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# Effect of ploidy on transcription levels in cultured rat aortic smooth muscle cells

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Aging and hypertension increase the number of polyploid smooth muscle cells (SMC) in a blood vessel. We assessed the effect of ploidy on the transcription of several genes in SMC cultures derived from newborn and adult rats. In diploid and tetraploid subcultures of SMC from newborn rats, RNA expression of the genes assayed is linked with ploidy. However, when phenotypically different SMC cultures derived from newborn and adult rats were compared, transcription levels varied from gene to gene and not linked with the ploidy. Thus, differences in gene expression due to polyploidy are superimposed on those due to other phenotypical features.

Ploidy; Vascular smooth muscle cell; Atherosclerosis; Rat

## 1. INTRODUCTION

Smooth muscle cell (SMC) proliferation is thought to play a major role in the development of both atherosclerosis and hypertension [1]. Velican and Velican [2] showed that SMC replication is the initial event in the formation of atherosclerotic plaques.

Folkow et al. [3] implicated thickening of the vessel wall in established hypertension as an adaptation to the increased peripheral resistance. Typically, DNA synthesis in the aorta and other large blood vessels consists of DNA replication without cell division, resulting in the accumulation of polyploid cells that account for most of the increased mass of hypertensive vessels [4,5]. In agreement with those observations, Owens et al. [6] found a fixed ratio between protein and DNA content in vascular SMC of Wistar–Kyoto rats and those of spontaneously hypertensive rats. Moreover, they noticed that polyploidy does not occur in SMC of prehypertensive spontaneously hypertensive rats. Polyploidization takes place after increase of blood pressure [7] and, once established, it is permanent. Reversal of hypertension failed to decrease the percentage of polyploid aortic SMC [8]. Only cell death is able to lower the DNA content. Therefore, anti-hypertensive therapy can prevent DNA ploidy increase only at an early stage [9].

*Abbreviations:* FCM, flow cytometry; PBS, phosphate buffered saline; SMC, smooth muscle cell(s).

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Polyploidy is a normal change in several types of cells during the lifetime of animals [10] and humans [11]. It is also seen in certain types of cells in culture at late passage [12]. Despite the general occurrence of polyploidy, the tacit assumption that the expression of genes is proportional to the ploidy of a cell has never been verified.

In this study, we show the relationship between ploidy and transcription of actin, platelet-derived growth factor A, collagen III and fibronectin in tetraploid and diploid late passage cultures of aortic SMC, isolated from newborn and adult rats, respectively, as well as in subcloned diploid and tetraploid cultures from newborn rats. Our results indicate a balanced relationship between ploidy and transcription levels in newborn SMC subcultures whereas the ratio between transcription levels in SMC derived from newborn and adult rats vary from gene to gene. These findings facilitate an interpretation of observed differences in gene expression in different phenotypes of vascular SMC.

## 2. MATERIALS AND METHODS

### 2.1. *Cell culture*

Thoracic aortas from 12-day-old (newborn) or 3-month-old (adult) male Wistar–Kyoto rats were removed. The tunica media was isolated and SMC were placed into culture as described [13]. SMC were grown in Waymouth's medium supplemented with 10% FBS (Hyclone, Logan, UT). To maximize the difference in ploidy between newborn and adult SMC, the cultures were used at very late passage (between the sixtieth and eightieth passage). Nearly pure diploid and tetraploid cultures of newborn SMC were obtained by growing cultures out of re-plated single cells in 96-well tissue culture plates (Costar, Badhoevedorp, The Netherlands) and were assayed by flow cytometry.

2.2. *Flow cytometry*

Cultures to be studied were analyzed in the confluent state after a growth arrest for 7 days in serum-free Waymouths medium. After trypsinization, the cells were centrifuged at 1,500 × *g* for 5 min. The cell pellet was resuspended in ice-cold 70% ethanol and incubated at −20°C for 12 h. The suspension was centrifuged at 1,500 × *g* for 5 min and the pellet was resuspended in 1 mM phosphate-buffered saline (PBS) with 20 μg/ml propidium iodide (Fluka, Brussels, Belgium) and 0.1 mg/ml RNase (Boehringer, Mannheim, Germany). The suspension was incubated for 1 h at room temperature and the lack of cell clumps was verified microscopically on the sample before being run on the flow cytometer. Chicken erythrocytes were used as an internal standard. The area of cell cycle compartments was estimated according to the method of Dean and Jett [14].

