# The effect of galectin-1 on the differentiation of fibroblasts and myoblasts in vitro

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## Summary

**Normal murine dermal fibroblasts implanted into the muscles of the *mdx* mouse, a model for Duchenne muscular dystrophy, not only participate in new myofibre formation but also direct the expression of the protein dystrophin which is deficient in these mice. We have reported that the lectin galectin-1 is implicated in the conversion of dermal fibroblasts to muscle. In the current work we confirm the presence of galectin-1 in the medium used for conversion. Furthermore we report that exposure of clones of dermal fibroblasts to this lectin results in 100% conversion of the cells. Conversion was assessed by the expression within the cells of the muscle-specific cytoskeletal protein desmin. We also investigate the effects of galectin-1 on cells of the C2C12 mouse myogenic cell line and on primary mouse myoblasts. Exposing both transformed and primary myoblasts to the lectin resulted in an increase in fusion of cells to the terminally differentiated state in both types of cultures. Galectin-1 does not cause the myogenic conversion of murine muscle-derived fibroblasts.**

Key words: Dermal fibroblast, Galectin-1, Myogenic conversion, Muscle differentiation, Myoblasts

## Introduction

Recent work in our laboratory has identified a factor that is capable of converting murine dermal fibroblasts to muscle cells (Goldring et al., 2000). This factor is the lectin galectin-1, previously known by several different names including β-galactoside binding protein (βGBP) (Barondes et al., 1994a). Galectin-1, a 14-15 kDa lectin secreted by myoblasts and myotubes in culture (Harrison and Wilson, 1992), belongs to the galectin subclass that has affinity for β-galactoside sugars (Barondes et al., 1994b; Cooper and Barondes, 1999), which upon binding to the appropriate glycoligands elicits a multitude of biological activities (Smetana et al., 1997).

The biological role of galectin-1 is still controversial. Yamaoka et al. (1991) reported that it caused 3T3 fibroblasts to exhibit a transformed phenotype and induce tumour formation. Sanford and Harris-Hooker have shown that it causes proliferation of vascular smooth muscle cells in vitro (Sanford and Harris-Hooker, 1990). By contrast, galectin-1 acts as a negative cell growth regulator in fibroblasts (Wells and Malluchi, 1991) acting to block the mitogenic MAP kinase cascade that is normally activated by binding of growth factor ligand to the appropriate receptor tyrosine kinase (Vespa et al., 1999; Walzel et al., 1999). In our previous study we added galectin-1 to cultures of primary mouse dermal fibroblast and observed that 40% of these cells converted to a myogenic lineage as determined by expression of muscle-specific markers (Goldring et al., 2000). Since galectin-1 appears capable of converting non-myogenic cells to a muscle lineage, it was of interest to determine its role in myogenesis.

Galectin-1 is abundant in all three forms of muscle: skeletal, cardiac and smooth (Poirier et al., 1992). It is activated during specific developmental or physiological stages (Poirier et al., 1992) and is present both within the cytoplasm and the extracellular environment (Barondes and Haywood-Reid, 1981; Cherayil et al., 1989; Cooper and Barondes, 1990; Roff and Wang, 1983). Although present within the cytoplasm of myoblasts in vitro, it is secreted from the cells as they terminally differentiate into myotubes (Cooper and Barondes, 1990; Harrison and Wilson, 1992). In skeletal muscle, galectin-1 inhibits the interaction between the cell and the extracellular matrix as it blocks the interaction between laminin and $\alpha_7\beta_1$ integrin, the major laminin receptor on myoblasts (Gu et al., 1994). Inhibition of this interaction may therefore have a role in muscle cell differentiation (Cooper et al., 1991; Gu et al., 1994). In chick muscle, the highest level of galectin-1 occurs between days 8 and 16 of development, the time of maximum myoblast fusion, further implicating its role in terminal muscle cell differentiation (Nowak et al., 1976). In addition, fusion of myoblasts derived from the rat L6 and L8 cell lines is increased in the presence of the lectin (Gartner and Podleski, 1975; Den and Malinzak, 1977) and it appears that galectin-1 may be involved in the myoblast recognition-fusion process (Nowak et al., 1976).

In the present work we have extended our initial findings on the effect of galectin-1 on the conversion of mouse dermal fibroblasts to a myogenic lineage. We have cloned primary mouse dermal fibroblasts to determine if the conversion of these cells to a myogenic phenotype was a cell autonomous characteristic. In addition we investigated whether growing myogenic cells in galectin-1, derived using the same methods as that used for conversion of dermal fibroblasts to muscle cells, had an effect on the proliferation or terminal differentiation of these cells. We used both primary mouse myoblasts and the mouse myogenic C2C12 cell line. Finally,

as there is also a population of fibroblasts present in muscle responsible for maintaining the connective tissue elements of skeletal muscle, we also investigated the action of galectin-1 on these cells.

## Materials and Methods

### Preparation and maintenance of cultures

#### C2C12 mouse myogenic cell line

C2C12 mouse myoblasts were obtained from the American Tissue Culture Collection (ATCC) and routinely maintained in tissue culture grade flasks (Triple Red Laboratory Technology) pre-coated with 0.01% sterile gelatin (Sigma-Aldrich). Although C2C12 cells do not require growth on a gelatin substratum, this step was included to ensure consistency with the culturing of all other cells used in the experimental procedures. Initially, cells were maintained in growth medium consisting of Dulbecco's minimal essential medium (DMEM) (Biowhittaker, Ltd) supplemented with 20% (v/v) fetal calf serum (PAA Laboratories), 100 IU/ml penicillin, 100 mg/ml streptomycin and 1% (w/v) L-glutamine (w/v) (Life Technologies). This medium was designated FCS-20.

#### Primary mouse myoblasts and mouse muscle fibroblasts

Primary mouse myoblasts and muscle fibroblasts were prepared by enzymatic disaggregation of neonatal skeletal muscle from the C57Bl/10 ScSn strain of mouse, as previously described (Watt et al., 1982). Cells released from the muscle tissue were resuspended in 5 ml FCS-20 medium. The yield of viable cells (i.e. those that excluded Trypan blue) was estimated by counting using a Mod-Fuchs haemocytometer. Cells were resuspended in the appropriate amount of FCS-20 and plated at $1\times10^5$ cells/ml in non-coated tissue culture grade flasks for 45 minutes in an incubator set at 37°C and delivering 5% $CO_2$. During this time the majority of muscle fibroblasts adhered to the flask. The cell suspension containing an enriched myoblast population was removed and placed in gelatin-coated tissue culture grade flasks in an incubator set at 37°C and delivering 5% $CO_2$. The differential adhesion step resulted in cultures that were enriched for myoblasts or those that contained almost exclusively muscle fibroblasts. Both primary myoblasts and primary muscle fibroblasts were grown in FCS-20. Muscle fibroblasts were also maintained in gelatin-coated flasks to maintain consistency in growing conditions with all other cell types used in the experimental procedures.

#### Primary mouse dermal fibroblasts

Primary dermal fibroblast cultures were obtained from explant cultures of neonatal C57Bl/10 ScSn mouse skin, as described (Goldring et al., 2000). Cells were maintained in gelatin-coated tissue culture grade flasks in FCS-20.

#### Cloning of primary mouse dermal fibroblasts

To achieve clones of primary mouse dermal fibroblasts, explant cultures of dermal fibroblasts, prepared as described above, were first grown to passage 3, 4 or 5. Such passage numbers were used to ensure that cells positive for myogenic markers were not present in the cultures (see Immunolabelling). To establish clones, cells were plated out at 100 cells per 100 mm gelatin-coated tissue culture grade petri dish (Triple Red Laboratory Technology) and allowed to adhere overnight. The position of individual attached cells was pinpointed on the outside of the petri-dish using a fine indelible marker. Growth of cells was continued for approximately 1 week. From the initiation of clones, cells were maintained in GAL-M (see below) diluted 1:100 with FCS-20, so that from the time of plating of the 100 cells in the 100 mm tissue culture plates, the cells were exposed to galectin-1. As controls, clones were grown in FCS-20 alone. Once the individual cells had divided to form small colonies (i.e. 12-15 cells), sterile cloning discs (Sigma-Aldrich) that had been pre-soaked in 0.25% trypsin-0.02% EDTA (Sigma-Aldrich) were placed on each isolated colony. Under these conditions, the majority (>80%) of cells detached from the tissue culture surface and adhered to the cloning disc. Each disc with attached cells was placed in an individual well of a gelatin-coated 6-well tissue culture grade plate (Triple Red Laboratory Technology). Each colony, now designated a clone, was grown until there were sufficient cells in each clone to transfer between the 10 wells of a Multitest slide (ICN-Flow). It took 18-21 days to establish enough cells to transfer to Multitests, and during this time they were maintained in either FCS-20 or in a 1:100 dilution of GAL-M:FCS-20. Once transferred to Multitest slides, cells were immunocytochemically labelled with muscle-specific markers in order to determine the myogenic conversion of the cells (see Immunolabelling).

