

Steroid RU 486 inducible myogenesis by 10T1/2 fibroblastic mouse cells

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Abstract For reconstruction or repair of damaged tissues, an artificially regulated switch from proliferation to differentiation would be of great advantage. To achieve conditional myogenesis, we expressed MyoD in mouse C3H 10T1/2 fibroblastic cells, using a gene regulation system based on the synthetic steroid RU 486. By stable co-transfection of a plasmid construct with the RU 486 dependent activator and an integrating inducible MyoD construct, a cell clone, designated 10T-RM, was obtained in which MyoD expression was stringently controlled by RU 486. 12 h after addition of 10 nM RU 486 to 10T-RM cells, saturation levels of MyoD mRNA were observed and ≥ 2 days later, mRNA for embryonal myosin heavy chain (MyHC_{emb}) was abundant and mononucleated cells fused into myotubes.

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Key words: Myogenic factor; MyoD; Myogenesis; Synthetic steroid RU 486; Inducible gene expression; C3H10T1/2 mouse fibroblast

1. Introduction

A hallmark in the formation of skeletal muscle during development is cell fate determination of mesenchymal cells and differentiation of myocytes to multinucleated myotubes by myogenic transcription factors. These comprise MyoD [1], myogenin [2–4], Myf-5 [5] and MRF4, also called Myf-6 or herculin [6–8]. Myogenic transcription factors are basic helix-loop-helix (bHLH) proteins. They are expressed at different times during skeletal muscle development [9–11] and serve different steps in muscle differentiation [12,13]. MyoD has originally been defined by its ability to convert cultured fibroblasts (C3H10T1/2 [14]) into a myogenic phenotype [1,15].

Reconstructing muscle is a major concern in the treatment of muscle diseases which result in a reduction of muscle mass. In mouse models many attempts have been made to reconstitute muscle tissue or to replace muscle fibres by the implantation of primary myoblasts or myogenic cell lines like C2C12 [16–19]. However cell therapy trials on patients suffering from Duchenne muscular dystrophy (DMD) with myoblasts have not been successful so far due to the low proliferation capacity of implanted myogenic cells [20,21]. An alternative approach could be a treatment based on the genetic conversion of *ex vivo* engineered fibroblasts involving compensation of the dystrophin deficiency and fibroblast-myoblast conversion by myogenic factors.

An inducible expression of myogenic factors would allow for a control of proliferation versus myogenic differentiation

in cell culture and presumably in the organism, provided that control of myogenic factor expression is not leaky. A variety of regulatory systems are available in which conditional expression is achieved by non-toxic ligands like tetracycline (tet) or by steroids. The tet-system is based on the bacterial tet repressor (*tetR*) whose ligand dependent DNA binding module has been fused to a transactivator VP 16 from Herpes simplex virus to act as a repressor, tTA [22] or as an activator, rtTA, which contains a mutated *tet* repressor [23]. In conjunction with a target promoter containing *tet* operator sites, transcription is either repressed (tTA) or induced (rtTA) by tet. Alternatively, steroid based inducible systems make use of ligand binding domains of steroid hormone receptors either from other species that bind ecdysone [24] or from mutated ligand binding domains with high affinity to antihormones tamoxifen [25] or antiprogesterone RU 486 [26–29]. In the latter case, the RU 486 dependent transactivator contains the progesterone binding domain of the human progesterone receptor (PR) with a 42 amino acid deletion in its C-terminus. This truncated domain binds only the progesterone antagonist RU 486 with high affinity [30]; when fused to a GAL4-DNA binding domain and to a VP16 transactivator, it mediates RU 486 dependent binding of the regulator to a target promoter containing multiple GAL4 binding sites. To circumvent potential enhancer effects due to integration of the chimaeric regulator and the target promoter, an episomal construct based on Epstein-Barr virus (EBV) is introduced in host cells, leading to high level expression of the RU 486 dependent transactivator (see Fig. 1a; [26]). The present study was initiated to explore the suitability of an RU 486 inducible system for conditional MyoD expression in 10T1/2 fibroblasts with the aim to control myogenesis in culture with the final aim to reconstruct muscle in experimental animals.