2.3. *Chromosome counts*

Chromosome counts were performed as described by Lawse et al. [15].

2.4. *RNA probes*

Antisense RNA probes were made using the Riboprobe Gemini System (Promega, Madison, WI) according to the manufacturers recommendations. The used DNAs were as follows: for actin, a 1.8-kb β-actin cDNA isolated from a rat SMC cDNA library, cloned in pBluescript (Stratagene, Heidelberg, Germany); for smooth muscle α-actin, a 25-bp oligonucleotide (5′ AGTGCTGTCCTCTTCTTCACACATA 3′), nucleotides −6 to 18 relative to the coding region of the human smooth muscle α-actin gene kindly provided by Dr. R. Meek (Department of Pathology, University of Washington, Seattle, WA, USA) [16]; for collagen III, a 600-bp *Eco*RI–*Pvu*II fragment from rat collagen III clone RGR-5, cloned in pBluescribe (kindly donated by J.K. Mäkelä and E. Vuorio, University of Turku, Turku, Finland); for fibronectin, a 500-bp rat cDNA, cloned in pGEM2, kindly donated by C. Giacelli (University of Washington, Seattle, WA, USA); for PDGF A, a 1,000-bp cDNA isolated from a rat SMC cDNA library, cloned in pBluescript.

2.5. *RNA isolation and determination of total RNA*

Total cellular RNA was isolated by the lithium–urea procedure as described by Auffray and Rougeon [17]. Cell pellets of newborn and adult SMC and cell pellets of newborn clone 1 and clone 10 were processed together giving a reproducible yield by this method.

The amount of total RNA per cell was determined as described by Merchant et al. [18], based on the measurement of the pentose content by the ornicol reaction. In brief, tissue culture cells were counted, pelleted and dounced. An aliquot was hydrolyzed in 0.3 M KOH and the mixture incubated for 30 min at 95°C in 5 vols. coloring reagent. The absorption at 665 nm was measured and compared to a yeast tRNA calibration curve. The coloring reagent was 0.18 g ornicol (Sigma, St. Louis, MO), 6 mg $FeCl_3$ (Merck, Darmstadt, Germany) in 30 ml 35% HCl (Merck, Darmstadt, Germany).

2.6. *Solution hybridization*

Solution hybridization was executed as described by Lee and Costlow [19]. In brief, fixed amounts of $^{32}$P-labelled antisense RNA were co-precipitated with increasing amounts of total RNA isolated from 80% confluent SMC cultures. The total amount of RNA was adjusted to 100 μg with yeast tRNA (Boehringer, Mannheim, Germany). After centrifugation the pellet was dried briefly, dissolved in 50% formamide, 0.4 M NaCl, 20 mM PIPES, pH 6.8, and 1 mM EDTA in a final volume of 20 μl and hybridized for 40 h at 60°C. 65 μg RNase A and 150 U RNase T1 (Boehringer, Mannheim, Germany) were added and incubated at 37°C for 1 h. Protected RNA duplexes were precipitated using 10% TCA, filtered through a Glasfaser Vorfilter (Schleicher and Schuell, Dassel, Germany) and the amount of protected antisense RNA probe was determined by counting in a liquid scintillation analyzer. Each experiment was repeated at least 3 times. Results were compared by Student's *t*-test and $P = 0.05$ or less was accepted as significant.