### Transfection of COS-1 cells with the galectin-1 containing CDM8 plasmid

The CDM8 plasmid containing a cDNA clone of murine galectin-1 was a gift from V. Wells (King's College London). The plasmid was transformed in *E. coli* MC1061 and selected using the p3 selection plasmid and banded on a caesium chloride gradient. The purity of the CDM8 plasmid was confirmed by cutting with appropriate restriction enzymes, as described (Goldring et al., 2000). As the galectin-1 plasmid does not contain a selectable marker or a reporter gene, COS-1 cells (ATCC) were separately transfected with the pcDNA3 plasmid containing the LacZ reporter gene under the same conditions as that used for galectin-1. This procedure enabled us to estimate the transfection efficiency (Goldring et al., 2000) by staining of the lacZ transfected cells using the X-gal colorimetric reaction (Dannenberg and Suga, 1981) and gave an efficiency of up to 70% (Goldring et al., 2000).

The CDM8 plasmid was used to transfect COS-1 cells. COS-1 cells have previously been reported to secrete expressed galectin-1 protein into the media (Wells and Malluchi, 1991). COS-1 cells were grown in 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin and 1% L-glutamine (w/v). Medium harvested from untransfected COS-1 cells was used as a control medium and was designated COS-1 medium. COS-1 cells were plated out at $1\times10^4$, $1\times10^5$ and $2\times10^5$/ml and 1.5 μg of CDM8 plasmid was used for each transfection. The plasmid was incubated with Lipofectamine (Life Technologies) at room temperature for 45 minutes after which it was added to the COS-1 cells for 24 hours at 37°C. After this time the cells were returned to COS-1 medium. Three days after transfection the media was harvested from the transfected COS-1 cells. The collected medium was then filtered and diluted as required in the various culture conditions (see below). The medium collected from the transfected COS-1 cells, enriched in galectin-1, was designated GAL-M.

### Confirmation of galectin-1 in transfected COS-1 medium

To ascertain that galectin-1 had been secreted into the COS-1 medium, samples of this medium were analysed using SDS-PAGE and western blotting. Samples of media were diluted 1:1 in sample buffer consisting of 65 mM Tris, pH 6.8 (Sigma-Aldrich), 2% (w/v) SDS (Sigma-Aldrich), 20% (v/v) glycerol (Merck), 0.7 M β-mercaptoethanol (Sigma-Aldrich), and 0.025% (w/v) bromophenol blue (Sigma-Aldrich) and boiled for 4 minutes. Samples were then run on 12.5% SDS-polyacrylamide gels (acrylamide/bisacrylamide, 37.5:1, 30% T, 2.67% C; Sigma-Aldrich) with a 4% stacking gel under standard conditions (Laemmli, 1970). Proteins were then transferred from the gel to nitrocellulose membranes for western blotting (Towbin et al., 1979). Non-specific binding was blocked using 5% dried milk and the membrane washed in 0.1% PBS-Tween (Merck) (Kanner et al., 1989; Schaller et al., 1992). The membranes were probed overnight at 4°C with a rabbit polyclonal antibody directed against

galectin-1 (kindly supplied by D. Cooper, UCSF). Blots were washed three times in 0.01% PBS-Tween, before probing with an anti-rabbit biotinylated secondary antibody (Amersham Pharmacia Biotec) for 1 hour at room temperature, washed three times in PBS-Tween and incubated at room temperature for 1 hour in streptavidin-horseradish peroxidase (Amersham Pharmacia Biotec). The bands were visualized using enhanced chemiluminescence ECL-western blotting detection reagents (Amersham Pharmacia Biotec) followed by exposure of the membrane to radiographic film. A reference purified recombinant galectin-1 provided by D. Cooper was used as a positive control.

### Quantifications of levels of galectin-1 in GAL-M

Samples of GAL-M and varying concentrations of purified recombinant galectin-1 were placed on a nitrocellulose membrane and analysed as described above for western blotting. The intensity of these dot blots were therefore used for quantification of the levels of galectin-1 in GAL-M, by comparing with known concentrations of purified recombinant galectin-1, also kindly provided by D. Cooper.

### Growth of C2C12 cells and primary myoblasts, muscle fibroblasts and non-cloned dermal fibroblasts in medium with or without galectin-1

Once C2C12, primary myoblast, muscle fibroblast and non-cloned dermal fibroblast cultures had been established, cells were introduced to the various experimental media to be used. Cells were detached from the initial culture substrata by incubating in 0.25% trypsin/0.02% EDTA in Hanks Basal Salt Solution (Biowhittaker, Ltd) for 5 minutes at 37°C. When re-plated, cells were seeded at a density of $1\times10^5$ cells/ml, either into the wells of 6-well tissue culture grade plates or into the wells of Multitest slides. Plating onto Multitest slides enabled immunocytochemical analysis, while plating into 6-well plates facilitated morphological studies.

Cells were grown in two types of media – either FCS-20, or DMEM supplemented with 2% horse serum (v/v), 100 IU/ml penicillin or 100 mg/ml streptomycin, and 1% L-glutamine (w/v). The latter medium, which encourages the fusion of myogenic cells to their terminally differentiated state (Freshney, 1994) was designated HS-2. Cells were grown in FCS-20 or HS-2 either in the absence or presence of galectin-1-enriched medium, designated GAL-M. Thus there were four experimental conditions in which the cells were grown; FCS-20; FCS-20+GAL-M; HS-2; HS-2+GAL-M. Where C2C12 cells, primary myoblasts and primary muscle fibroblasts were exposed to galectin-1, GAL-M was diluted 1:1 with either FCS-20 or HS-2. Although a 1:1 dilution was used for the growth of such cells, this proved detrimental to the growth of dermal fibroblasts. Therefore for dermal fibroblast cultures various dilutions of GAL-M were used initially: 1:10, 1:50, 1:100 or 1:200 with either FCS-20 or HS-2. These cells exhibited optimal growth in GAL-M diluted 1:100 and therefore this concentration was used for all further experiments.

As controls, cells were grown in FCS-20 or HS-2 diluted 1:1 with COS-1 medium. COS-1 medium alone was not used as a control because this medium had already been used for the growth of COS-1 cells for 3 days and would therefore have been lacking in nutrients. Therefore for control regimes, COS-1 medium diluted 1:1 with either FCS-20 or HS-2 was used, which was the same regime used with the experimental GAL-M medium. Cells grown in 6-well plates achieved confluency 5-7 days after plating and 3-4 days in the wells of Multitest slides. Once all wells had reached confluency, all cultures were analysed, as detailed below.

### Analysis of cells in culture

#### Immunolabelling

When confluency of C2C12, primary myoblasts, muscle and dermal fibroblasts had been achieved, the wells of multitest slides were immunolabelled for the cytoskeletal markers desmin, skeletal muscle-specific myosin II heavy chain (MHC) and vimentin. Desmin and MHC are muscle specific markers, whereas vimentin is present in all cells of mesenchymal origin, including both myoblasts and fibroblasts (Stewart, 1990) and was used in the present study as a control to ensure efficacy of immunolabelling regimes. Cells were fixed in 1:1 methanol/acetone for 10 minutes and air-dried. 50 μl primary antibody – either 1:10 dilution of anti-desmin (Sigma-Aldrich D1033); 1:5 dilution of anti-skeletal muscle myosin II heavy chain (Novocastra Laboratories Ltd, NCL-MHCn); or 1:20 dilution of anti-vimentin (Sigma-AldrichV5255), was added to selected wells and incubated at room temperature for 1 hour in PBS containing 1% (v/v) Bovine Serum Albumin (BSA) and 0.01% sodium azide (w/v) (Sigma-Aldrich). Wells were washed 3 times in PBS prior to the addition of a 1:100 dilution of FITC conjugated goat-anti-mouse IgG (Sigma-Aldrich F0257) for 1 hour. Slides were then washed three times in PBS followed by a 1 minute addition of 0.02% (w/v) 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) in PBS to enable visualisation of cell nuclei. In control wells the primary antibody was omitted. Slides were washed in PBS, mounted in glycerol-aqueous mountant and viewed on a Zeiss Axioskop fluorescent microscope fitted with appropriate filter sets. Results were recorded using a Zeiss MC100 camera and Kodak Ektachrome 160T film. In relation to cloned dermal fibroblasts, cells seeded onto Multitest slides were stained with antibodies directed against desmin and vimentin, using the same labelling regimes as for the other cell types.

It should be noted that when preparing primary muscle and dermal fibroblasts for culture, there is a possibility that both types of culture could contain ‘contaminating’ myogenic cells. This would bias results for the conversion of non-myogenic cells to a myogenic lineage in subsequent experiments. Therefore, to detect any possible contamination from myoblasts, samples were taken from early passages of muscle fibroblast and dermal fibroblast cultures and stained with antibodies directed against desmin. Although occasional desmin-positive cells were observed at very early passage, later cultures were observed to be myoblast-free and it was these cultures that were used in all subsequent assays.

