AND NONDIALYZED FETAL CALF SERUM ON [^{3}H]TdR INCORPORATION (0- TO 4- AND 16- TO 18-hr PULSE) INTO DNA IN CALVARIA CELLS

	% Inhibition[a]			
	0- to 4-hr pulse		16- to 18-hr pulse	
% FCS	Dialyzed	Nondialyzed	Dialyzed	Nondialyzed
1	−4 ± 31	45 ± 7[b]	0 ± 8.9	20 ± 12.9[c]
5	3 ± 20	71 ± 4[b]	5.7 ± 11.1	52 ± 4.5[b]
10	31 ± 12[b]	82 ± 3[b]	16.4 ± 8.4[c]	73 ± 2.5[b]

[a] [^{3}H]TdR incorporation for no addition control (5419 ± 964 cpm) was used to calculate the percentage inhibition during the 0- to 4-hr pulse. Because the fetal calf serum stimulates [^{3}H]TdR incorporation at 16–18 hr, the untreated control cannot be used to determine the percentage inhibition. Instead the percentage inhibition was calculated by assuming the 1% dialyzed fetal calf serum (11,805 ± 1050 cpm) treatment gave no inhibition. Values are means ± SD of six observations.

[b] Statistically different from control, $P < 0.001$, by Student's *t* test.

[c] Statistically different from control, $P < 0.02$, by Student's *t* test.

6000 as described in the earlier study, we should have been able to detect it in the void volume of Sephadex G-10 and G-25. In our original study, IS was found to be specific for bone cells, however, the results of the present study are in conflict with those original data. The earlier study also revealed that conditioned medium did not influence cellular [^{3}H]TdR pool size, whereas in the present study, there was a marked effect of conditioned media to decrease [^{3}H]TdR pool size. We are unable to completely reconcile our results with the earlier observations about the specific inhibitor and have concluded that the inhibitory effect of conditioned medium is due to TdR.

Our results also emphasize that TdR in conditioned medium is a fairly widespread occurrence. The significance of TdR in conditioned medium is that it could give the appearance of an inhibitor or mask the appearance of a stimulator if one uses only a [^{3}H]TdR incorporation assay to assess proliferation. Fetal calf serum probably contains TdR based on our results and on other published data, and this could artifactually decrease the effect of mitogenic components of serum by decreasing the uptake of [^{3}H]TdR. Our studies indicate that this effect is prominent in a 4-hr incubation assay, but it can probably be ignored for low concentrations of serum (<1%) in an 18-hr incubation assay. In conclusion, a [^{3}H]TdR incorporation assay is a sensitive and rapid assay for cell proliferation and will continue to be used probably more than ever as our search continues for local factors effecting cell proliferation. While being very useful, this assay has several pitfalls, the one disclosed in this study being that many test substances may contain TdR which can reduce the specific activity of [^{3}H]TdR and thereby result in spurious results. Independent assessment of cell proliferation by cell count, mitotic index, or DNA content should be done to substantiate results based only on [^{3}H]TdR incorporation (5).

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1. Puzas JE, Drivdahl RH, Howard GA, Baylink DJ. Endogenous inhibitor of bone cell proliferation. Proc Soc Exp Biol Med **166**:113–122, 1981.
2. Howard GA, Bottemiller BL, Baylink DJ. Evidence for the coupling of bone formation to bone resorption *in vitro*. Metab Bone Dis Relat Res **2**:131–135, 1980.
3. Merril GF, Witter EB, Hauschka SD. Differentiation of thymidine kinase deficient mouse myoblasts in the presence of 5′-bromodeoxyuridine. Exp Cell Res **129**:191–199, 1980.
4. Shall S. In: Kruse PF, Patterson MK, eds. Tissue Culture. New York, Academic Press, p195, 1973.
5. Maurer HR. Potential pitfalls of [^{3}H]Thymidine techniques to measure cell proliferation. Cell Tissue Kinet **14**:111–120, 1981.

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