



Response of the porcine *MYH4*-promoter and *MYH4*-expressing myotubes to known anabolic and catabolic agents *in vitro*

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ABSTRACT

Myosin heavy chain-IIB (MyHC-IIB; encoded by *MYH4* or *Myh4*) expression is often associated with muscle hypertrophic growth. Unlike other large mammals, domestic pig breeds express MyHC-IIB at both the mRNA and protein level.

Aim: To utilise a fluorescence-based promoter-reporter system to test the influence of anabolic and catabolic agents on increasing porcine *MYH4*-promoter activity and determine whether cell hypertrophy was subsequently induced.

Methods: C2C12 myoblasts were co-transfected with porcine *MYH4*-promoter-driven ZsGreen and CMV-driven DsRed expression plasmids. At the onset of differentiation, treatments (dibutyryl cyclic-AMP (dbcAMP), Des(1–3) Insulin-Like Growth Factor-1 (IGF-I), triiodo-L-thyronine (T3) and dexamethasone (Dex)) or appropriate vehicle controls were added and cells maintained for up to four days. At day 4 of differentiation, measurements were collected for total fluorescence and average myotube diameter, as indicators of *MYH4*-promoter activity and cell hypertrophy respectively.

Results: Porcine *MYH4*-promoter activity increased during C2C12 myogenic differentiation, with a marked increase between days 3 and 4. *MYH4*-promoter activity was further increased following four days of dbcAMP treatment and average myotube diameter was significantly increased by dbcAMP. Porcine *MYH4*-promoter activity also tended to be increased by T3 treatment, but there were no effects of Des(1–3) IGF-I or Dex treatment, whereas average myotube diameter was increased by Des(1–3) IGF-I, but not T3 or Dex.

Conclusion: Porcine *MYH4*-promoter activity responded to dbcAMP, Des(1–3) IGF-I and T3 treatment *in vitro* as observed previously in reported *in vivo* studies. However, we report that increased *MYH4*-promoter activity was not always associated with muscle cell hypertrophy. The fluorescence-based reporter system offers a useful tool to study muscle cell hypertrophic growth.

1. Introduction

Skeletal muscle tissue demonstrates a high level of plasticity and the ability to dynamically respond to its environment. The basic molecular structure of skeletal muscle is highly conserved across species and comprises of contractile motor units called sarcomeres [1]. Sarcomeres are composed of two major proteins: actin and myosin. Myosin makes up the largest proportion of the sarcomeres in the form of myosin heavy

chains (MyHC), essential myosin light chains and regulatory myosin light chains [2]. The four main MyHC isoforms expressed in adult skeletal muscle are MyHC-I, -IIA, -IIX and -IIB, and each confers different morphological, enzymatic and functional characteristics [3]. With the exception of rodents, mammalian muscle fibres generally express multiple MyHC isoforms and are able to transition sequentially in response to mechanical stimuli (I ↔ IIA ↔ IIX ↔ IIB). MyHC-I (encoded by *MYH7* gene) is indicative of a slow-twitch oxidative muscle fibre phenotype,

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whereas MyHC-IIB (encoded by *MYH4* gene) indicates a fast-twitch glycolytic phenotype [4]. Interestingly, all mammals carry the *MYH4* gene but most large mammals (e.g. humans, cattle, sheep) do not express MyHC-IIB at the protein level [5–7]. Pigs are the exception, particularly domestic pig breeds which have been reported to express much higher levels of MyHC-IIB in their skeletal muscle than wild pigs [8]. Increased expression of MyHC-IIB is often associated with muscle hypertrophy, therefore this characteristic has most likely developed as a result of intensive breeding for enhanced muscle growth traits [8,9]. Similarly domestic pigs administered with Ractopamine, a beta-adrenergic receptor agonist (BA), demonstrate significant increases in both muscle weights and *MYH4* mRNA expression, whilst *MYH1* (encodes MyHC-IIX) and *MYH2* (encodes MyHC-IIA) mRNA are reduced [10,11].