2.7. *DNA isolation*

Chromosomal DNA was isolated as follows: cells, scraped in PBS, from a 150-cm$^2$ tissue culture flask (Costar, Cambridge, UK) in PBS were centrifuged for 5 min at 1,500 × *g*. The pellet was washed with PBS and dissolved in 10 ml 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE). 400 μl 5 M NaCl and 500 μl 10% sodium dodecyl sulphate were added and the cells were lysed at room temperature. Proteinase K (Boehringer, Mannheim, Germany) was added to a final concentration of 50 μg/ml and incubated for 2 h at 37°C. After an extraction with TE-saturated phenol, the water phase was removed to a clean tube and extracted with chloroform. The water phase was removed to a clean tube and 2.5 vols. ethanol were added. The DNA was spooled, dried briefly and dissolved in 2 ml TE. 200 μg RNase was added and incubated for 20 min at 37°C. 100 μl 10% sodium dodecyl sulphate and proteinase K were added to a final concentration of 50 μg/ml. After a phenol and chloroform extraction a 2.5 vol. of 96% ethanol was added. The DNA was spooled, dried briefly and dissolved in 0.5 ml TE.

2.8. *Southern blotting*

Southern blots were made using Hybond N$^+$ (Amersham, 's Hertogenbosch, The Netherlands) following manufacturers recommendations and hybridized with 1–2 × 10$^6$ cpm per ml of $^{32}$P-labelled DNA probes according to the method described by Church and Gilbert [20]. Membranes were washed at 65°C in 0.1 M sodium phosphate, pH 7.2, 1% SDS, 1 mM EDTA, pH 8.0, for 2 × 30 min.

2.9. *Densitometry*

Intensities of hybridization signals were quantified using a LKB Brumma 2202 Ultrascan laser densitometer (LKB, Uppsala, Sweden) following manufacturers guidelines.

## 3. RESULTS AND DISCUSSION

### 3.1. *Culture morphology*

Aortic SMC from 3-month-old (adult) Wistar–Kyoto rats in culture have a spindle shape morphology and grow in overlapping bundles organized in multilayers at confluence as described by Gordon et al. [13] (Fig. 1a). In contrast, SMC of 12-day-old (newborn) rats grow in a monolayer and have an endothelial-like morphology (Fig. 1b), yet they do contain mRNA coding for α-actin and lack Von Willebrand factor (results not shown), clearly indicating the SMC nature of these cells. α-Actin and Von Willebrand factor are established markers for SMC and endothelial cells, respectively [13].

### 3.2. *Flow cytometry*

Flow cytometry (FCM) revealed that 86% of the late passage adult SMC had a DNA content corresponding to a diploid rat cell. 14% of the cells were tetraploid (Fig. 2a). Most of these cells are true tetraploid cells and not diploid cells in the G2 phase because [$^3$H]thymidine incorporation experiments indicate that less than 1% of the adult cells replicate after 1 week serum starvation (result not shown). FCM of late passage newborn SMC revealed that 15% of the cells were diploid, 66% tetraploid and 16% of the cells had a DNA content corresponding with an octaploid rat cell (Fig. 2b). Instead of true tetraploid and octaploid cells, these cells could also be dividing diploid and tetraploid cells, respectively. In order to obtain more homogeneous cultures of diploid