2. Materials and methods

2.1. Vector construction

The cDNA for MyoD ([1]; gift of E.-M. Füchtbauer, Freiburg, Germany) was cloned in pSBC-2 [31]; gift of H. Hauser, Braunschweig, Germany) as an *EcoRI* fragment. For construction of an RU 486 inducible MyoD target vector, the SV 40 promoter fragment of pSBC2-MyoD was cleaved by *Clal*, filled-in with Klenow DNA polymerase, and digested with *XhoI*. The RU 486 target promoter p17x4-tk containing four copies of GAL4 consensus sequence linked to the thymidine kinase (tk) gene promoter [26], was digested by *HindIII*, filled-in, redigested with *XhoI* and ligated into the respective sites of pSBC2-MyoD. This construct is designated pSBC2-tk-MyoD. To allow for selection of 10T1/2 cell clones using G418, we cloned the chimaeric regulator hPRB891/GAL4/VP16 (GL-VP) by *BamHI* and *KpnI* digestion and ligation into the respective sites of pCEP9 (Invitrogen, Groningen, Netherlands) to yield GL-VPneo.

2.2. Cell culture and transfections

C3H 10T1/2 fibroblast cells (subline CCL-226) were obtained from ATCC (Rockville, MD, USA) and cultured under 5% CO₂ in

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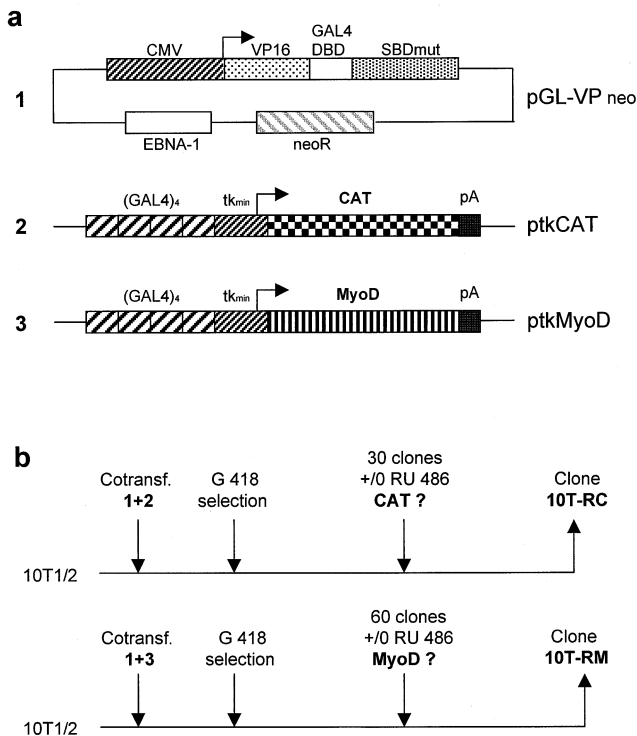


Fig. 1. Constructs and experimental procedures for the production of RU 486 inducible 10T1/2 cell clones and regulated myogenesis. a: Constructs: (1) EBV vector carrying the RU 486 responsive domain ('SBD_{mut}', ligand binding domain of the human PR hPRB891), the GAL4 DNA binding domain ('GAL4DBD'), and the VP 16 transactivation domain from Herpes simplex ('VP 16'); (2) target construct with four GAL4 consensus sequences oriented in sense, a minimal tk promoter, and CAT gene; (3) the same for MyoD. b: Steps to produce from 10T1/2 cells the RU 486 dependent CAT expression clone 10T-RC and the corresponding MyoD expressing clone 10T-RM.

Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) foetal calf serum (FCS). 10T1/2 cells of low passage numbers (≤ 5) were frozen in aliquots. For transfection, usually 1.5×10^5 cells were seeded into 60 mm plates and vector constructs were added 24 h later for 6 h along with Transfectam (Promega, Madison, WI, USA) in serum-free medium. Usually, cotransfections were done in ratios of 1:5, 1:10, and 1:20 of GL-VPneo to pSBC2-GAL4-MyoD. 2 days after transfection, transfectants were selected with 400 $\mu\text{g}/\text{ml}$ G418 (Gibco BRL). For fusion experiments, 10T-RM or control C2C12 cells were seeded onto collagenised cover slips and kept in DMEM containing 5% FCS or 2% horse serum (HS), respectively. In addition, along with changing to differentiation medium, the solvent ethanol alone or RU 486 in ethanol was added.

2.3. Quantification of chloramphenicol acetyltransferase (CAT)

The amount of CAT protein was determined using the enzyme linked immunosorbent assay (ELISA) system supplied by Boehringer (Ingelheim, Germany). Cell cultures were washed twice with phosphate buffered saline and lysed by addition of 1 ml lysis buffer supplied by the manufacturer. The colourimetric assay yields the concentration of CAT protein in ng/mg protein by reference to a standard curve.

