The pharmacology of cell differentiation.
R.A. Rifkind, editor

Effects of *myc* expression on mouse myoblasts are reversed in mixed culture with normal cells

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INTRODUCTION

The relationship between transformation and terminal differentiation is generally one of mutual exclusion. This is true both *in vivo*, for animal tumors, and *in vitro*, for differentiating cellular systems. The differentiating skeletal muscle *in vitro* system offers several advantages for the study of the reciprocal interference between transformation and differentiation.

Many mammalian skeletal muscle cell lines are available that can be induced to undergo differentiation, usually by growth factor withdrawal. Several of these lines are also exquisitely sensitive to transformation by known oncogenes. One of such cell lines is C2C12 [1], derived from adult mouse skeletal muscle. It proliferates at a fast rate in the presence of standard growth medium, and differentiates efficiently shortly after serum withdrawal. Skeletal muscle differentiation classically involves several steps. Proliferating cells, when induced to differentiate, irreversibly withdraw from the cell cycle (commitment) and express muscle-specific genes (biochemical differentiation) [2]. Next, biochemically differentiated cells form multinucleated, syncitial myotubes (fusion), that often exhibit spontaneous contractile activity *in vitro*.

In recent years, a family of muscle regulatory genes has been identified. This includes four known members, in mammals: MyoD [3], myogenin [4], myf-5 [5], and MRF4 (also called herculin or myf-6) [6, 7]. The members of this family are transcription factors and share common regulatory regions, the so-called basic and helix-loop-helix domains [8]. Functionally, each of these factors is able to convert mouse fibroblasts and other cell types into myoblasts. Moreover, no exception has been found so far to the rule that at least one of these factors must be expressed for a cell to differentiate into skeletal muscle. Members of the family can cross-activate one another [9], although this ability seems to vary, depending on the cell line in which the phenomenon is investigated [10].

A number of cellular and viral oncogenes are known to transform C2C12 cells, and transformation, in turn, strongly inhibits differentiation [11, 12]. In principle, this might be due to the impaired ability of transformed cells to regulate their proliferation. In this view, a transformed cell would not stop proliferating even in the absence of exogenous growth factors. Differentiation would be inhibited since irreversible growth arrest is a necessary prerequisite for it. However, at least in some instances, inhibition of differentiation appears to take place through transcriptional down-regulation of muscle regulatory genes of the MyoD family [13, 14]. This would suggest that continued proliferation is not the only mechanism for oncogene-provoked inhibition of differentiation.

The *myc*-oncogene has been shown to transform and block differentiation of quail myoblasts [15]. It was also previously shown that contact with normal cells could induce reversion of the *myc*-induced phenotype [16]. That is, the myoblasts could now differentiate, despite the continued expression of *myc*. We define this phenomenon, in which normal cells can suppress the transformed phenotype of a *myc*-expressing cell, as "normalization".

In this contribution, we report on our investigation of the effects produced by forced expression of the *myc* oncogene in C2C12 cells. We describe that despite clear evidence of transformation, *myc*-expressing C2C12 myoblasts were not inhibited from differentiating. However, fusion did not take place and biochemically differentiated cells remained mononuclear. In a series of attempts at correcting this phenotype, we found that *myc*-transformed cells could form heterologous myotubes together with fusion-competent myoblasts. Moreover when these cells were put in cocultivation with untransformed fibroblasts, the former showed a normalized phenotype, in that the *myc*-expressing cells readily formed myotubes. This last finding provides evidence that normalization of the *myc*-induced phenotype is not restricted to growth control. Other aspects of such phenotype—fusion competence in this case—are also normalized.

MATERIALS AND METHODS

General procedures

Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (growth medium, GM) in a humidified incubator in a 5% CO_2 atmosphere Differentiation was induced by replacing GM with DMEM + 10 μg/ml insulin + 5μg/ml transferrin (serum free medium, SF).

Differentiation index was defined as percentage of nuclei in differentiated cells. It was calculated by scoring at least 100 nuclei in differentiated cells. Fusion index was defined as percentage of nuclei belonging to cells with three or more nuclei.