### Analysis of myogenic conversion of cloned dermal fibroblasts

The number of cells within a dermal fibroblast clone that converted to the myogenic lineage was assessed by counting the cells that expressed desmin relative to those that failed to express this muscle-specific marker. Three clones were analysed, and for each clone four wells of the Multitest slide were labelled for desmin, four for vimentin and the remaining two wells were used as secondary antibody controls. For wells labelled with desmin, three random areas of the four separate wells were counted. To ensure that the three areas did not overlap each other, the Vernier scale on the microscope was used. Incubating the cells with the nuclear marker DAPI enabled us to count all cells within a field of view. Therefore the number of cells expressing muscle-specific markers could be calculated as a percentage of the total number of cells within a field of view.

### Immunocytochemical analysis of C2C12 cells, primary myoblasts, muscle and dermal fibroblasts

To examine whether any muscle or dermal fibroblasts within cultures had converted to the myogenic lineage, cells on multitest slides were analysed for the presence of desmin-positive cells, in a similar manner to that described for dermal fibroblast clones. Desmin was used as a positive control for the C2C12 and primary myoblast cultures.

### Analysis of myotube formation in Giemsa-stained cultures

Cultures grown in 6-well plates were stained with Giemsa in order to determine the number of nuclei that had contributed to myotube

formation. This method also enabled us to morphologically distinguish between mononuclear myoblasts and fibroblasts present in any of the cultures. The method involved washing the cells three times in PBS before fixing for 10 minutes in 100% methanol. Cells were stained with 3% Giemsa for 30 minutes and again washed in PBS. To qualify as a myotube, three or more nuclei had to be present within the cell to avoid including counts of dividing cells where two nuclei could have been present within one cell. Nine random areas were counted per well using a 19 $mm^2$ grided eyepiece graticule and the mean value for each well calculated. These counts enabled us to determine the exact number of nuclei present within fibroblasts, myoblasts and myotubes within a given field. The number of nuclei present in myotubes was calculated as a percentage of the total number of nuclei in C2C12 cultures and as a percentage of the total number of myogenic cells in primary myoblast cultures.

### Proliferation assays

For proliferation assays, 100 μl aliquots of cell samples grown in the various media were placed into individual wells of a 96-well tissue culture plate (Triple Red Laboratory Technology). Plates were placed in an incubator set at 37°C and delivering 5% $CO_2$. At various time points between 1 and 5 days after plating, 20 μl of Cell Titer 96 Aqueous One Solution Reagent (Promega) was added to the wells. This colourimetric assay is based on a tetrazolium salt that is bio-reduced by cells into a formazan product, the intensity of which is directly proportional to the number of viable cells in culture. The absorbance was read at 492 nm on a 96-well plate reader two hours after addition of the reagent.

## Results

### Confirmation and quantification of galectin-1 in GAL-M

Fig. 1A shows the results of western blotting analysis using an antibody directed against galectin-1. As a positive control, recombinant galectin-1 was applied to lane 3. Lane 7 contained medium from untransfected COS-1 cells, lane 5 medium from C2C12 cells and in lane 6 GAL-M was applied to the gel. Galectin-1 was detected in control lanes but not in the lane loaded with medium from untransfected COS-1 cells. Galectin-1 was present in C2C12-conditioned medium, although this band was fainter than that observed for the GAL-M lane. The molecular markers in lane 1 show the positive bands to be at the correct molecular weight. Fig. 1B shows the results of the dot blot analysis. From this result the concentration of galectin-1 in GAL-M is calculated to be equivalent to a 1:100 dilution of purified recombinant galectin-1. As the concentration of the purified protein is 1.85 mg/ml, (Cooper, personal communication), the concentration of galectin-1 in GAL-M is of the order of 18.5 μg/ml.

### Effect of galectin-1 on clones of dermal fibroblasts

The previous result verified the presence of galectin-1 in GAL-M and C2C12-conditioned medium, the two media types we have previously shown convert dermal fibroblasts to the myogenic lineage (Wise et al., 1996; Goldring et al., 2000). It is of interest that the GAL-M medium contained more galectin-1 than the C2C12-conditioned medium, as our previous work has shown that when cells are grown in GAL-M medium the number of dermal fibroblasts that convert to the myogenic lineage is higher (Goldring et al., 2000) than when cells are grown in C2C12-conditioned medium (Wise et al., 1996).

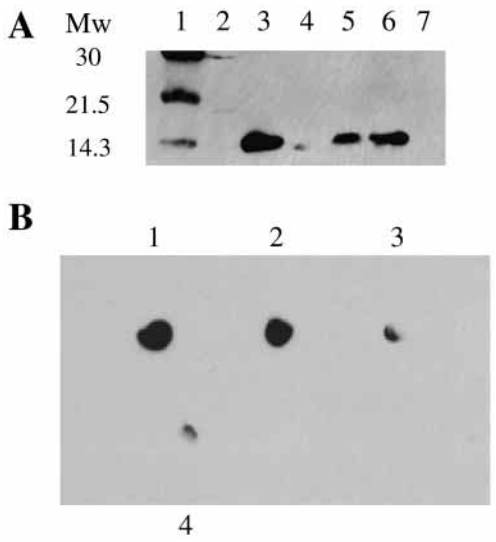


**Fig. 1.** (A) Western blot developed using an anti-galectin-1 antibody. Lane 1, molecular weight markers; lanes 2 and 4, no samples were run; lane 3, galectin-1 positive control; lane 5, C2C12-conditioned media; lane 6, media from COS-1 cells transfected with the galectin-1 construct; lane 7, the non-transfected COS-1 media. The positive galectin-1 band is at approximately 14.5 kDa in lanes 3, 5 and 6. (B) Dot-blot developed using an anti-galectin-1 antibody. Samples 1, 2 and 3 are purified recombinant galectin-1 (1.85 mg/ml) diluted 1:10, 1:50 and 1:100, respectively. Sample 4 is non-diluted GAL-M.

When dermal fibroblasts were cloned and grown in C2C12-conditioned medium, up to 40% of cells converted to the myogenic lineage (Goldring et al., 2000) compared with 8-10% when uncloned cells were grown in this medium (Wise et al., 1996). In the present investigation we determined whether growth of cloned dermal fibroblasts in GAL-M medium would result in more cells entering the myogenic lineage in comparison to non-cloned dermal fibroblasts. Three clones were cultured in GAL-M diluted 1:100 in FCS-20. To detect the expression of a myogenic marker, cells from each clone were distributed between the wells of a Multitest slide. Four of the wells from each clone were stained for desmin and all of the cells in each of these 12 wells were positive for this marker (Fig. 2; Table 1). Four of the remaining wells were stained with a vimentin antibody, with all cells being vimentin positive. Where the primary antibody was omitted, cells were negative for both desmin and vimentin.

The results strongly suggest that cloning of primary mouse dermal fibroblasts and their subsequent growth in GAL-M medium resulted in all of the cells from each clone converting to the myogenic lineage. As controls, four clones were grown

**Table 1. The number of dermal fibroblasts that have converted to a myogenic lineage**

| Well number | Number of desmin-positive cells in field | | |
|---|---|---|---|
| | Culture 1 | Culture 2 | Culture 3 |
| 1 | 14, 23, 13 | 14, 24, 5 | 4, 4, 17 |
| 2 | 8, 4, 32 | 4, 6, 7 | 3, 9, 4 |
| 3 | 16, 8, 6 | 12, 10, 8 | 24, 3, 8 |
| 4 | 4, 8, 6 | 9, 23, 11 | 16, 10, 8 |