We previously showed that the MyHC isoforms are expressed in two distinct cohorts during C2C12 myogenic differentiation, where *Myh4* mRNA expression was associated with mature, well differentiated myotubes following 3–4 days of differentiation [12]. We also previously reported the use of a porcine *MYH4*-promoter-reporter system that can be utilised in C2C12 cells to study the transcriptional regulation of the porcine *MYH4*-promoter activity [13] and changes in myotube diameter [14]. BA, growth hormone (GH), triiodothyronine (T3) and dexamethasone (Dex) are well known anabolic and catabolic agents. In this study we aimed to utilise these agents to characterise the response of the porcine *MYH4*-promoter-reporter system, and the association with average myotube diameters, as a potential *in vitro* model system for identifying novel anabolic and catabolic agents for pigs.

As indicated above, BAs like Ractopamine have previously been shown to induce both muscle hypertrophy and a shift to a fast-glycolytic MyHC-IIB phenotype in pigs [10,11,15,16], cows [17,18], sheep [19], rats [20–23] and mice [14,24]. In the present study, dibutyl 3',5'-cyclic adenosine monophosphate (dbcAMP), the cAMP analogue, was used to mimic the downstream intracellular signalling response to BA treatment, as previous studies have shown variable responses of cultured muscle cells to BA treatment [25]. We hypothesised that dbcAMP would increase both *MYH4*-promoter activity and average myotube diameters in the porcine *MYH4*-promoter reporter system.

Treatment of adult pigs with GH has been shown to increase muscle fibre size, but with no effect on fibre-type composition [26–30]. We previously reported that GH administration also had no effect on the proportion of MyHC isoforms in both pigs [11] and sheep [19]. Insulin-like Growth Factor-I (IGF-I) is considered the main active component of the GH-IGF axis [31]. In this study we used Des(1–3) IGF-I as it is reported to induce similar responses to IGF-I treatment *in vitro* [32]. Des(1–3) IGF-I has a similar binding affinity as IGF-I to the IGF-type 1 receptor [33], but has a much lower binding affinity for IGF binding proteins (IGFBPs) [34]. Hence using Des(1–3) IGF-I avoided any possible effects of IGFBPs present, and we hypothesised that it would increase average myotube diameters, whilst having no effect on *MYH4*-promoter activity.

The active form of thyroid hormone (3,3',5-Triiodo-L-thyronine or T3) has previously been shown to induce a fibre-type shift in adult rats administered T3 for 4 weeks, with an increase in the proportion of fast-twitch type-II fibres and a reduction in slow-twitch type-I fibres [35]. Prolonged treatment of rats with T3 for 6 weeks resulted in a reduction in body weight, muscle weights and muscle fibre cross sectional area [36–38]; however the decrease in muscle mass could be due to the increase in whole body energy expenditure observed, rather than a direct effect of T3 alone on muscle atrophy. Thus, we hypothesised that T3 would increase *MYH4*-promoter activity, but either have no effect or possibly decrease average myotube diameters.

Lastly, Dex was used as a potential catabolic agent. Dex is a synthetic glucocorticoid that is more potent than endogenous corticosterone and concentrations up to approximately 120 nM are thought to be within the physiological range [39]. Chronic, prolonged exposure to glucocorticoids has been reported to induce muscle atrophy [40,41]. Dex treatment for 5 days caused a reduction in both body weight and EDL muscle

mass in rats, associated with a decrease in cross sectional area of both type-I and type-II fibres [42]. Similarly, long-term Dex treatment of growing pigs resulted in significantly reduced growth rates, body weight and lean mass percentage [43]. Therefore, we hypothesised that Dex would reduce average myotube diameters, but have no effect on *MYH4*-promoter activity in the porcine *MYH4*-promoter reporter system.