For soft agar assays, cells were seeded in GM containing 0.35% agar. Dishes were fed twice weekly after the first seven days and colonies were scored after two to three weeks.

RNA extraction, gel separation, transfer to nitrocellulose filter, and Northern blot hybridization were carried out according to standard procedures [17].

Viruses and infections

MMCV is a replication-defective recombinant retrovirus carrying the avian v-*myc*^{OK10} [18].

The replication-defective Babe-c-*myc* and Babe-c/v-*myc* viruses direct the expression of chicken c-*myc* and a chimaeric chicken c-*myc*/v-*myc* protein. The details of their construction will be described elsewhere [19].

Infections were carried out in 60-mm tissue culture dishes. C2C12 cells ($2 \cdot 10^5$) were incubated for one to three hours in the presence of 2.5 μg/ml polybrene and infected overnight with one ml of viral stock.

Immunofluorescence

Immunofluorescence (IF) for the following antigens was carried out employing the reagents listed below.

5-bromo-2'-deoxyuridine (BrdU): monoclonal antibody/DNAse mixture (Amersham).

myc: rabbit polyclonal antiserum (kind gift of K. Moelling).

MyoD: rabbit antiserum raised against the carboxy-terminal half of the bacterially expressed molecule.

Myogenin: monoclonal antibody IF5D7 from W. Wright.

Myosin heavy chain (MHC): monoclonal antibody MF20 from D. Fischman [20].

Troponin T: monoclonal antibody (Amersham).

Secondary antibodies: fluorescein- or rhodamine-conjugated goat antisera against rabbit or mouse IgG (Organon Teknika).

DNA was stained after IF procedures by a three minute's incubation with a 0.1 μg/ml solution of Hoechst 33258 in phosphate-buffered saline.

Cocultures

c/v-*myc*-expressing C2C12 cells were cocultured with either rat L8 myoblasts or mouse C3H-10T1/2 fibroblasts. Cocultures with L8 myoblasts were set up by seeding equal numbers (10^5) of *c/v-myc*-transformed C2C12 and L8 into 35-mm Petri dishes. Cultures were shifted to SF on the next day and stained (MHC and DNA) three days later. At least 100 MHC-positive, multinucleated myotubes were scored as possessing rat only, mouse only, or rat and mouse nuclei.

Cocultures with fibroblasts: 100 to 1000 *c/v-myc*-transformed C2C12 cells were seeded alone or along with $5 \cdot 10^5$ C3H-10T1/2 or Rat-2 fibroblasts into 60-mm dishes in GM. Five to 7 days later myoblast colonies were stained by Giemsa and the presence of multinucleated myotubes (three or more nuclei) was evaluated.

RESULTS

Phenotype of *myc*-expressing C2C12

C2C12 cells were infected with the replication-defective, chicken v-*myc*-transducing retrovirus MMCV and the MLV helper virus. Because of the presence of the helper virus, the great majority of the cells was infected. This resulted in phenotypical changes. Cells rounded up, became smaller, and showed prominent nucleoli. Their growth rate became faster and their saturation density higher. Unselected cell lines thus obtained were named C2C12(MMCV). These cells also exhibited anchorage-independent growth in soft agar assays. In a series of experiments, growth efficiency in soft agar ranged from 40.5 to 80%.

When the differentiative capacity of C2C12(MMCV) cells was tested, we found a surprising phenotype. Despite the presence of the transformation-associated traits described above, C2C12(MMCV) cells were capable of efficient differentiation, as assessed by morphological changes and MHC expression. However, the differentiated cells did not fuse and remained mononucleated even when grown at high cell density to favor fusion (Fig. 1). Typically, the differentiation indices of both C2C12 and C2C12(MMCV) cells were around 40%.