2.4. Isolation of RNA and Northern blot analysis

Total RNA from cultured cells was isolated by the RNeasy preparation method (Qiagen, Hilden, Germany). For each sample, 10 μg of total RNA was fractionated by electrophoresis on a 1% agarose formaldehyde gel and transferred in $20\times$ standard saline citrate buffer (SSC) to nylon membranes (Pall Biodyne B, Pall, Dreieich, Germany). The blots were subsequently hybridised with [α - ^{32}P]dATP random

prime labelled *Eco*RI fragments of MyoD cDNA or an 18S rRNA DNA fragment, respectively.

2.5. RT-PCR analysis

1 μg of total RNA in 20 μl reaction volume was subjected to reverse transcription (RT) using SuperScript reverse transcriptase (Life Technologies GmbH, Eggenstein, Germany). After RT, 5 μl of this mixture were used for PCR amplification in a total volume of 50 μl containing 10 pmol of each primer and 1 Unit of AmpliTaq (Perkin-Elmer GmbH, Überlingen, Germany). For embryonic myosin heavy chain, MyHC_{emb}, the primers were: MYHCs: 5'-GCAAGGAATGCA-GAGGAGAAGG-3'; MYHCas: 5'-CCTGCCTCTTGTAGGACTT-GA-3' [32]; for ribosomal protein L7 mRNA: L7s: 5'-AGATG-TACCGCACTGAGATTC-3'; L7as: 5'-ACTTACCAAGAGACCG-AGCAA-3' [33]. Primers were obtained from TIB MolBiol (Berlin, Germany). PCR conditions were: 4 min 94°C, 1 min 64°C, 1 min 72°C, 1 min 92°C for 20–30 cycles. The resulting amplification products (403 bp for MyHC_{emb} and 352 bp for L7) were separated by electrophoresis on a 1% agarose gel and visualised with ethidium bromide.

2.6. Documentation

Photodocumentation on cells grown on collagen coated coverslips was done on a Zeiss Axiophot microscope with phase contrast microscopy. Images were processed by Adobe Photoshop, version 5.0.

3. Results

Stable 10T1/2 cell clones expressing constitutively MyoD, Myogenin, or Myf-4 were generated and analysed for their differentiation potential. At cell densities of $> 60\%$ confluency most of the cell clones showed spontaneous differentiation to multinucleated myotubes, even in proliferation medium. Con-

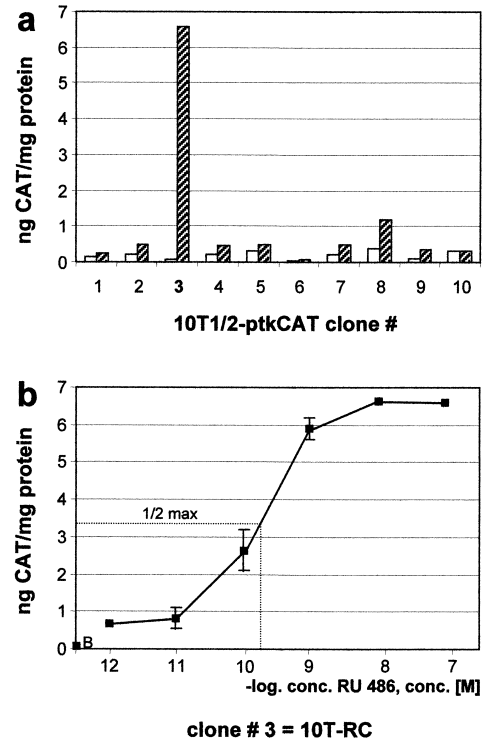


Fig. 2. RU 486 inducibility in 10T1/2 cell clones of the CAT reporter gene. a: Concentration of CAT protein in 10 representative 10T1/2 cell clones without RU 486 (open columns) or 24 h after addition of 10 nM RU 486 (hatched columns). b: RU 486 dependent expression of CAT protein in clone 10T-RC (= clone #3 of upper panel). B = Basal value in the absence of RU 486; Mean values \pm S.D. of triplicate experiments.