2. Materials and methods

2.1. Materials and compounds

High glucose (25 mM) Dulbecco's modified eagle's medium (DMEM; D5796), cell culture grade water, N6,2'-O-Dibutyladenosine 3',5'-cyclic monophosphate sodium salt (dbcAMP), dexamethasone (Dex) and 3,3',5-Triiodo-L-thyronine sodium salt (T3) were all purchased from Sigma-Aldrich (Poole, UK). Human Recombinant Des(1–3) IGF-I was purchased from SourceBioscience and other cell culture reagents were purchased from Fisher Scientific (Loughborough, UK). Compounds were prepared as follows: dbcAMP was dissolved in cell culture grade water at 100 mM, Des(1–3) IGF-I in 0.1 M acetic acid at 100 µg/ml, T3 in 0.1 M sodium hydroxide at 100 nM and Dex in absolute ethanol at 10 mM. Compounds were filter-sterilised through a 0.2 µm filter and aliquots stored at –20 °C.

2.2. Cell culture methods

C2C12 cell stocks were originally purchased from the European Collection of Authenticated Cell Cultures (ECACC; 91031101) and checked regularly for mycoplasma. C2C12 cells were cultured in a humidified incubator at 37 °C with 5% CO₂ and were used between passages 14–18 for experiments. C2C12 myoblasts were seeded into growth media (High glucose (25 mM) DMEM supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin, P/S) at 25,000 cells per well onto 24-well plates. Approximately 1 kb of the porcine *MYH4*-promoter (–961bp to +32bp, relative to the transcriptional start site) was cloned into a ZsGreen1-1 expression vector (Clontech) as described previously [13]. The day after seeding (~50–60% confluence), C2C12 myoblasts were co-transfected with *MYH4*-ZsGreen promoter-reporter plasmid (200 ng per well) and CMV-DsRed-Express-N1 (100 ng per well) at a ratio of 3:1 for plasmid DNA:FuGENE® (E2311, Promega). The following day, cells (~80% confluence) were switched to differentiation media (DM; DMEM supplemented with 2% horse serum and 1% P/S) with or without treatments, determined as day 0.

The following doses used were based on previous studies, 1 mM for dbcAMP [44], 20 ng/ml for Des(1–3) IGF-I [32,45], 10 nM for T3 [46] and 10 µM for Dex [47]. Immediately prior to treatment, compounds and respective vehicle controls were diluted to working concentrations either directly into pre-warmed DM (dbcAMP and T3) or initially in phosphate buffered saline (for Des(1–3) IGF-I and Dex) followed by a further dilution in DM. Culture media and treatments were refreshed every 48 h. Measurements for total fluorescence per well and average myotube diameter were performed on consecutive days or on day 4 of differentiation only.

2.3. Measurement of porcine *MYH4*-promoter activity

Porcine *MYH4*-promoter activity was measured as total fluorescence

Table 1
Typhoon Trio + parameters for total fluorescence per well quantification.

Channel	Laser	Fluorophore detected	Excitation (nm)	Emission (nm)	Sensitivity
1	Blue	ZsGreen	488	520	Medium
2	Green	DsRed	532	580	Medium

per well using a Typhoon Trio + scanner (GE Healthcare). Cell culture plates were scanned as indicated in Table 1, with focal plane set to +3 mm and image quality captured at 100 μm . Scan time was approximately 20 min per cell culture plate (1 \times 24-well plate). ‘Integrated Density’ for the set area of each well was quantified using ImageJ software. The fluorescence intensity of ZsGreen for each well was normalised to DsRed (i.e. ZsGreen \div DsRed) to account for differences in transfection efficiency. Wells seeded with C2C12 cells, but not transfected, were used as a ‘background control’ to account for autofluorescence. All data related to porcine *MYH4*-promoter activity can be found in Supplementary Datafile 1.

2.4. Measurement of myotube diameters

Immediately after the scan of total fluorescence per well (on day 4 differentiation), fluorescence images of live cells were captured using a Leica inverted fluorescence microscope and Leica Application Suite software. Three to five fields of view were captured per well, then from each view average diameter of *MYH4*-expressing myotubes was determined for 10 randomly selected myotubes. Using ImageJ, the diameter was measured at approximately 25, 50 and 75% along the total length of each myotube as described previously [14]. Images were blind labelled to remove bias for the analysis. All data related to myotube diameter measurements can be found in Supplementary Datafile 2.