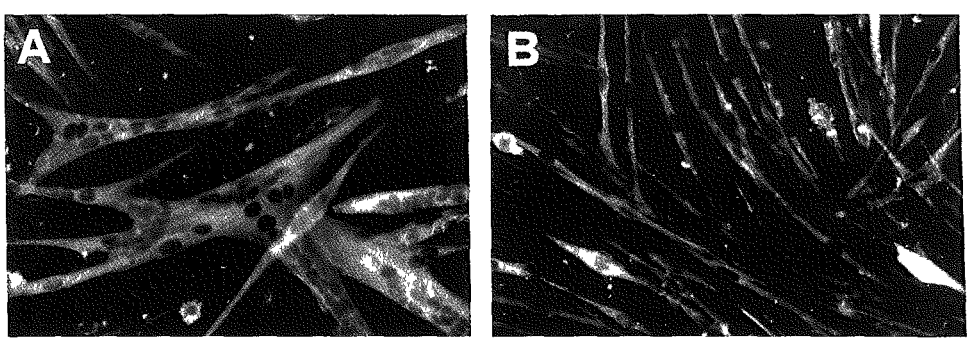

Figure 1. Immunofluorescence for myosin heavy chain detection.
Cells were stained after three days in SF medium. A: wild-type C2C12 cells; B: C2C12(MMCV). Notice expression of MHC in mononucleated cells in B.

The unexpected coexistence of transformation and differentiative potential within the same cell line might be explained by the presence of two subpopulations within the C2C12(MMCV) cells. One of these could be transformed by *myc*, while the other might express low levels of the oncogene and be capable of differentiating. To test this possibility we randomly isolated 14 independent clones from soft agar. All the clones showed differentiation indices similar to that of the parental

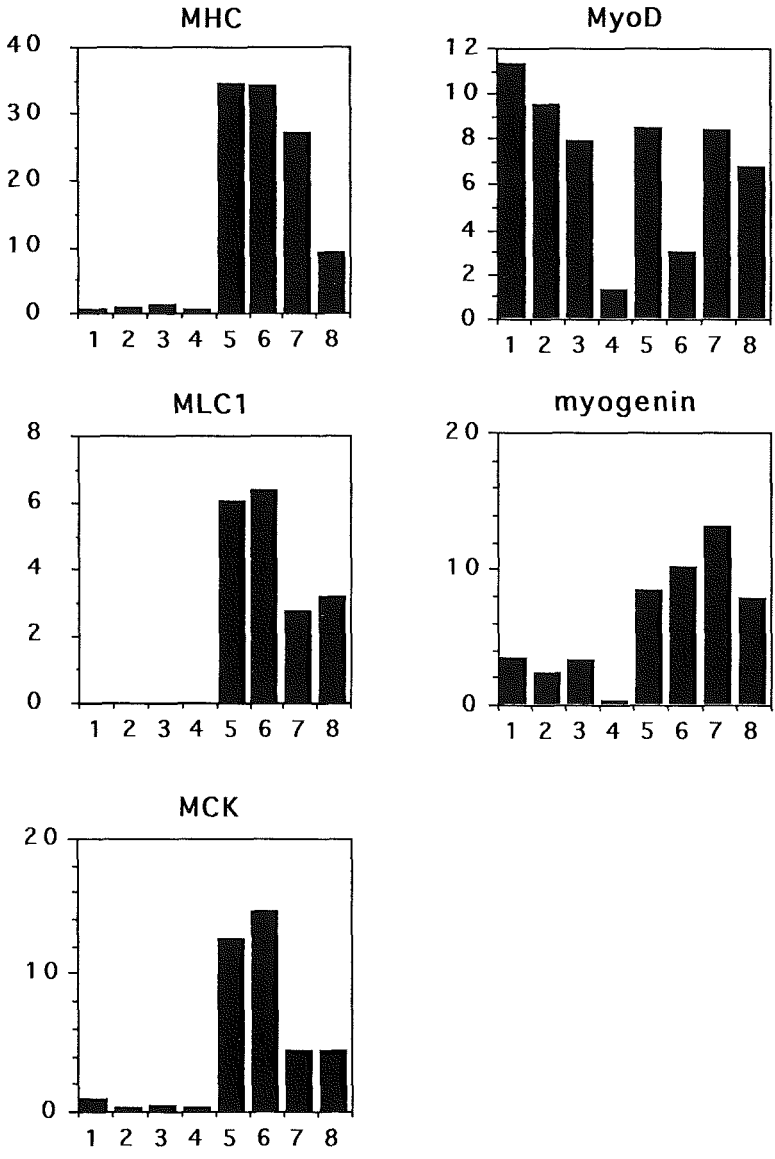

Figure 2. Densitometer-aided evaluation of northern blots.
RNA from proliferating (1-4) and differentiated (5-8) cells. 1, 5: C2C12;
2, 6: C2C12(Babe-c-myc); 3, 7: C2C12(Babe(c/v-myc); 4, 8: C2C12(MMCV).