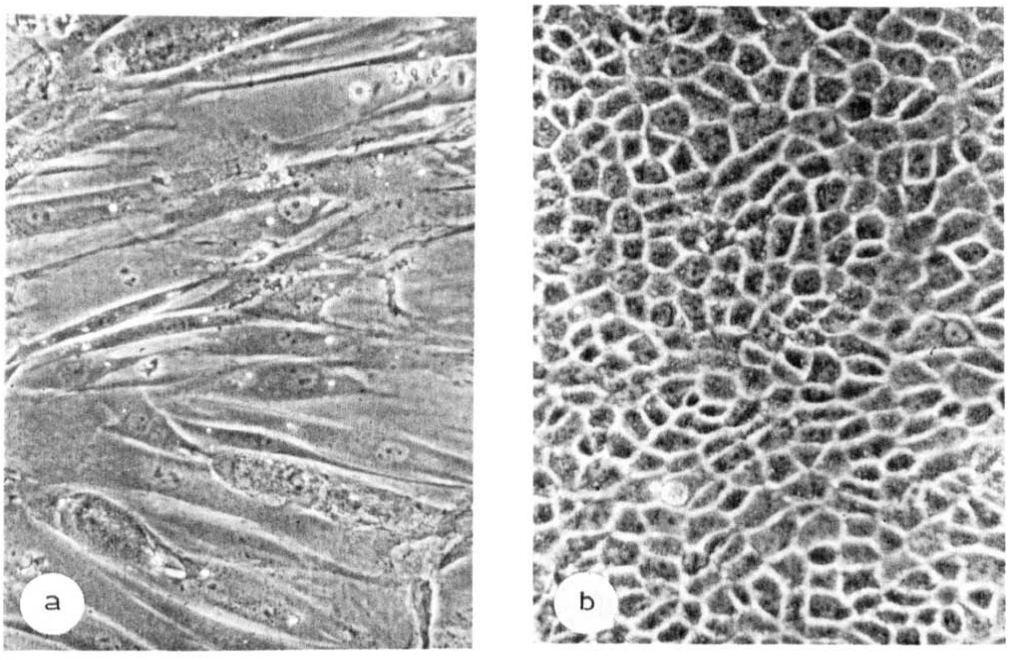


Fig. 1. Morphology of rat aortic SMC in vitro. Adult (a) and newborn (b) rat aortic SMC were grown to confluence and photographed by phase-contrast light microscopy.

and tetraploid newborn SMC, we subcloned the initial culture of newborn SMC, re-plating out cells at an average cell density of 1 cell per well. Thus we obtained an almost pure diploid (clone 1) and tetraploid (clone 10) newborn SMC subculture, as assayed by FCM (Fig. 2c and d, respectively).

### 3.3. *Chromosome counts*

Chromosome counts were performed on metaphase spreads of the 4 rat aortic SMC cultures. In general, the chromosome counts fit our FCM data for the different cultures (Table I). Diploid and tetraploid spreads were clearly present but with some dispersion in the actual counts, caused by errors when counting many very small rat chromosomes.

Since chromosomes are easily lost or rearranged in long term tissue culture, relative copy numbers of the genes used for measuring transcription prevalence were determined. Southern blots containing equal amounts of *Eco*RI-digested chromosomal DNA were hybridized with DNA probes for actin, collagen III, fibronectin and PDGF A. Densitometric scanning of the autoradiogram revealed equal intensities in all lanes, indicating a linear relationship between the hybridization signal intensities and the amount of DNA used (results not shown). This observation, together with FCM analysis and chromosome counts, indicates that the gene dose of the genes included in this study is directly proportional to the ploidy of a particular culture of SMC.

### 3.4. *Quantitation of transcription of several genes in adult and newborn SMC does not match ploidy*

In each of the SMC cultures the amount of total RNA was determined using a quantitative RNA-isolation procedure (see Materials and Methods). It appeared

Table I

Chromosome counts on metaphase spreads of aortic SMC in cultures of adult and newborn rats

| SMC culture | Percentage of spreads in cluster | | | Total number of spreads |
|---|---|---|---|---|
| | Diploid[a] | Tetraploid[b] | Octaploid[c] | |
| Adult | 92 | 8 | – | 47 |
| Newborn | 3 | 89 | – | 37 |
| Newborn clone 1 | 89 | 5 | – | 38 |
| Newborn clone 10 | – | 91 | – | 34 |

Metaphase spreads were made and assayed as described in Materials and Methods.
[a]The diploid (= 42 chromosomes) cluster consisted of counts in the 39–45 chromosomes range.
[b]The tetraploid cluster consisted of counts in the 78–90 chromosomes range.
[c]The octaploid cluster consisted of counts in the 156–180 chromosomes range.