2.5. Statistical analysis

Statistical analysis was performed using GenStat (17th edition) statistical software. For total fluorescence measurements taken on the same samples for consecutive days, a repeated measures two-way analysis of variance (ANOVA) was performed (time \times treatment). For total fluorescence measurements collected at a single time-point, a Student’s *t*-test was performed to compare the treatment to its vehicle control. For myotube diameter measurements collected at a single time-point, one-way ANOVA was performed and blocked according to the individual ‘well’ for different fields of view. Data are presented as means \pm

standard error of the mean (SEM).

3. Results

3.1. Effect of dbcAMP on porcine *MYH4*-promoter activity and diameter of *MYH4*-expressing myotubes

There was a significant time \times treatment interaction for *MYH4*-promoter activity during myogenic differentiation ($P < 0.001$; Fig. 1a). Porcine *MYH4*-promoter activity (i.e. ZsGreen fluorescence) was only observed in differentiated myotubes and increased gradually with time as myotubes developed and matured, being low at day 2 of differentiation but with a slight increase by day 3 and followed by a marked increase between days 3 and 4. Treatment with dbcAMP increased *MYH4*-promoter activity at day 4 of differentiation (32%) relative to the water vehicle control. This was supported by images shown in Fig. 1b. Similarly, average myotube diameter was also significantly increased (43%; $P < 0.001$; Fig. 1c) after four days of treatment with dbcAMP compared to water.

As dbcAMP was observed to significantly increase both *MYH4*-promoter activity and average myotube diameter of *MYH4*-expressing myotubes compared to water, dbcAMP and water were used in subsequent experiments to act as positive controls for both measurements.

3.2. Effect of Des(1–3) IGF-I on porcine *MYH4*-promoter activity and diameter of *MYH4*-expressing myotubes

As before, treatment with dbcAMP significantly increased both *MYH4*-promoter activity (40%; $P = 0.006$) and average myotube diameter (30%; $P = 0.006$) relative to water. In contrast, treatment with Des(1–3) IGF-I for four days had no effect on *MYH4*-promoter activity ($P = 0.823$; Fig. 2a), but did increase average myotube diameter of *MYH4*-expressing myotubes (20%; $P = 0.021$; Fig. 2b) compared to its vehicle control, 0.1 M acetic acid, indicating a hypertrophic-like growth effect, although this was to a lesser degree compared to dbcAMP treatment.

