A human Myogenin promoter modified to be highly active in alveolar rhabdomyosarcoma drives an effective suicide gene therapy

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Abstract
Rhabdomyosarcoma is a rare childhood soft tissue cancer whose cells resemble poorly differentiated skeletal muscle, expressing myogenic proteins including MYOGENIN. Alveolar rhabdomyosarcoma (ARMS) accounts for ~40% of cases and is associated with a poorer prognosis than other rhabdomyosarcoma variants, especially if containing the chromosomal translocation generating the PAX3-FOXO1 hybrid transcription factor. Metastasis is commonly present at diagnosis, with a five-year survival rate of <30%, highlighting the need for novel therapeutic approaches. We designed a suicide gene therapy by generating an ARMS-targeted promoter to drive the herpes simplex virus thymidine kinase (HSV-TK) suicide gene. We modified the minimal human MYOGENIN promoter by deleting both the NF1 and MEF3 transcription factor binding motifs to produce a promoter that is highly active in ARMS cells. Our bespoke ARMS promoter driving HSV-TK efficiently killed ARMS cells in vitro, but not skeletal myoblasts. Using a xenograft mouse model, we also demonstrated that ARMS promoter-HSV-TK causes apoptosis of ARMS cells in vivo. Importantly, combining our suicide gene therapy with standard chemotherapy agents used in the treatment of rhabdomyosarcoma, reduced the effective drug dose, diminishing deleterious side effects/patient burden. This modified, highly ARMS-specific promoter could provide a new therapy option for this difficult-to-treat cancer.

Introduction
Rhabdomyosarcoma is the commonest form of childhood soft tissue cancer, affecting 1:150,000 children [1], with an overall favourable prognosis. However, prognosis correlates with classification into two major subtypes: embryonal rhabdomyosarcoma (ERMS, >70% five-year survival rate) and alveolar rhabdomyosarcoma (ARMS, <30% five-year survival rate) [2]. ERMS frequently displays mutations in common oncogenes, such as members of the RAS family, FGFR4, PIK3CA and CTNNB1, while such mutations leading to either gain- or loss-of-function are rarely consistently present in ARMS [3].

Currently, treatment for ARMS is predominantly restricted to surgery, together with conventional radiation therapy and chemotherapy. A combination of vincristine, actinomycin and cyclophosphamide (VAC) is the common chemotherapy regime used to treat ARMS in North America. According to both the international classification of paediatric sarcomas and the World Health Organisation classification of skeletal muscle tumours, ARMS is classified as ‘High-Risk Malignant’ [4]. Partially due to the high degree of metastasis at diagnosis, the five-year survival rate remains poor (<30%), highlighting the urgent need for novel therapeutic approaches.

Another factor correlating with low survival is the expression of a novel and ARMS-specific hybrid transcription factor generated through an inframe chromosomal translocation. While a small subset of histologically classified ARMS tumours do not express this hybrid transcription factor, these cases are genetically more aligned with ERMS than fusion-positive ARMS [5]. The DNA binding domain
of PAX proteins subfamily III members PAX3 or PAX7 fuses in frame with the transactivation domain of FOXO1, generating highly potent chimeric transcription factors, termed PAX3-FOXO1 (chromosomes 2 and 13) or PAX7-FOXO1 (chromosomes 1 and 13) [4]. PAX3-FOXO1 is associated with a poorer prognosis. PAX3 is essential for embryonic/foetal development of skeletal muscle [6], while PAX7 controls specification/regulation of the resident stem cell pool of postnatal muscle as satellite cells [7, 8]. PAX3 and PAX7 operate with members of the myogenic regulatory factor family (Myf5, MyoD, Myogenin and Myf6/Mrf4) of transcription factors in controlling myogenesis [9]. However, PAX3/7-FOXO1 suppress the transcriptional activity of some MyoD-target genes in muscle stem cells [10]. Interestingly, PAX3-FOXO1 has significantly altered DNA binding properties compared to PAX3, even though the DNA recognition sequences remain identical. PAX3 can bind to an E5 target sequence (adjacent homeodomain ATTA motif and paired GTTCC domain) with higher affinity than PAX3-FOXO1, but even with this lower binding affinity, PAX3-FOXO1 is the more potent transcriptional activator [11].