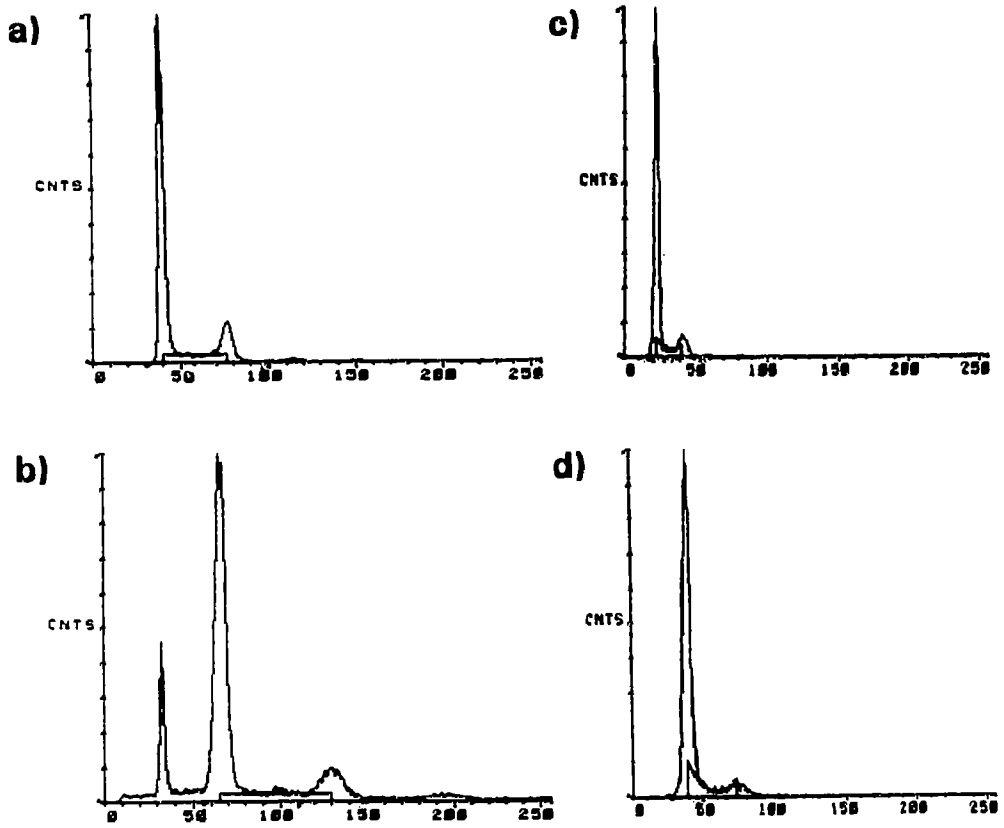


Fig. 2. Flow cytometry (FCM) of cloned populations of smooth muscle cells. (a) Adult SMC culture showing 86% 2C and 14% 4C (including diploid cells in the G2 phase) cells. (b) Newborn SMC culture showing 15% 2C, 66% 4C (including diploid cells in the G2 phase) and 16% 8C (including tetraploid cells in the G2 phase). (c) Diploid newborn SMC subclone (clone 1) showing 92% 2C and 8% 4C (including diploid cells in the G2 phase). (d) Tetraploid newborn SMC subclone (clone 10) showing 92% 4C and 8% 8C (including tetraploid cells in the G2 phase). Ploidy was determined using chicken erythrocytes as an internal standard. The experiments were carried out in 2 sets: a + b is one set, c + d is another set.

that the amount of total RNA per SMC matched the ploidy of the cell; in tetraploid cultures, cells contained about 2-fold more RNA than diploid cultures (Table II). To quantitate transcription levels of single genes in the newborn and adult cultures, solution hybridization assays were conducted using a panel of genes (Fig. 3).

Table II

Quantitation of the total amount of RNA per cell in SMC cultures of adult and newborn rats

| SMC cultures | Ploidy percentage of cells | | | pg RNA/cell[a] |
|---|---|---|---|---|
| | 2C | 4C | 8C | |
| Adult | 86 | 14 | – | 26 ± 5 |
| Newborn | 15 | 66 | 16 | 56 ± 10 |
| Newborn clone 1 | 92 | 8 | – | 38 ± 4 |
| Newborn clone 10 | – | 92 | 8 | 58 ± 2 |

Total RNA was isolated from $10^8$ cells as described in Materials and Methods. The ploidy percentage of the cultures was determined by flow cytometry.

[a] Values represent mean of 3 separate experiments performed in duplicate.

Our results show that the ratio of transcription in newborn and adult SMC varies from gene to gene and does not depend on gene doses (ploidy) alone. Therefore, we conclude from the experiments specified below that newborn and adult SMC represent clearly distinct phenotypes.