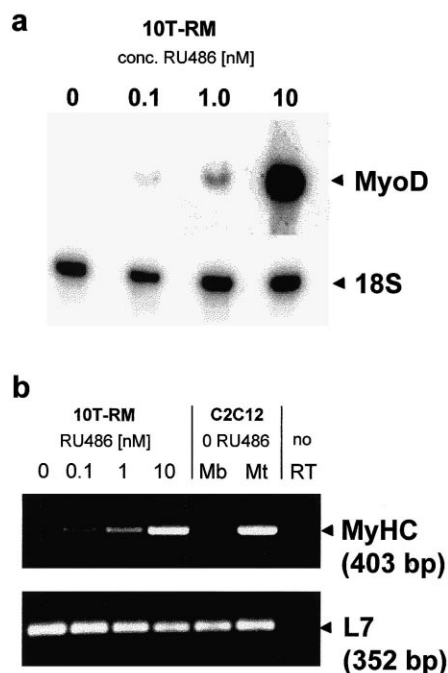


Fig. 3. RU 486 dependent expression of MyoD (A) and embryonic myosin heavy chain (B). a: Northern-blot of total RNA from cell clone 10T-RM, 24 h after addition of ethanol control (0) and various concentrations of RU 486. For each lane, 10 μ g total RNA were applied and after electrophoresis and blotting hybridised against the probes indicated to the right; MyoD, cDNA probe; 18S, cDNA probe for 18S ribosomal RNA. b: Secondary induction of myosin heavy chain (embryonal), 'MyHC', as determined after 25 cycles RT-PCR. 10T-RM cells, either uninduced or induced with RU 486 for 2 days; C2C12 cells, undifferentiated myoblasts ('mb') or myotubes, 2 days after change to differentiation medium ('mt'). 'no RT', control without RT. Internal standard, RT-PCR for ribosomal protein L7 mRNA. The amplificate sizes in bp are given below.

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stitutive overexpression of myogenic factors in these cell clones obviously bypasses the requirement for an external stimulus (e.g. low serum) probably due to a high copy number of myogenic factor genes present in these cell clones. Consequently, these clones could not be grown to large cell numbers.

For controlled myogenesis we used the inducible expression system based on the synthetic steroid RU 486 as a ligand (Fig. 1; [26]). To test whether this system is feasible in 10T1/2 cells, we co-transfected the CAT reporter target construct (ptkCAT) together with the chimaeric regulator and selected

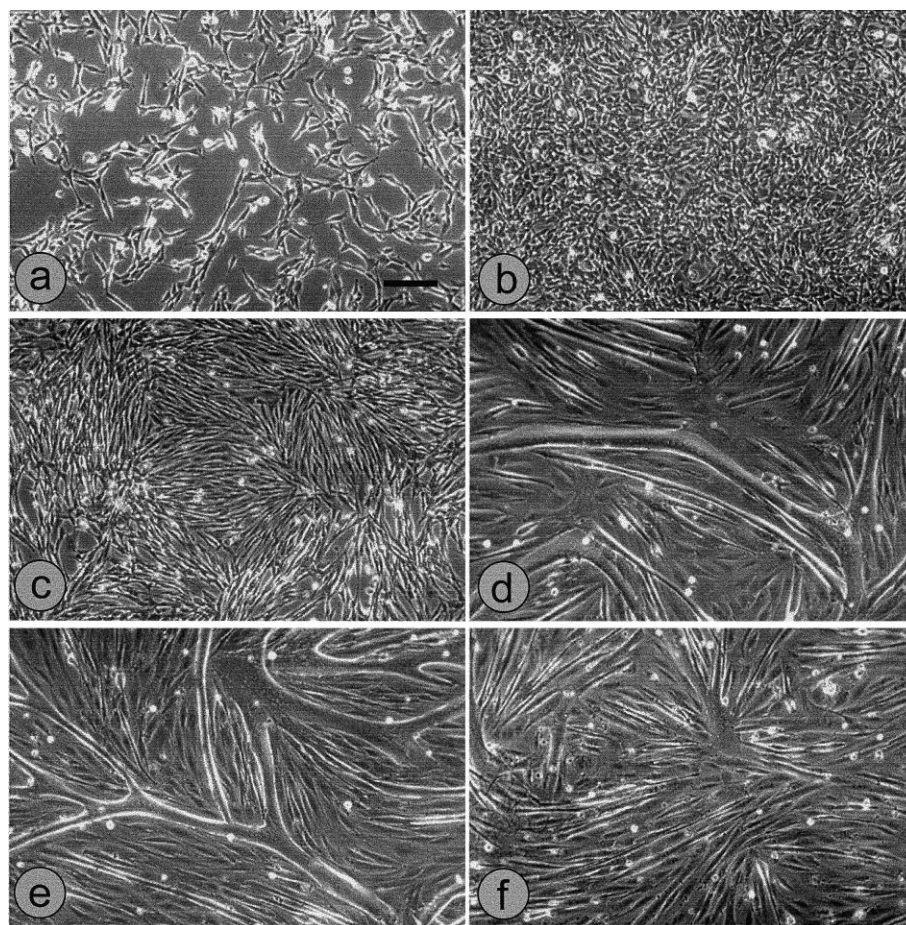


Fig. 4. RU 486 dependent differentiation of 10T-RM cells in culture. a and b: without inducer, subconfluent (a) or confluent (b); with inducer 1 day (c), 4 days (d) and 7 days (e) after addition of 10 nM RU 486. f: 7 Days after addition of 1 nM RU 486. Bar in (a) 10 μ m, valid for whole panel. In d and e, large myotubes are seen; in f, myotubes are less mature.