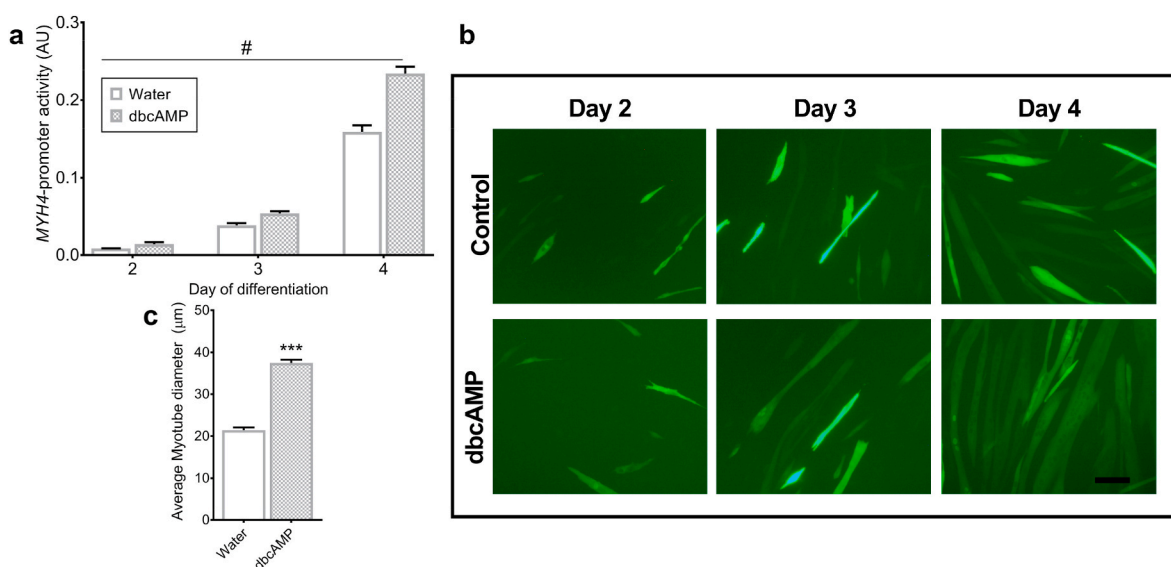


Fig. 1. Effect of dbcAMP on porcine *MYH4*-promoter activity and average myotube diameters.

C2C12 cells were transfected with a ZsGreen expression plasmid driven by a porcine *MYH4*-promoter on day –1 of differentiation, then treated with 1 mM dbcAMP or water (vehicle control) from day 0. (a) *MYH4*-promoter activity, determined as total fluorescence per well (ZsGreen) normalised to CMV-DsRed, was quantified every 24 h for three consecutive days from day 2 of differentiation. # $P < 0.001$ (time \times treatment interaction). Data shown as means \pm SEM, from four and seven wells at each time-point for control and dbcAMP-treated cells respectively. (b) Fluorescence images captured at days 2, 3 and 4 of differentiation. Magnification: 10X. Scale bar: 100 μm . (c) Average myotube diameter of *MYH4*-expressing myotubes was quantified at day 4 of differentiation following treatment from day 0 with water or 1 mM dbcAMP. Data shown as means \pm SEM, from three fields of view per well using four wells for water and six wells for dbcAMP ($n = 12$ and $n = 18$ respectively). *** $P < 0.001$.