Actin expression is raised significantly in newborn SMC (Fig. 3a). Newborn cells contain 3-fold more actin transcripts than adult cells. Corrected for ploidy, this represents a 1.5-fold relative overexpression in newborn SMC. Due to the high homology amongst members of the actin gene family, the $\beta$-actin probe used hybridized to all actin transcripts giving no information about only smooth muscle-specific $\alpha$-actin expression. However, Northern analysis with a SMC $\alpha$-actin-specific oligonucleotide suggests that this differentiation marker is more abundant in adult SMC, again indicating that these newborn and adult SMC represent different phenotypes (result not shown). On the other hand solution hybridizations to RNA from the diploid newborn SMC subclone (clone 1) and the tetraploid newborn SMC subclone (clone 10) show that in these cells, for all genes assayed, the transcription level is linked with ploidy

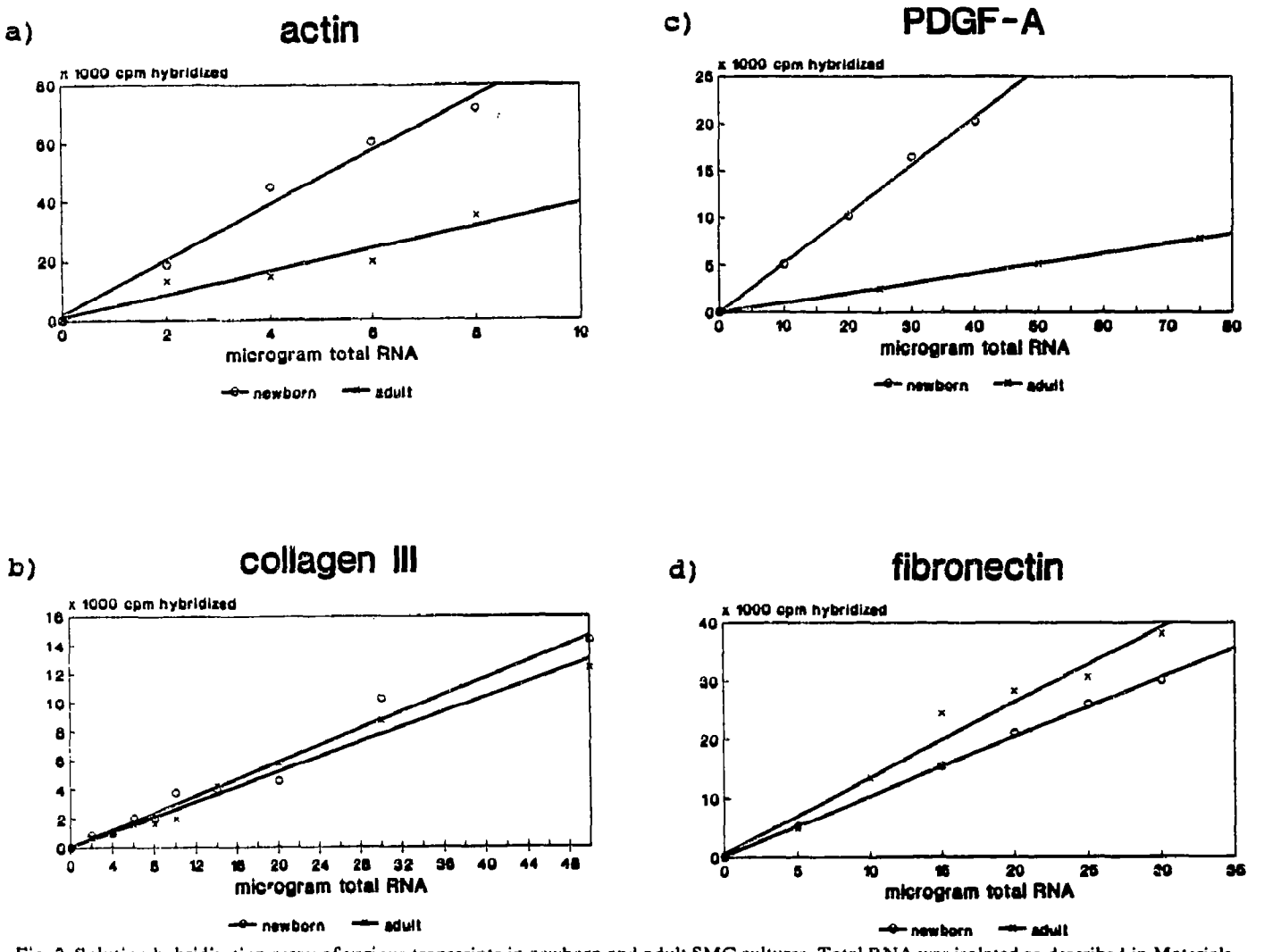


Fig. 3. Solution hybridization assay of various transcripts in newborn and adult SMC cultures. Total RNA was isolated as described in Materials and Methods and hybridized in solution to the following probes and concentrations: (a) $\beta$-actin, 4 ng; (b) collagen III, 1 ng; (c) platelet-derived growth factor A, 0.15 ng; and (d) fibronectin, 1 ng. (○) Experiments with total RNA from newborn smooth muscle cells. (+) Experiments with total RNA from adult smooth muscle cells. For other details see Materials and Methods.