co-transfected cell clones. Of four representative clones, 10T-RC (clone #3 in Fig. 2a) showed a basal expression of 0.06 ng CAT/mg protein in the absence of the inducer and a >100-times higher CAT concentration after addition of 10 nM RU 486 (Fig. 2a). The dose response curve showed a half-maximal induction at 0.2 nM RU 486 and a maximal induction at ≥ 10 nM RU 486 (Fig. 2b). After induction with 10 nM RU 486, more than 90% of 10T-RC cells stained for CAT protein with a chicken anti-CAT antibody (not shown).

Similarly, we generated 10T1/2 cell clones containing ptkMyoD and the episomal regulator GL-VP (Fig. 1a). Selection, however, was difficult: many cell clones already showed myogenic differentiation in the absence of RU 486, probably due to a high basal rate of MyoD expression. Another fraction of cell clones did not respond to RU 486, i.e. there was no induction of MyoD mRNA.

Only one out of 60 clones characterised, designated 10T-RM, proliferated in the absence of RU 486 and, 24 h after addition of 10 nM RU 486, showed a >50-fold induction of MyoD mRNA as determined by Northern blot analysis (Fig. 3a). MyoD dependent myogenic differentiation was monitored by mRNA levels for myosin heavy chain (embryonal; MyHC_{emb}). MyHC specific amplicates of 403 bp size were only seen in RNA from RU 486 induced 10T-RM cells (Fig. 3b). As a positive control we triggered myogenic differentiation in C2C12 cells by serum withdrawal and determined mRNA levels for MyHC_{emb}. In the myoblast stage, C2C12 cells did not express MyHC_{emb} mRNA, whereas 1 day after serum withdrawal the MyHC_{emb} mRNA level was upregulated (Fig. 3b).

Without RU 486, we never observed any morphological changes in 10T-RM cells which would be indicative for myogenic differentiation, independent of the degree of confluency (Fig. 4a,b). After addition of 10 nM RU 486, morphological changes in 10T-RM cells were observed already after 1 day (Fig. 4c) and differentiation proceeded to myotubes within 7 days (Fig. 4d,e). Myogenic differentiation also occurred with 1 nM RU 486 (Fig. 4f).

In a total of 25 experiments with 10T-RM cells of different passage numbers (4–10) we always observed stringent control of myotube formation by RU 486.

4. Discussion

Constitutive myogenisation of skin fibroblasts and their subsequent participation in muscle fibre formation in the mouse has previously been achieved [34]. Here we demonstrate the feasibility of a regulated expression of a myogenic factor leading to conditional myogenesis in cell culture. As a model cell line we used C310T1/2 fibroblasts that can be converted into myogenic cells as demonstrated by azacytidine treatment [1]. Anti-estrogen and glucocorticoid inducible chimaeric myogenic factors have been constructed for conditional myogenesis of 10T1/2 cells, but no clearcut data on the regulation in this system have been presented [35,36]. Furthermore these inducers, at the concentrations required, would interfere with metabolism and immune defence in the organism.

Our approach to construct RU 486 responsive fibroblastic cells has faced some technical difficulties: First, a substantial fraction of 10T1/2 cell clones transfected with target promoter and regulator failed to respond by MyoD expression in the

presence of the inducer. Second, many other cell clones underwent premature differentiation presumably due to a high basal activity of the RU 486 dependent target promoter. For this reason, a large number of cell clones had to be grown and a subset of these characterised in order to obtain a cell line tightly regulated in culture. Under these conditions, myogenesis was reproducible and clear-cut and proceeded up to the myotube stage which approximately corresponds to gestational day 12–16 in the mouse.

Future investigations will have to deal with myogenesis by 10T-RM cells in the environment of the organism and the possibility to repair muscle damage. These experiments require an appropriate labelling system to distinguish donor from host cells [37,38] and long-term immunological tolerance. We are presently constructing recipient mice that fulfil these conditions.

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