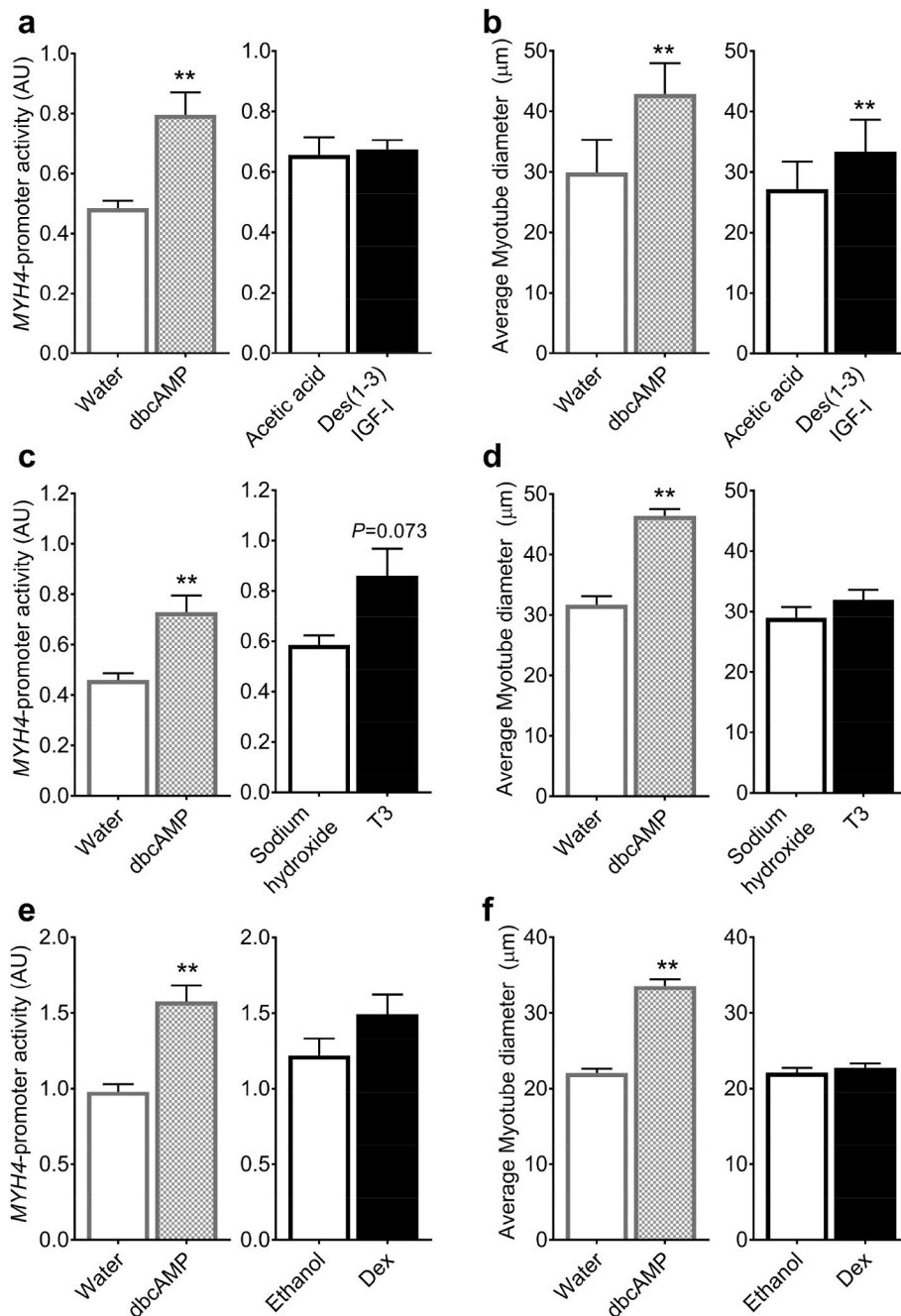


Fig. 2. Effects of dbcAMP, Des(1–3) IGF-I, T3 and Dex on porcine *MYH4*-promoter activity and average myotube diameters.

C2C12 cells were transfected with porcine *MYH4*-promoter-ZsGreen and CMV-DsRed expression plasmids on day –1, then treated from day 0 of differentiation with the following compounds and appropriate vehicle controls: (a, b) Des(1–3) IGF-I (20 ng/ml) or 0.1 M acetic acid, (c, d) T3 (10 nM) or 0.1 M sodium hydroxide, and (e, f) Dex (10 μM) or absolute ethanol. For each experiment, treatment with 1 mM dbcAMP or water was used as controls for the assay. Measurements were taken on day 4 of differentiation for (a, c, e) *MYH4*-promoter activity, determined as total fluorescence per well (ZsGreen) normalised to CMV-DsRed, and (b, d, f) average myotube diameter of *MYH4*-expressing myotubes. Data shown as means ± SEM from n = 3 or 4 wells per treatment. To measure average myotube diameter, n = 3 or 5 fields of view per well were used for each treatment. To compare each treatment group with the appropriate vehicle control, data were analysed by either Student's t-test (a, c, e) or one-way ANOVA, blocking for well (b, d, f). ***P* < 0.01.

3.3. Effect of 3,3',5-Triiodo-L-thyronine (T3) on porcine *MYH4*-promoter activity and diameter of *MYH4*-expressing myotubes

As expected dbcAMP treatment again resulted in a significant increase in both *MYH4*-promoter activity (35%; *P* = 0.009) and average myotube diameter (32%; *P* = 0.003) relative to water. T3 treatment tended to increase *MYH4*-promoter activity (32%; *P* = 0.073; Fig. 2c) relative to the vehicle control, sodium hydroxide, and although this was not quite significant, this was possibly due to the low number of replicates (n = 3). Average myotube diameter of C2C12 cells expressing *MYH4* was not affected (*P* = 0.294; Fig. 2d) after four days of treatment with T3 compared to 0.1 M sodium hydroxide, indicating that T3 potentially induced a change in the expression of the MyHC isoforms, but had no effect on myotube size.