(Fig. 4). Therefore, the observed difference in actin gene expression must be viewed as a difference between newborn and adult SMC rather than a difference between diploid and tetraploid SMC. Clearly, the expression of actin genes in newborn and adult SMC is controlled in a different manner.

The solution hybridization experiments show a ploidy-dependent expression of collagen III mRNA in adult and newborn SMC (Fig. 3b). The expression in the newborn subclones also is ploidy-dependent (Fig. 4b). Collagen III is a component of both the normal vessel wall and the atherosclerotic plaque in which no significant rise in the amount of collagen III is observed [21].

PDGF A is expressed 4-fold higher in newborn SMC than in adult SMC (Fig. 3c). Since an adult SMC contains half the amount of DNA compared to a newborn SMC, this reflects a 2-fold raise in transcriptional activity per gene copy. In solution hybridizations to RNA from newborn SMC clone 1 and clone 10 the transcription level correlates with ploidy (Fig. 4c). PDGF A is a mitogen for cultured SMC [22]. In the vascular system PDGF A-expressing cells found in the intima were endothelial cells and neointimal SMC showed PDGF A expression. Medial SMC did not express PDGF A [23]. Majesky et al. [24] hybridized Northern blots with equal amounts of RNA isolated from newborn and adult SMC cultures using a probe for PDGF A. They showed an equal hybridization intensity in both lanes. It is unknown whether the difference in amount of mRNA we observe is related to the late passage number of the cultures or, more likely, reflects the better accuracy of the solution hybridization technique.

Fibronectin expression correlates with ploidy (Fig.