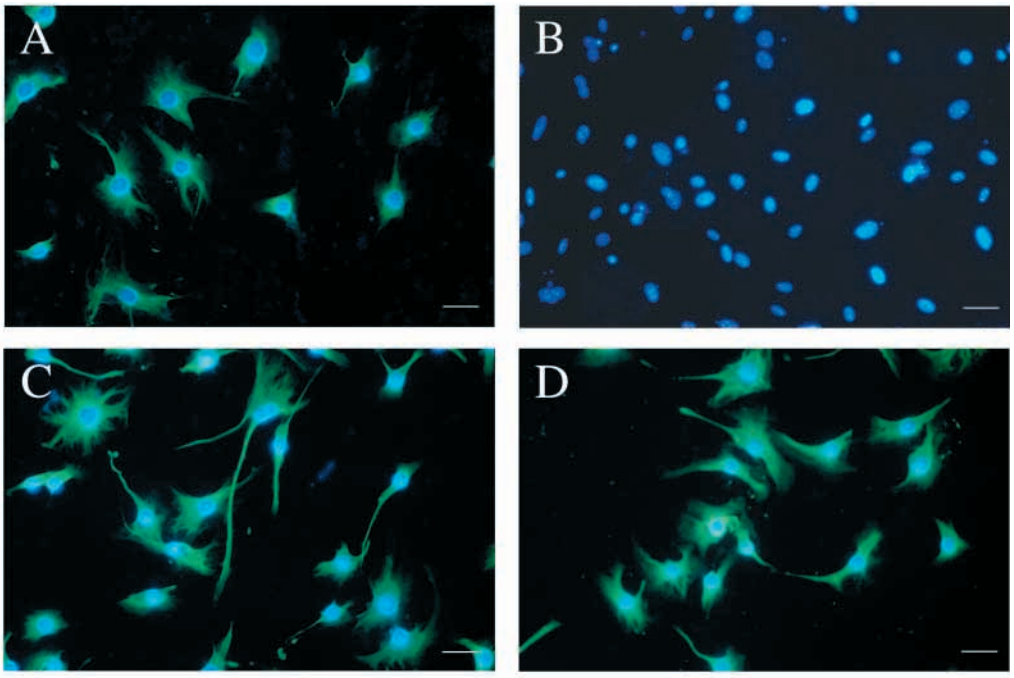


**Fig. 2.** Cloned dermal fibroblasts grown in 100:1 FCS-20:GAL-M. All cells in this field are desmin positive (A) indicating 100% conversion to a myogenic lineage. Cloned dermal fibroblasts grown in FCS-20 alone are desmin negative (B). Cloned cells are all positive for the mesenchymal cell marker vimentin both in the presence (C) and absence (D) of galectin-1. Nuclei are stained with DAPI. Bars, 50 μm.

in FCS-20 alone under exactly the same conditions as those grown in GAL-M. Again, four multitest wells from each clone were stained with anti-desmin antibody. None of the cells in any of these 16 wells were positive for desmin indicating that in FCS-20 cloned cells were incapable of converting to the myogenic lineage.

## Effect of galectin-1 on the terminal differentiation of C2C12 cells

The consequence of growing C2C12 cells in GAL-M medium was investigated. For each experiment cells were plated into the wells of a 6-well plate and grown in the following six media conditions (i.e. HS-2; FCS-20; HS-2+GAL-M; FCS-20+GAL-M; HS-2+COS-1 medium; FCS-20+COS-1 medium). This was repeated seven times resulting in seven wells for each media condition being examined. For each of these cultures a sample of cells was also plated onto Multitest slides under all media conditions, these slides being used for immunolabelling with muscle-specific markers. All cultures of C2C12 cells were positive for desmin and vimentin, and terminally differentiated C2C12 cells were also positive for myosin II heavy-chain (Fig. 3A-C). Cultures on 6-well plates were observed daily, and those in HS-2+GAL-M were seen to reach confluency before the control. However, galectin-1 exposed cultures were allowed to continue their growth until control cultures had also become confluent. This therefore meant that analyses were performed at plateau phase of cell growth on all cultures. All required media changes were routinely performed on all cultures at the same time intervals. In the light of this finding, proliferation assays were performed (see below). At plateau phase of cell growth on 6-well plates, cells in the six different media types were stained with Giemsa (Fig. 4A-F). The number of nuclei contributing to mononuclear myoblasts or multinucleate myotubes was also investigated in all of these cultures. Such counts gave a ratio of cells that had entered the terminally

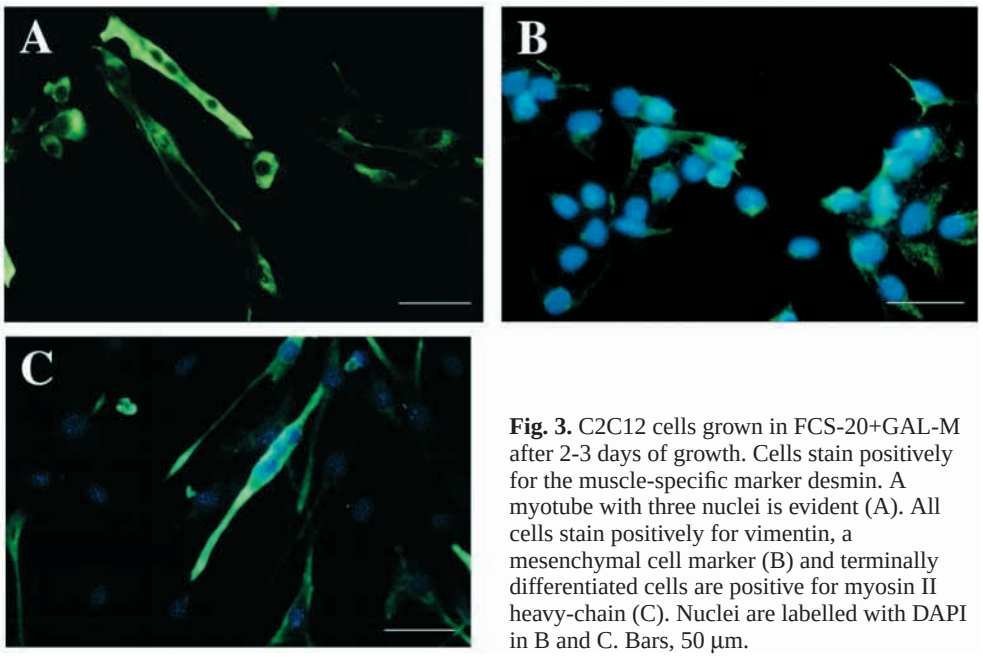


**Fig. 3.** C2C12 cells grown in FCS-20+GAL-M after 2-3 days of growth. Cells stain positively for the muscle-specific marker desmin. A myotube with three nuclei is evident (A). All cells stain positively for vimentin, a mesenchymal cell marker (B) and terminally differentiated cells are positive for myosin II heavy-chain (C). Nuclei are labelled with DAPI in B and C. Bars, 50 μm.

differentiated state, in the presence or absence of galectin-1. For each well, nine random fields of view were counted. There were differences in the number of nuclei present within each field and although the number counted over all the fields ranged from 50 to 709 the high and low numbers were outliers seen in only a few fields. The average nuclear counts for all fields were 241. Table 2 shows the average percentage of nuclei present within multinucleate myotubes within the wells of each of the cultures. An average number of 16.87% of nuclei were observed within myotubes in cultures grown in HS-2 which increased to 25.63% in the presence of GAL-M, and 4.26% of nuclei were present within myotubes when cells were grown in FCS-20, which increased to 11.60% in the presence of GAL-M. A summary of this data is displayed in Fig. 5. Using a one-way ANOVA and post-hoc multiple paired comparison ($P<0.05$), there were statistically significant difference between cells incubated in the following: (a) FCS-20 and HS-2; (b) FCS-20 and HS-2+GAL-M; (c) FCS-20 and HS-2+COS-1; (d) FCS-20 and FCS-20+GAL-M; (e) FCS-20+COS-1 and FCS-20+GAL-M; (f) HS-2+COS-1 and FCS-20+COS-1; (g) HS-2 and FCS-20+COS-1; and (h) HS-2+GAL-M and FCS-20+COS-1. These findings suggest that the addition of galectin-1 to HS-2 medium resulted in more nuclei contributing to myotube formation than in any other media combination used. In the absence of galectin-1, the number of nuclei within myotubes appeared greater in HS-2 media than in FCS-20 medium. With the addition of galectin-1, both groups showed an increased number of nuclei within myotubes, as shown by Geimsa staining. We also observed a greater alignment of myoblasts in galectin-1-supplemented cultures. There was no significant difference in the number of nuclei present in myotubes between cultures in FCS-20 and FCS-20+COS-1 or between those in HS-2 when compared with HS-2+COS-1. These results indicate that the increase in the number of nuclei present in myotubes in GAL-M cultures are due to the presence of galectin-1, as opposed to any other factor in the COS-1 medium.