C2C12(MMCV) cells. Moreover, IF studies demonstrated that in differentiated C2C12(MMCV) cells exogenous *myc* was still expressed (not shown).

We tested the completeness of biochemical differentiation in C2C12(MMCV) cells by analyzing the expression of a number of muscle-specific genes. MHC, myosin light chain 1 (MLC1), muscle creatine kinase (MCK), MyoD, and myogenin mRNA expression was comparable in differentiated wild-type C2C12 and C2C12(MMCV) cells (Fig. 2). MHC, MyoD, and myogenin protein expression was verified by IF. Expression of troponin T was also assessed by IF.

C2C12 cells were infected with two other *myc*-transducing, G418-selectable, replication-defective retroviruses: Babe-c-*myc*, and Babe-c/v-*myc*. In the cell lines so derived, the transformed and differentiated phenotypes and the patterns of mRNA expression were virtually indistinguishable from those of C2C12(MMCV). We analyzed *myc* mRNA expression levels in cell lines derived from C2C12 infected with both of the Babe viruses. Fig. 3 shows that steady-state *myc* mRNA levels did not vary significantly between proliferating and differentiated cells. Again, this strongly indicated that differentiation did not take place selectively in cells expressing low levels of *myc*.

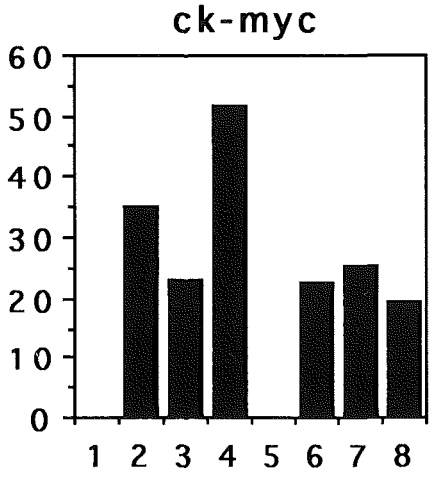

Figure 3. Exogenous mRNA *myc* levels in proliferating and differentiated cells. Exogenous levels of *myc* detected by a chicken probe. Sample order as in Fig. 2.

Normalization of the *myc*-induced phenotype

A series of cocultivation experiments were designed to correct the fusion-defective phenotype. Initially, we cocultivated equal numbers of Babe-c/v-*myc*-

transformed C2C12 cells and untransformed rat L8 myoblasts. Three days after induction of differentiation, the cultures were stained to detect MHC and DNA. As table 1 shows, C2C12 (infected and not) and L8 efficiently formed heterologous myotubes, containing nuclei from both species. However, c/v-*myc*-expressing C2C12 were also induced to form homologous myotubes with remarkable frequency.

Table 1
L8/C2C12 cocultivation: homologous and heterologous myotubes

	% rat + mouse	% mouse only	% rat only
L8 + C2C12	58	42	0
L8 + C2C12(Babe-c/v-*myc*)	30	70	0

Results are shown as percentage of multinucleated myotubes possessing rat and mouse, mouse only, or rat only nuclei.
In this experiment, C2C12(Babe-c/v-*myc*) cells plated alone showed a fusion index of 1%

These results prompted us to ask whether simply the presence of untransformed cells in cocultivation was sufficient to cause normalization of the *myc*-induced phenotype. To this end we set up cocultures of C2C12(Babe-c/v-*myc*) cells and either mouse C3H-10T1/2 or rat Rat-2 fibroblasts In these experiments untransformed fibroblasts far outnumbered C2C12(Babe-c/v-*myc*) cells, in order to ensure that virtually every *myc*-expressing cell was in contact with fibroblasts. Cultures were allowed to become confluent and were either stained with Giemsa stain or processed for MHC IF. In the presence of fibroblasts, C2C12(Babe-c/v-*myc*) formed multinucleated myotubes (Fig. 4). Control cultures in which C2C12(Babe-c/v-*myc*) had been seeded alone showed either no differentiation (in GM) or biochemical differentiation only (in SF).