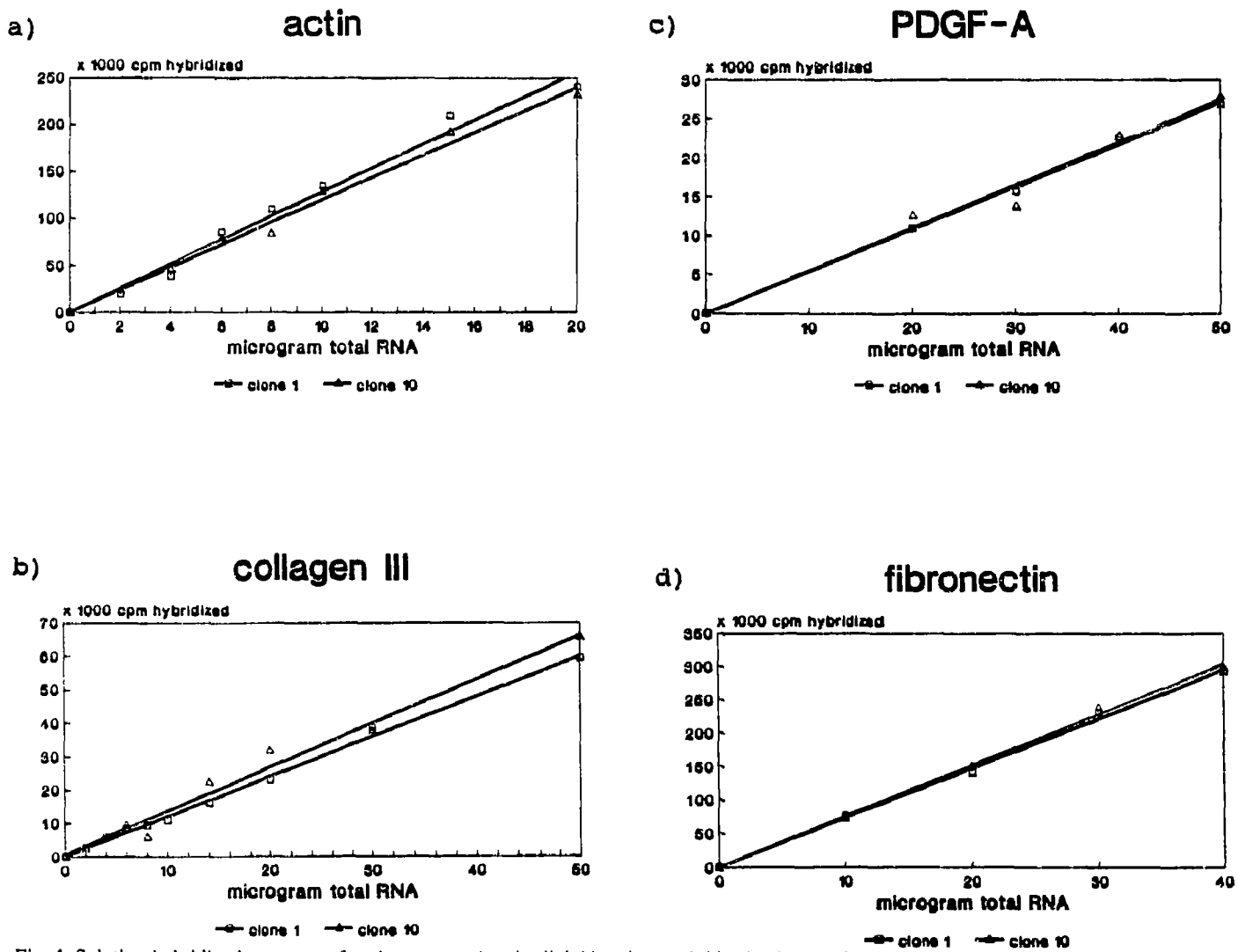


Fig. 4. Solution hybridization assays of various transcripts in diploid and tetraploid subcultures of newborn SMC. Total RNA was isolated as described in Materials and Methods and hybridized in solution to the following probes and concentrations: (a) $\beta$-actin, 4 ng; (b) collagen III, 1 ng; (c) platelet-derived growth factor A, 0.15 ng; and (d) fibronectin, 1 ng. (□) Experiments with total RNA from clone 1 (diploid newborn smooth muscle cells). (△) Experiments with total RNA from clone 10 (tetraploid newborn smooth muscle cells). For other details see Materials and Methods.

3d). On a per cell basis, newborn SMC contain 2-fold more fibronectin mRNA than adult SMC. Fibronectin expression in clone 1 and clone 10 also matches ploidy (Fig. 4d). Fibronectins are large glycoproteins that play a role in many biological processes including cellular adhesion, morphology and cytoskeletal organization [25].

All together these results indicate a different control of gene expression in cultures of SMC derived from newborn and adult rats which is not strictly linked with ploidy. Neonatal SMC in culture are able to produce the peptide growth factors required for their own growth [26]. The reduced expression of the genes coding for contractile proteins suggests that neonatal SMC, compared to adult SMC, represent a lesser differentiated SMC [27]. The phenotype of newborn SMC is also seen when neointimal SMC are brought in culture [28] or when adult SMC are cultured in platelet-deprived serum [29]. These observations suggest that the coat of a blood vessel may be heterogeneous, containing SMC with different functions and phenotypes [30]. Less differentiated SMC could serve as the stem cell population maintaining the regenerative potential whereas differentiated SMC controls the contractile properties of a blood vessel. The newborn and adult SMC culture system provides a useful tool for studying those differences that are relevant to the development of atherosclerosis and hypertension.

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