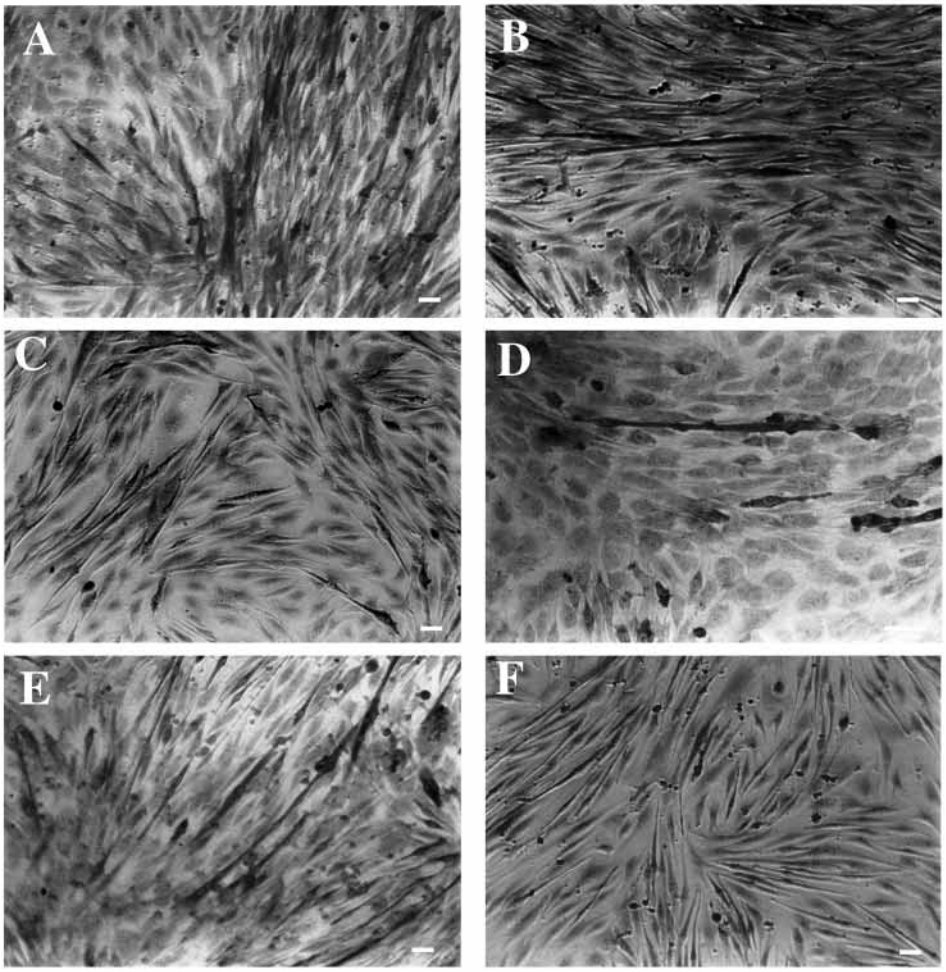


**Fig. 4.** C2C12 cells in HS-2 (A); HS-2+GAL-M (B); HS-2+COS-1 (C); FCS-20 (D); FCS-20+GAL-M (E); or FCS-20+COS-1 (F). Giemsa stained cultures showing the presence of mononuclear cells and varying numbers of multinucleate myotubes. Bars, 50 μm.

## Proliferation assays

Proliferation assays were performed on triplicate samples of seven separate C2C12 cultures and were corrected for media

**Table 2. Percentage of nuclei present in myotubes in C2C12 cultures**

| | Percentage of nuclei in myotubes in seven different C2C12 cultures (mean±s.e.m.)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Conditions | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Mean |
| HS-2 | 6.15±1.04 | 11.85±5.86 | 11.76±5.67 | 15.32±4.77 | 19.96±5.67 | 24.48±7.45 | 28.54±5.98 | 16.87±3.22 |
| FCS-20 | 0.48±0.22 | 1.30±0.91 | 0.60±0.56 | 4.58±3.55 | 5.10±2.80 | 9.91±3.16 | 7.68±5.13 | 4.26±1.51 |
| HS-2+GAL-M | 9.52±0.63 | 12.75±4.90 | 13.40±6.57 | 34.95±7.84 | 30.81±8.53 | 36.77±10.55 | 41.19±8.93 | 25.63±5.41 |
| FCS-20+GAL-M | 3.56±0.49 | 5.08±2.80 | 2.89±1.44 | 19.01±5.98 | 15.61±5.72 | 18.44±4.70 | 16.63±4.68 | 11.60±3.01 |
| HS-2+COS-1 | 6.50±1.12 | 7.24±2.81 | 6.43±3.25 | 17.02±8.42 | 20.83±7.81 | 24.91±6.62 | 26.10±6.72 | 15.58±3.59 |
| FCS-20+COS-1 | 1.35±0.24 | 3.42±2.17 | 5.41±2.97 | 5.73±2.74 | 4.61±2.14 | 9.03±3.46 | 11.75±4.93 | 5.90±1.42 |

*The percentages are the mean±s.e.m. for nine counts in each well.

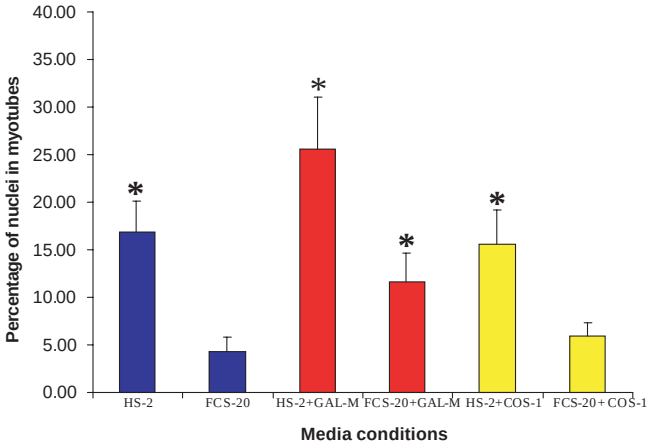


**Fig. 5.** Averages from seven separate experiments of C2C12 cells grown in HS-2; HS-2+GAL-M; HS-2+COS-1; FCS-20; FCS-20+GAL-M, or FCS-20+COS-1. Results show percentage of nuclei present in myotubes. Asterisk (*) indicates a significant difference from FCS-20 or FCS-20+COS-1 tested by one-way ANOVA and post-hoc multiple paired comparison ($P$<0.05). Higher percentages of nuclei in myotubes were observed in galectin-1-treated cultures.

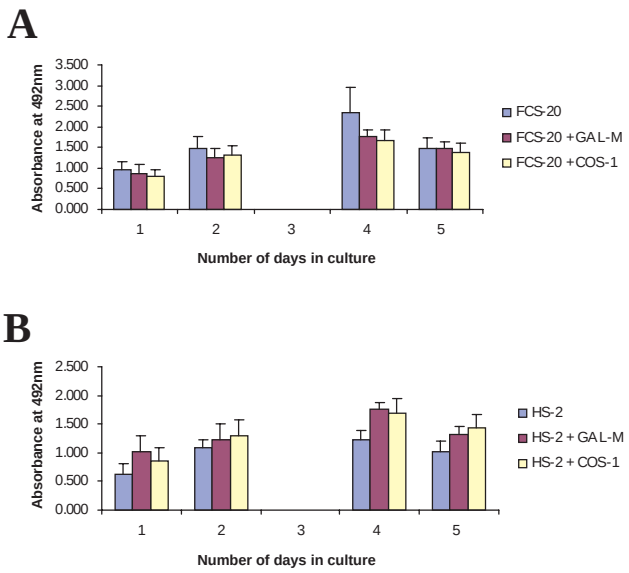


**Fig. 6.** Proliferation assays were undertaken on seven separate C2C12 cultures grown in FCS-20; FCS-20+GAL-M, or FCS-20+COS-1 (A) and HS-2; HS-2+GAL-M; HS-2+COS-1 (B). No significant differences were observed in proliferation of cells at any of the time points examined between the various media types.

and media plus supplement controls. Absorbance was read for each culture at 1, 2, 4 and 5 days after plating with increased absorbance being indicative of increased proliferation rate. Cells maintained in FCS-20 showed an increased proliferation rate up to 4 days after which no further increase was observed (Fig. 6A). Cells grown in FCS-20+COS-1 medium or FCS-20+GAL-M displayed no significant increase in their proliferation when compared with FCS-20 alone. Cells grown in HS-2 alone (Fig. 6B) proliferated less at all time points recorded, in comparison with those grown in FCS-20. Cultures grown in HS-2+COS-1 medium or HS-2+GAL-M showed an increased proliferation when compared with HS-2 alone (Fig. 6B). However there was no difference in proliferation between cultures grown in HS-2+GAL-M when compared with those in HS-2+COS-1 medium or between cultures maintained in FCS-20+GAL-M and those in FCS-20+COS-1 medium (Fig. 6A,B). As no difference in proliferation was observed between cells grown in GAL-M compared with untransfected COS-1 medium, these results indicate that the greater number of myotubes observed in GAL-M compared with untransfected COS-1 is not a secondary consequence of increased proliferation and earlier cell crowding in these cultures.