Rat-2 cells were consistently more efficient than C3H-10T1/2 in correcting the phenotype. Occasionally, myotubes formed by C2C12(Babe-c/v-*myc*) in cocultivation with fibroblasts displayed spontaneous contractions, demonstrating full integrity of the contractile apparatus.

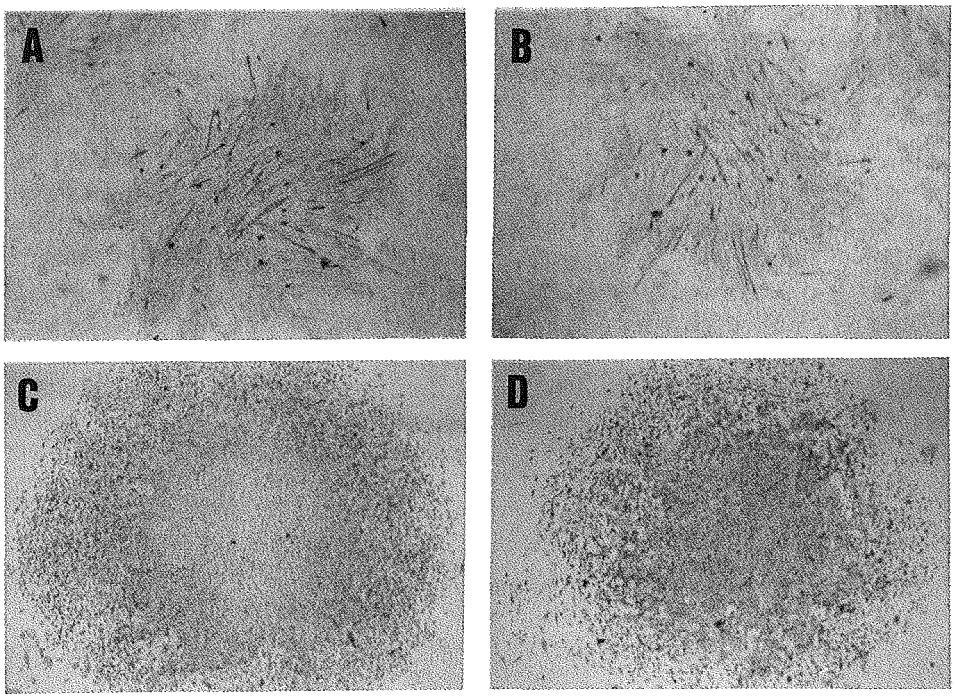

Figure 4. Cocultivation of Babe-c/v-*myc*-transformed C2C12 and C3H-10T1/2 fibroblasts.
Giemsa-stained dishes. A, B: colonies of *myc*-transformed cells grown on a monolayer of fibroblasts. C, D: control Babe-c/v-*myc*-transformed C2C12 growing alone in GM. Notice a number of multinucleated myotubes (some of which show a darker stain) in A and B.

DISCUSSION

In the present contribution, we describe the effects of expressing *myc* oncogene in the mammalian myoblast cell line C2C12. The cells were infected with three retroviruses transducing different forms of avian c- or v-*myc*. The phenotype obtained with each of the viruses was virtually indistinguishable from the others. Two features of such phenotype are remarkable. First, that differentiation could efficiently take place despite the presence of unmistakable transformation. Second, the absence of fusion in the presence of apparently unimpaired biochemical differentiation. We also performed an initial series of experiments aimed at

correcting the *myc*-induced phenotype by means of cocultivation with untransformed cells.