3.4. Effect of Dexamethasone (Dex) on porcine *MYH4*-promoter activity and diameter of *MYH4*-expressing myotubes

As observed previously, treatment with dbcAMP significantly increased both *MYH4*-promoter activity (37%; *P* = 0.007) and average myotube diameter (34%; *P* = 0.009) relative to water. However, treatment with dexamethasone (Dex) for four days had no effect on either *MYH4*-promoter activity (*P* = 0.187; Fig. 2e) or average diameter of *MYH4*-expressing myotubes (*P* = 0.69; Fig. 2f) compared to its vehicle control, ethanol.

4. Discussion

Here we have further characterised the porcine *MYH4*-promoter ZsGreen reporter system described previously [13,14] and for the first time report the response of the porcine *MYH4*-promoter to known

anabolic and catabolic agents (dbcAMP, Des(1–3) IGF-I, T3, Dex).

Treatment with dbcAMP significantly increased both average myotube diameter of *MYH4*-expressing myotubes and *MYH4*-promoter activity. This supports previous observations following treatment of C2C12 cells with dbcAMP from day 0 of differentiation leading to a dramatic upregulation of *Myh4* mRNA and increased myotube size [48]. It also reflects both the muscle-specific hypertrophy and shift to a fast-glycolytic MyHC-IIB phenotype observed with BA treatment in pigs [10,11,15], cows [17,18], sheep [19], rats [20–23] and mice [14,24]. The reproducibility of the response to dbcAMP treatment was highly consistent, therefore dbcAMP and water (vehicle control) were used as positive controls for the *MYH4*-promoter activity and average myotube diameter measurements in subsequent experiments.

Next, we investigated the effects of Des(1–3) IGF-I, an IGF-I analogue, on *MYH4*-promoter activity and myotube diameters. In the current study, Des(1–3) IGF-I treatment increased average myotube diameter but had no effect on *MYH4*-promoter activity. This indicates a hypertrophic-like growth response, but with no direct effect on *MYH4* expression, in agreement with previous reports of GH treatment *in vivo* [10,17]. Interestingly, a previous *in vitro* study showed that IGF-I treatment of C2C12 cells resulted in an increase in both *Myh4* mRNA and murine *Myh4*-promoter activity [49]. They transfected C2C12 cells with a 3 kb murine *MYH4*-promoter construct and identified a putative IGF-I responsive element in the murine *Myh4*-promoter located approximately 1.2 kb upstream of the murine *Myh4* gene [49]. This contrasts with our finding for the pig promoter, but it should be noted that Shanely et al. [49] treated C2C12 cells with high concentrations (250 ng/ml) of IGF-I in DM from day 0 for 2–4 days, which resulted in clear increases in differentiation that coincided with the increase in *Myh4* promoter activity (and endogenous *Myh4* mRNA). In fact there was no effect of IGF-I on *Myh4* promoter activity at day 1 (24 h treatment [49]), suggesting that the effect may be indirect (as a direct effect on transcription might be expected within 24 h) and may well relate to the observed induction of differentiation, rather than a direct effect of IGF-I per se. Indeed Shanely et al. [49] suggest that the responsiveness of the *Myh4*-promoter to IGF-I was via a T-cell factor/lymphoid enhancer factor-response element (Tcf/Lef-RE) that was conserved across species (rat, human, chimp). However, shorter promoter constructs lacking the putative Tcf/Lef-RE still responded to IGF-I and none of the signalling pathway inhibitors nor mutation of the putative Tcf/Lef-RE actually blocked the response to IGF-I [49]. It has also previously been reported that IGF-I KO mice have reduced body weight and reduced diaphragm muscle fibre size and fibre number, but no changes in fibre type proportions [50], indicating that IGF-I has no effect on MyHC expression in mice *in vivo*. Hence, we believe the lack of response of the pig promoter to Des(1–3) IGF-I treatment is reflective of what happens in the pig and agrees with previous *in vivo* studies that also showed no change in MyHC mRNA expression when plasma IGF-I levels were increased by GH treatment [11].