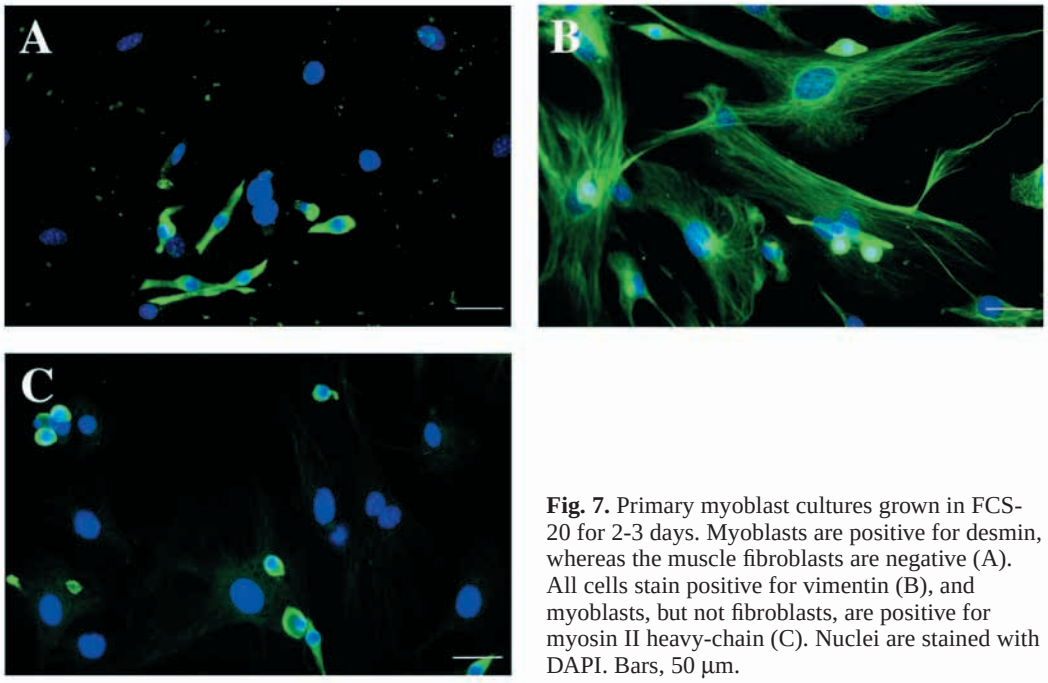


**Fig. 7.** Primary myoblast cultures grown in FCS-20 for 2-3 days. Myoblasts are positive for desmin, whereas the muscle fibroblasts are negative (A). All cells stain positive for vimentin (B), and myoblasts, but not fibroblasts, are positive for myosin II heavy-chain (C). Nuclei are stained with DAPI. Bars, 50 μm.

### Effect of galectin-1 on the terminal differentiation of primary myoblasts

In all cultures, primary myoblasts were positive for the myogenic markers desmin and myosin II heavy chain, and for the mesenchymal marker vimentin (Fig. 7A-C). Three distinct cell types could be identified: myotubes, myoblasts and fibroblasts.

The number of nuclei present in fibroblasts, myoblasts or myotubes could be counted from the results of immunolabelling or analysis of Giemsa stained cultures. However, owing to the large number of each cell type present in each field, counting using fluorescently stained cells was made difficult by bleaching of the photochrome. Therefore, as Giemsa enables permanent visualisation of all three cell types simultaneously, this method was used for accurate cell counting.

Primary myoblasts grown under the different culture conditions and stained with Giemsa are shown in Fig. 8A-F. There were a greater number of myotubes present in cultures grown in HS-2 alone compared with FCS-20 (Fig. 8A,D). Addition of galectin-1 to both these media resulted in an increase in number of nuclei present within myotubes, as well as an increase in both myotube number and size. Myotubes in cultures supplemented with galectin-1 also appeared to be more aligned relative to each other, in comparison to cultures that lacked galectin-1 (Fig. 8A,B,D,E). Such effects were more prominent with HS-2 than FCS-20. Only a few myotubes were present in cultures grown in HS-2+COS-1 medium and FCS-20+COS-1 medium (Fig. 8C,F).

Counting of Giemsa stained cultures showed that galectin-1 had a similar effect on primary myoblasts as that observed on C2C12 cells. Since muscle fibroblasts are unable to fuse, the number of nuclei present in myotubes was calculated as a percentage of the total number of myogenic cells in the primary myoblast cultures and not as a percentage of the total number of cells in culture. The percentage of nuclei contributing to myotubes was lower in both FCS-20 and HS-2 in the absence of the lectin (Table 3; Fig. 9). The average percentage of nuclei observed in myotubes cultured in HS-2 was 7.06%, which was significantly greater than the 1.52% observed with FCS-20 ($P<0.05$, $n=7$, one-way ANOVA followed by post-hoc multiple paired comparison). When galectin-1 was added to HS-2 media, the average number of nuclei in myotubes increased from 7.06% to 27.29%, and when added to FCS-20 the average number of nuclei in myotubes increased from 1.52% to 14.52% ($P<0.05$, $n=7$, one-way ANOVA followed by post-hoc multiple paired comparison). As a control, primary myoblasts were grown in either a 1:1 dilution of HS-2 and medium harvested from untransfected COS-1 cells or a 1:1 dilution of FCS-20 with medium harvested from untransfected COS-1 cells (Fig. 8; Fig. 9). No statistical differences were observed between the percentage of nuclei present in myotubes in cultures grown in HS-2 alone compared with cultures grown in HS-2+COS-1 medium or between cultures of FCS-20 alone compared with FCS-20+COS-1 medium. Consequently, when the data from

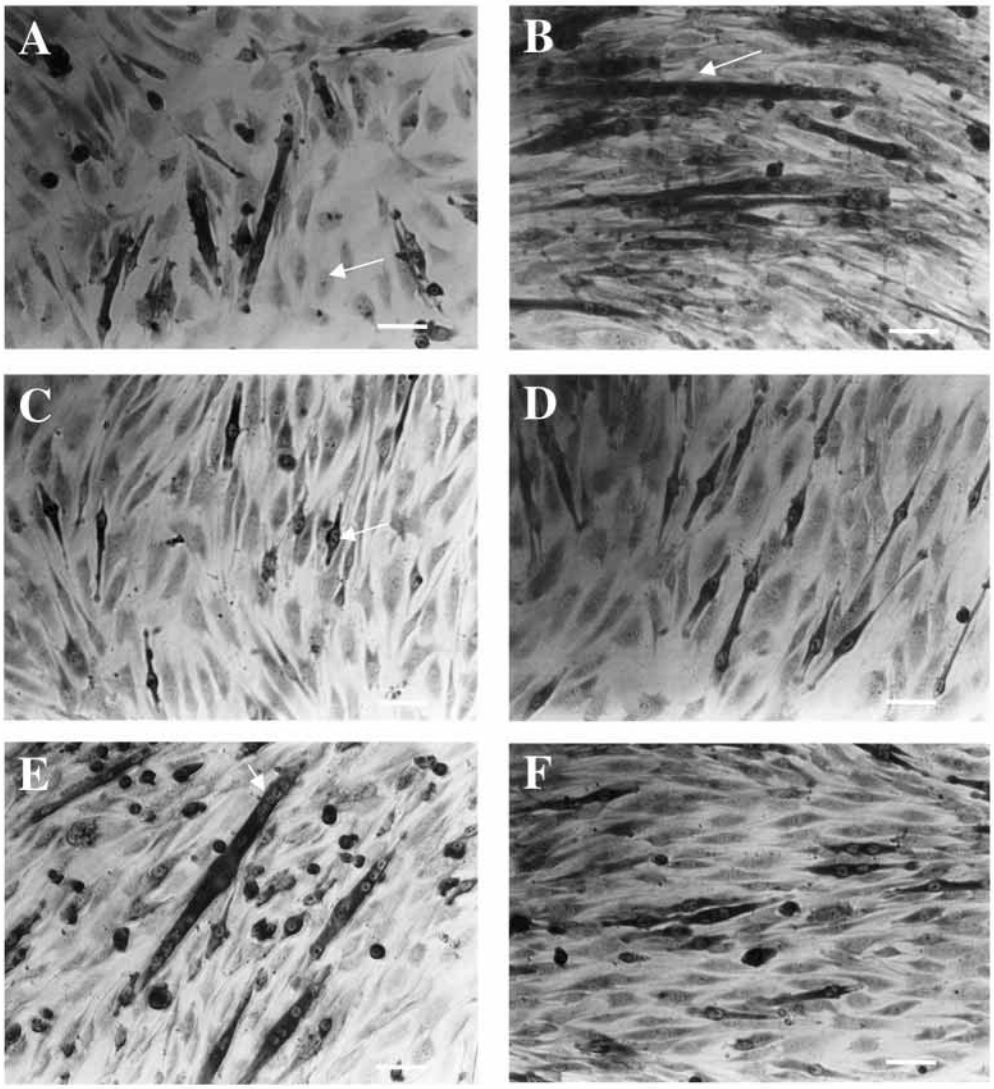


**Fig. 8.** Giemsa stained primary myoblast cultures grown for 5-6 days in HS-2 (A); HS-2+GAL-M (B); HS-2+COS-1 (C); FCS-20 (D); FCS-20+GAL-M (E); or FCS-20+COS-1 (F). Three different cell types, myoblasts, fibroblasts and myotubes, are present in these populations. Fibroblasts (A, white arrow), myotubes (B, white arrow) and myoblasts (C, white arrow) are evident in these cultures. Myotubes in GAL-M-treated cultures appeared more numerous and larger than in other media types (arrows, B,E). Bars, 50 μm.