Transformation is incompatible with terminal differentiation in general and with skeletal muscle differentiation in particular. Transformation by a number of oncogenes has been shown to prevent differentiation in both primary myoblasts and established myoblastic cell lines. However we showed that 14 clones of *myc*-expressing cells, selected in soft agar for showing a transformed phenotype, all differentiated efficiently in the appropriate conditions. Moreover, the differentiated cells still expressed levels of exogenous *myc* comparable to those of proliferating cells. We conclude that in *myc*-expressing C2C12 cells transformed state and differentiative potential coexist within the same cell.

A second, remarkable characteristic of the *myc*-transformed cells is their inability to fuse to form syncitial myotubes. It is possible to separate fusion from biochemical differentiation by means of various *in vitro* manipulations, such as lowering calcium concentration in culture medium [21]. However, these treatments most likely alter effector molecules that are necessary to carry out fusion. The fact that *myc* can reproduce such a defective phenotype suggests–although does not prove–that an oncogene can selectively impair a dedicated regulatory mechanism specifically controlling fusion, without altering the preceding steps.

After defining the *myc*-induced phenotype, our efforts have focused on inducing its reversion. We cultivated c/v-*myc*-transformed C2C12 cells together with fusion-competent, L8 rat myoblasts. We hoped that fusing cells could recruit the differentiated, *myc*-expressing ones. In these conditions, *myc*-expressing cells readily formed heterologous myotubes containing both mouse and rat nuclei. However, we noticed that myotubes containing only mouse nuclei were also induced. We had previously shown that *myc*-transformed, unestablished quail myoblasts were inhibited from differentiating. These cells could be induced to revert to a differentiation-proficient phenotype by cocultivating them with untransformed cells. Because in such conditions transformed cells revert to an apparently normal phenotype, we refer to the phenomenon as to "normalization". In consequence, we cocultivated *myc*-transformed C2C12 with mouse or rat untransformed fibroblasts. The presence of these cells induced myotube formation. This is a meaningful finding for several reasons. In the first place, it extends the phenomenon of normalization to a mammalian system. Moreover, C2C12 cells can be manipulated far more easily than primary quail myoblasts. This should allow to explore the normalization phenomenon with the aim of understanding its molecular basis. Finally, we stress that in the present case the phenotype being corrected by normal fibroblasts is that of fusion incompetence. At the moment, we cannot distinguish whether correction takes place before or after terminal differentiation of *myc*-transformed C2C12. Both normal and *myc*-transformed cells withdraw permanently from the cell cycle in order to express biochemical differentiation. Therefore the present experiments indicate that the correction of the fusion-defective phenotype of *myc*-transformed myoblasts in mixed culture is not

accounted for by growth restriction by surrounding normal cells, but involves a more profound phenotypic reversion of *myc*-expressing cells.

ACKNOWLEDGMENTS

The present work has been supported by grants from: Associazione Italiana per la Ricerca sul Cancro (progetto Oncosoppressori); Consiglio Nazionale delle Ricerche (progetto finalizzato Applicazioni Cliniche della Ricerca Oncologica); Fondazione Cenci-Bolognetti.

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Discussion - EFFECTS OF *MYC* EXPRESSION ON MOUSE MYOBLASTS ARE REVERSED IN MIXED CULTURE WITH NORMAL CELLS

R. Benezra

It has been shown with overexpression of two antagonists of muscle differentiation, namely Jun and Id, that you can block differentiation for as long as the protein is in the nucleus. I was wondering if you had looked at protein levels in your myc differentiated cells to see whether or not the protein is on the nucleus.

M. Crescenzi

Yes, we have looked at protein levels and there are no differences between proliferating cells and differentiating cells in mRNA levels. The other thing is that you can clearly see myc in the nuclei of the differentiated mononuclear cells. As far as you can tell from immunofluorescence it is as high as in the non differentiated cells.

R. Benezra

Is there any way you can reconcile your results with the results of Wold who shows that overexpression of myc completely blocks differentiation?