Next, we determined the response of the porcine *MYH4*-promoter to treatment with T3. There was no effect of T3 on average myotube diameter of *MYH4*-expressing myotubes, but there was a tendency to induce *MYH4*-promoter activity. This indicates T3 treatment induces a fibre-type switching-like response by potentially altering the expression of the MyHC isoforms. The T3-induced increase in *MYH4*-promoter activity agrees with the current *in vivo* literature. It has been reported that T3 treatment of rats (175 µg/kg) for 6 weeks increased the expression of MyHC-IIB protein (with decreased MyHC-IIX expression) in isolated fibres from the posterior cricoarytenoid muscle [51]. Conversely, hypothyroidism induced by thyroidectomy in adult rats resulted in a reduction in fast-twitch muscle fibres and an induction in slow-twitch, oxidative fibres [36,52]. Similarly, hypothyroidism in new-born piglets, induced by the addition of inhibitory compounds (methimazole and iopanoic acid) to their feed, reduced the proportion of fast-twitch oxidative/glycolytic type-II fibres and increased the proportion of slow oxidative type-I fibres [53]. Despite the consistency with *in vivo*

observations reported here, a higher dose (30 nM) of T3 has previously been shown to reduce myotube diameters in C2C12 cells [54]. However, T3 treatment was added after two days of differentiation compared to at the onset of differentiation (day 0) in the present study, which may explain the discrepancy.

Finally, the relatively short-term (i.e. 4 day) treatment with Dex (10 µM) had no effect on either average myotube diameter or *MYH4*-promoter activity. This was unexpected as Dex is known as a catabolic agent *in vivo* and is frequently used to induce atrophy and protein catabolism in C2C12 cells to model muscle wasting [39,47,55]. However, in those studies C2C12 cells had been differentiated for 3+ days prior to Dex treatment, whereas we treated myoblasts at the onset of differentiation (from day 0). It has also recently been reported that proliferating C2C12 myoblasts transiently treated with Dex (10 µM) prior to differentiation actually enhanced myogenic differentiation, and increased both myotube diameter and the abundance of total MyHC protein [56]. This suggests that proliferating myoblasts might respond differently to Dex treatment compared with differentiated myotubes.

5. Conclusions

Here we report the response of the porcine *MYH4*-promoter activity and *MYH4*-expressing C2C12 myotubes to known anabolic and catabolic agents (dbcAMP, Des(1–3) IGF-I, T3 and Dex) *in vitro*. For most treatments (except Dex), these observations correspond to previous *in vivo* studies, and help to further understand the regulation of the porcine *MYH4*-promoter. This study also indicates that increased *MYH4*-promoter activity is not always associated with muscle fibre hypertrophy.

Declaration of competing interest

I have read the journal's policy and the authors of this manuscript have the following competing interests: This research was funded by the BBSRC and Zoetis (formally Pfizer Animal Health).

Acknowledgements

MCB, DML, JMB, TP and PTL conceived the experimental designs. DML undertook the preliminary and validation experiments associated with methods utilised in this study, including cloning the *MYH4*-ZsGreen plasmid and determining methods to quantify changes in *MYH4*-promoter activity and myotube diameters. MCB performed all cell culture procedures and measurements collected in this study. MCB was the major contributor in writing the original manuscript draft, preparing the figures and performing data and statistical analyses. JMB and TP edited the manuscript. JMP, TP and PTL carried out supervision and funding acquisition for this project. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2021.100924>.

CRedit author statement

MCB: Conceptualisation, Investigation, Formal analysis, Visualization, Writing – Original Draft Preparation, Review & Editing. DML: Conceptualisation, Methodology, Validation. PTL: Supervision, Funding acquisition, Conceptualisation. TP: Supervision, Funding acquisition,

Conceptualisation, Writing – Review & Editing. JMB: Supervision, Funding acquisition, Conceptualisation, Writing – Review & Editing.

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