**Table 3. Percentage of nuclei present in myotubes in primary myoblasts**

| Conditions | Percentage of nuclei in myotubes in seven different primary myoblasts cultures (mean±s.e.m.)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Mean |
| HS-2 | 7.68±2.85 | 1.39±1.47 | 4.04±4.28 | 1.15±1.22 | 8.93±7.40 | 4.62±3.66 | 21.60±7.59 | 7.06±2.87 |
| FCS-20 | 4.45±2.34 | 0±0 | 0±0 | 0±0 | 3.27±2.63 | 0±0 | 2.94±3.12 | 1.52±0.80 |
| HS-2+GAL-M | 27.61±3.71 | 43.81±3.14 | 14.10±3.60 | 17.83±2.95 | 8.61±2.84 | 44.00±4.00 | 35.09±7.47 | 27.29±5.83 |
| FCS-20+GAL-M | 17.10±1.89 | 26.01±12.89 | 0±0 | 8.71±3.85 | 9.44±3.38 | 33.26±7.25 | 7.15±5.04 | 14.52±4.76 |
| HS-2+COS-1 | 1.85±1.96 | 3.60±2.80 | 5.80±4.08 | 0±0 | 3.33±2.34 | 5.21±5.52 | 18.19±8.12 | 5.43±2.43 |
| FCS-20+COS-1 | 2.36±2.03 | 0±0 | 0±0 | 0±0 | 2.22±2.36 | 9.03±3.46 | 0.24±0.03 | 0.69±0.45 |

*The percentages are the mean±s.e.m. for nine counts in each well.

HS-2 or FCS-20+COS-1 media was compared with data from cultures of HS-2 or FCS-20 supplemented with galectin-1, the increase of nuclei within myotubes that occurred with the galectin-1 treatment was found to be statistically significant ($P$<0.05, $n$=7).

Proliferation assays were not carried out on multiple primary myoblast cultures, in view of the vast number of primary cells that would be required and the technical difficulties in producing such cultures. However, assays were performed on triplicate samples of two separate primary myoblast cultures and the proliferation of these cells followed a similar trend to that of C2C12 cells (data not shown).

### Effect of galectin-1 on the conversion and terminal differentiation of muscle fibroblasts

As we had previously observed the conversion of skin fibroblasts to muscle in the presence of galectin-1 (Goldring et al., 2000), we chose to monitor the effect of galectin-1 on muscle fibroblasts. When muscle fibroblasts were grown in FCS-20 and HS-2 in the presence or absence of galectin-1, all cells were desmin negative (Fig. 10). In addition, no myotubes were observed in muscle fibroblast cultures in any of the media types (Fig. 11). The fact that muscle fibroblasts do not convert to a myogenic lineage further supports the argument that in the primary myoblast cultures it was the myoblasts (and not converted muscle fibroblasts) that were involved in the formation of myotubes.

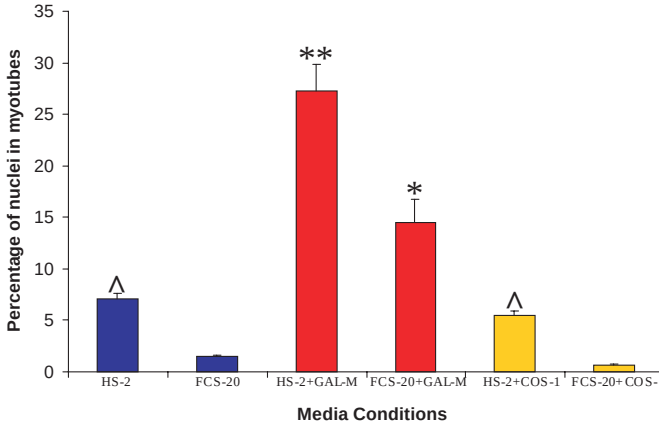


**Fig. 9.** Averages from seven separate experiments of primary myoblast cultures grown in HS-2; HS-2+GAL-M; HS-2+COS-1; FCS-20; FCS-20+GAL-M; or FCS-20+COS-1. GAL-M cultures produced visibly higher counts. Double asterisk (**) indicates a significant difference from all other cultures. Asterisk (*) indicates a difference from all other cultures apart from cultures grown in HS-2 alone. Inverted v (^) indicates a significant difference from FCS-20 and FCS-20+COS-1. Tested by one-way ANOVA followed by post-hoc multiple paired comparison ($P$<0.05).

## Discussion

There is increasing evidence for a role of the lectin galectin-1 in a number of cellular processes. Galectin-1 has been shown to have an effect on cell adhesion (Mahanthappa et al., 1994; Cooper et al., 1991; Gu et al., 1994), regulation of the cell cycle and cell proliferation (Wells and Malluchi, 1991; Yamaoka et al., 1991) and also on immune functions (Levi et al., 1983; Offner et al., 1990; Lutomski et al., 1995; Allione et al., 1998). The concentration of galectin-1, its presence as a monomer or dimer, as well as a number of other factors, would appear to affect whether galectin-1 has a positive or negative action on these processes (Adams et al., 1996). Our own previous work implicated galectin-1 in the conversion of mouse dermal fibroblasts to a myogenic lineage (Goldring et al., 2000). When grown in medium harvested from COS-1 cells transfected with a plasmid containing a galectin-1 construct, up to 30% of such cells in culture expressed the muscle-specific marker, desmin. SDS-PAGE analysis indicated an increased level of a 14-15 kDa protein in this medium. In the present work western blot and dot blot analysis confirm the presence of galectin-1 in the transfected medium (GAL-M) and lower amounts were also found in medium conditioned by C2C12 muscle cells. Cells only converted when cultured in either of these two media, the conversion being greater in GAL-M than in C2C12-conditioned medium. A former study using muscle cell-conditioned medium showed that the extent of conversion of dermal fibroblasts to a myogenic lineage can be increased by cloning of these cells (Goldring et al., 2000). The current work shows that GAL-M causes a further increase in the myogenic conversion of cloned dermal fibroblasts, with 100% of cloned cells converting. We have therefore successfully achieved a method for producing high numbers of converted cells in low passage cultures.

There are a number of possible explanations as to how 100% conversion can be achieved in dermal fibroblast clones. It is unlikely that fibroblasts are pluripotent, as a mixed population of fibroblastic/myogenic cells would be expected in a proliferating population. Conversion could occur by transdifferentiation of the dermal fibroblast from its original lineage to another cell type or, alternatively, by the differentiation of a subpopulation of cells residing in the dermis that have stem cell characteristics. From our results both these outcomes are feasible. If all fibroblasts are capable

of conversion, then 100% conversion of cloned cells is a viable result. If only a subpopulation of converting cells exist, then in the presence of galectin-1 the progeny of these cells are all likely to be myogenic. If there is a subpopulation of converting cells, it would also be expected that some clones will grow up from the fibroblast population that cannot convert. Although we did not find clones that remained fibroblastic in the presence of galectin-1 this may reflect the fact that the cloning procedure favours the survival of a subpopulation of cells that have the capacity to convert. Further studies are underway to clarify this point. We are also currently establishing more clones, a time consuming procedure, to determine whether galectin-1 causes cells to fuse to the terminally differentiated state in these highly converting cultures.

In relation to committed myogenic cells, there is a certain amount of controversy on the action of galectin-1 with regard to terminal differentiation. Fusion of myoblasts derived from the rat L6 and L8 cell lines has been shown to be increased in the presence of the lectin (Gartner and Podleski, 1975; Den and Malinzak, 1977). Contrary to these results, MacBride and Przybylski showed an inhibition of fusion when a 14 kDa lectin derived from the chick was added to chick primary myoblast cultures and rat myoblast cell lines (MacBride and Przybylski, 1980). However this lectin is non-mammalian and may therefore differ from galectin-1. Owing to differing evidence on the effect of galectin-1 on terminal differentiation of myoblasts we have investigated the effect of galectin-1 on primary mouse myoblasts and also on the C2C12 mouse myogenic cell line.