M. Crescenzi

I don't have a full answer to that. Barbara Wold has shown that in NIH3T3 converted by MyoD into myoblasts, myc prevents differentiation, even in the presence of MyoD. NIH3T3 converted by MyoD are not necessarily myoblasts under all respects, they exhibit some differences in comparison with regular myoblasts. For example, they don't fuse and this is definitely one difference. Second, NIH3T3 have some kind of restriction in allowing MyoD to elicit the other muscle regulatory genes, so it is possible that the presence of several self regulatory genes together is necessary to achieve cell fusion, or it is necessary for myc to be unable to block differentiation.

Wold suggested that perhaps myc interacts, more or less directly, with MyoD and blocks altogether its action. I would say that probably our experiments tend to

disprove that notion, or at least they indicate that notion is not entirely correct, in the sense that the C2C12 cells express most likely only MyoD when they are proliferating, so MyoD is the only muscle determination gene present there and it's obviously functional in the presence of myc, because these cells can undergo differentiation. I would say that even though MyoD mRNA levels go down to some extent in proliferating cells transformed by myc, the amount of MyoD that is left is most likely enough to induce differentiation.

K. Nasmyth

Have you observed changes in MyoD when the cells differentiate?

M. Crescenzi

In wild type cells there is no major change in the levels of MyoD, it may increase to some extent but not so much. What changes dramatically is its ability to transactivate genes. It appears to be almost inactive as a transcription factor in proliferating cells, it becomes dramatically active when cells differentiate. It is not the mere presence of the protein, what changes is its activity but we do not know exactly what its activity is regulated by.

K. Nasmyth

What happens to endogenous myc during the differentiation process?

M. Crescenzi

It goes down, it basically disappears on differentiation.

J. León

As a comment to the previous question, you should probably look first at the max levels to compare both systems. Looking at them you could probably reconciliate your results with those of Wald, because myc works only when it binds max.

Concerning endogenous myc, it is worth recalling that in many systems when you overexpress myc, especially in not very transformed cell systems, the endogenous

myc downregulates. You have just said that in your system the endogenous myc disappears, and I wonder whether it is just because of the differentiation or because of the exogenous myc you are overexpressing there.

M. Crescenzi

Of course, max is important. I would assume that max is expressed and working in the differentiated cells because myc is supposed to need max in order to exert its effects, and it's obviously doing something in those cells in that it prevents differentiation. Nevertheless, max should certainly be investigated. As far as the endogenous myc is concerned, it disappears upon differentiation in wild-type cells. I would assume, although it is not true for all cell lines, that, in fact, as you are saying, the exogenous myc down regulates the endogenous in C2C12 cells. This is not the case, however, in cell lines such as NIH3T3, where myc does not majorly overregulate itself.

H.F. Lodish

In your experiments, rat myoblasts could induce fusion of these myc transformed mouse cells, but you mentioned that there were cells that had only mouse nuclei. This leads me to the question: Is it possible that what is missing in these cells is a secreted factor? I remember a metalloprotease that various groups had postulated was involved in some way in cell-cell fusion. Is anything more known about this whole process?

M. Crescenzi

Not that much. What I can say is that we have tried, at length, to detect anything in the conditioned media and we have found nothing. Of course, this is a negative result which means little, but it really appears that if you separate these cells by even a little space normal cells are unable to correct the phenotype any more. That is, even though we postulate that cell to cell communication is needed, at least cell to cell contact really appears to be needed. Almost for sure it is not something that gets secreted.

I.B. Weinstein

Have you measured cell to cell communication between the non myc cells, the myc cells and a mixture of myc plus non myc cells?

M. Crescenzi

The myc cells are, as far as we can tell, able to communicate with themselves as efficiently as the parental cells, they are also able to communicate with other cells, including the rat cells we have used in co-cultures. So myc does not appear to affect cell to cell communication.

H.F. Lodish

Can you explain that a bit more? What do you mean by these cells being unaffected by cell to cell cycle communication - they don't fuse?

M. Crescenzi

By cell to cell communication I mean the ability of cells to exchange at least small molecules through common gaps in their membranes, that is by gap junctions or similar organelles. This kind of cell to cell communication is not blocked by myc, it is blocked by transformation by a number of other oncogenes. The phenotype induced by these other oncogenes cannot be corrected in mixed culture, while the myc induced phenotype can be corrected.