The addition of galectin-1 to culture media had an effect on both C2C12 cells and primary mouse myoblasts compared with cultures of cells that had not been exposed to galectin-1. The percentage of nuclei within

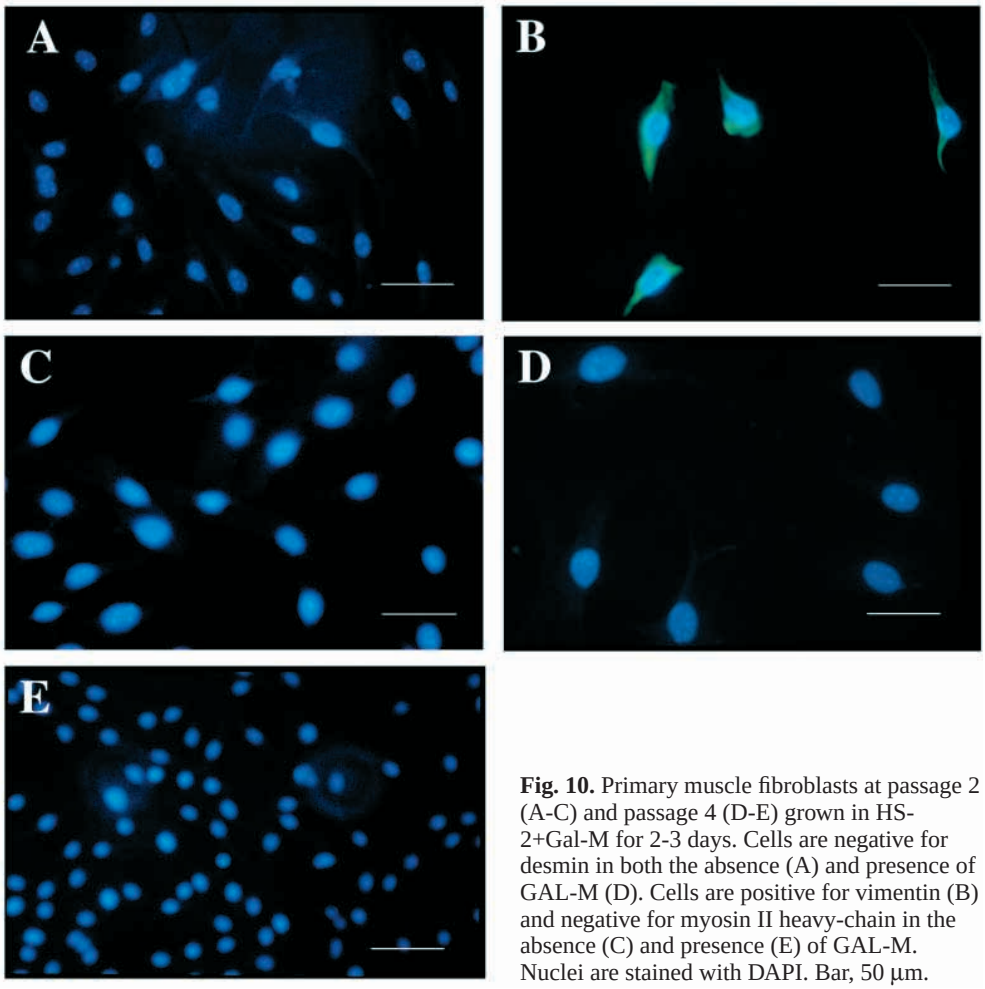


**Fig. 10.** Primary muscle fibroblasts at passage 2 (A-C) and passage 4 (D-E) grown in HS-2+Gal-M for 2-3 days. Cells are negative for desmin in both the absence (A) and presence of GAL-M (D). Cells are positive for vimentin (B) and negative for myosin II heavy-chain in the absence (C) and presence (E) of GAL-M. Nuclei are stained with DAPI. Bar, 50 μm.

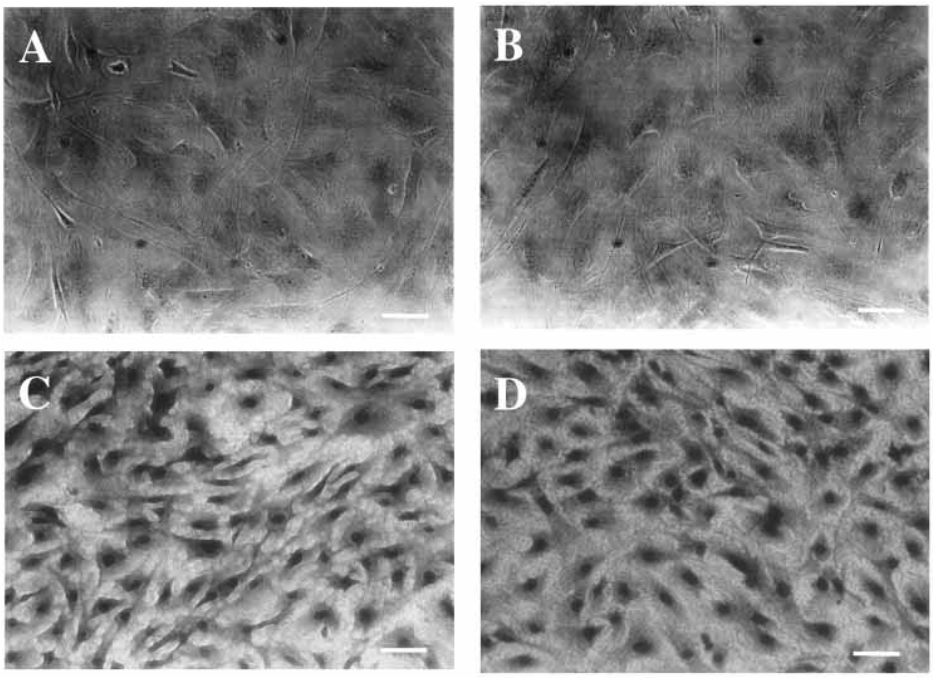


**Fig. 11.** Giemsa stained primary muscle fibroblasts grown for 5-6 days in HS-2 (A); HS-2+GAL-M (B); FCS-20 (C); and FCS-20+GAL-M (D). No myotubes are present in any of these culture conditions. Bars, 50 μm.

myotubes was greater in galectin-1-treated cultures, both using the C2C12 cell line and primary myoblasts. One explanation for the greater number of nuclei within myotubes in galectin-1-treated cultures may have been the result of an enhancement of cell proliferation that occurred in the presence of galectin-1, thereby making more cells available for fusion. However, cell proliferation assays indicated that this was not the case as exposure to GAL-M did not cause an increase in proliferation in either of these cell types. We would therefore suggest that GAL-M is having a direct effect on the fusion of myogenic cells. In relation to primary myoblasts, the cultures used in the current work did not consist of pure myogenic populations, but contained a high proportion of fibroblasts. It could therefore be argued that the fibroblasts may have had some effect on the proliferation and terminal differentiation of myoblasts. However, similar numbers of fibroblasts are present in both galectin-1 and non-galectin-1-treated cultures, yet a difference in proportion of myogenic cells present between the treated and non-treated cultures was observed.

Our results with both primary muscle cultures and the C2C12 cell line would appear to indicate that galectin-1 increases the fusion of muscle cells to their terminally differentiated state. This would support the evidence that it is expressed at high levels during muscle development when cell fusion is at its highest (Poirier et al., 1992). In addition, there is some evidence that differentiating myoblasts release galectin-1 and that proliferating ones do not (Cooper and Barondes, 1990; Harrison and Wilson, 1992), which again would suggest a role for galectin-1 in the terminal differentiation of these cells. There is also evidence that constitutive and differentiation-induced expression of galectin-1 is regulated by different factors (Lu and Lotan, 1999). It is possible that galectin-1 could be released from regenerating muscle thereby acting as a mechanism for recruitment of satellite cells in the formation of new muscle fibres. This possibility may receive support from the discovery of Chen et al. of an unidentified factor present in crushed muscle extracts that is mitogenic to myoblasts (Chen and Quinn, 1992; Chen et al., 1994).

In the present study we did not find any evidence that galectin-1 is capable of converting muscle fibroblasts to a myogenic lineage. As muscle fibroblasts have a specific role in forming the connective tissue barriers within muscle, their inability to convert may be key in maintaining the complex structure of muscle. These results support the previous findings of our group that muscle fibroblasts did not enter the myogenic lineage when grown in the presence of myoblast-conditioned medium (Wise et al., 1996), but it is the first time that it has been shown that galectin-1 fails to convert these cells. There are reports that galectin-1 is released from fibroblasts derived from certain tissues but not from others. It is released at low levels from mouse embryonic fibroblasts and the 3T3 fibroblastic cell line (Barondes and Haywood-Reid, 1981; Wilson et al., 1989) but not from fibroblasts derived from the lung (Clerch et al., 1988; Chaudhuri et al., 1989). The synthesis of galectin and its action on some fibroblasts and not others may reflect their diverse embryonic origin and functions within tissues. Evidence from cell types other than fibroblasts may corroborate this. For example, galectin-1 has been shown to be released from both smooth muscle cells and from cancer cells and causes proliferation of both these cell types (Adams et al., 1996; Sanford and Harris-Hooker, 1990). In addition, it is present in the thymus where it has a role in the selection of T cells (Perillo et al., 1995; Perillo et al., 1997). Further, neurons express galectin-1 and it has been shown to have a role in nerve outgrowth following axotomy (Horie et al., 1999).

It is clear that galectin-1 has a number of important actions in modulating cell processes. Here we confirm its role in converting non-myogenic cells to the myogenic lineage and show it affects the terminal differentiation of myogenic cells. Given its abundance in muscle, galectin-1 could be an important factor that is released by regenerating muscle in order to recruit cells and cause their fusion to existing muscle fibres. This area certainly warrants further study and is being actively investigated in our laboratories.

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