PAX3/7-FOXO1 target genes are enriched in pathways controlling mesodermal development, neural-related gene expression, and myogenic signalling and differentiation [12]. Specifically, MYOD and MYOGENIN are upregulated by PAX3/7-FOXO1 [13] and PAX3-FOXO1 interacts directly with the MYOGENIN promoter in a MYOD independent way [14]. MYOGENIN is normally tightly controlled during myogenesis, being at negligible levels in proliferating myoblasts, but increasing on cell cycle exit, with peak expression during the fusion phase of the myogenic differentiation program. In contrast, MYOGENIN expression in ARMS cells is constitutive, and considered a reliable marker for diagnosis [15, 16], yet is unable to drive myogenic differentiation in such sarcoma cells.

Dysregulated and constitutive expression of MYOGENIN in ARMS highlights the MYOGENIN promoter as a potential tool to regulate an ARMS-specific suicide gene therapy. A transgene comprising a minimal MYOGENIN promoter (−130 to +18 bp) driving a lacZ reporter replicates temporal and spatial expression of MYOGENIN during embryonic myogenesis in mouse [17, 18]. This minimal MYOGENIN promoter transgene contains several well-described and evolutionarily conserved DNA binding motifs, including the TATA box, half a palindromic NF1 site, a MEF3, MEF3 and PBX site, and two E-boxes [19], that control its dynamic regulation. The MEF2 binding motif for example, is required for expression in cells in a subset of somites and the limb buds at embryonic day (E) 11.5 in mouse [17, 18].

Considering differences in expression profile and regulation of the MYOGENIN promoter in healthy skeletal muscle compared to ARMS, we hypothesised that a modified MYOGENIN promoter could generate a novel ARMS-specific promoter, less active in healthy skeletal muscle. This would allow development of gene therapies driven by such an ARMS-specific promoter. The use of tissue-specific promoters to target cancer cells is not novel, having been demonstrated in multiple cancers, such as the cholecystokinin type A receptor promoter in colorectal cancer [20] or the HTERT promoter active in >90% of human cancers [21]. A promoter specific for ARMS, ERMS or rhabdomyosarcoma in general has yet to be described.

An ARMS-specific promoter could drive a suicide gene that delivers an apoptosis-inducing therapy into cancer cells. This strategy using ubiquitously active promoters such as CMV has been tested in various cancer types (reviewed in [22]), including ARMS [23], and targets chemotherapy-resistant cell lines efficiently [24]. A commonly used suicide gene therapy is combination of herpes simplex thymidine kinase (HSV-TK) with ganciclovir (GCV). A non-toxic prodrug capable of penetrating dense tumour, GCV is converted into a cytotoxic drug only through phosphorylation by HSV-TK. Monophosphorylated GCV is then converted to tri-phosphorylated GCV by host cell kinases, creating an adenosine analogue incorporated into DNA during synthesis, causing a delay in S and G2-phase, accompanied by induction of apoptosis [25]. In addition, caspase-8, Chk1 activation [26] and mitochondrial damage [25] occur. HSV-TK/GCV is characterised by high safety, efficacy of mediated cell suicide and an abundant choice of alternatives to GCV with reduced side-effects and increased specificity to cell kinases [27].

By modification of transcription binding motifs, we generated a custom minimal human MYOGENIN promoter by deleting the MEF3 and NF1 sites to drive HSV-TK with enhanced specificity for ARMS. Upon GCV treatment, our custom miniMg-ΔMEF3/NF1-HSV promoter decreased viability in ARMS cells, but not viability in skeletal muscle cells. In vivo, tumour size was significantly reduced by miniMg-ΔMEF3/NF1-HSV in an ARMS xenograft mouse model treated with GCV. In addition, the miniMg-ΔMEF3/NF1-HSV promoter effectively targeted tumour cells and lowered chemotherapy dose, and so could be employed in combination with a chemotherapeutic regime. In summary, miniMg-ΔMEF3/NF1-HSV is a potential supplement to conventional therapies for this difficult-to-treat cancer.

Materials and methods

Mice

Animal work was performed in accordance with British law under the provisions of the Animals (Scientific Procedures)
Act 1986, as approved by the Ethical Review Process Committee of King’s College London.

12-week-old immuno-compromised female Swiss Nude mice (Crl:NU(lco)-Foxn1nu) were purchased from Charles River Laboratories and then housed in ventilated cages to acclimatize for 2 weeks. Mice were assigned randomly to different experimental groups, and no blinding was implemented for data analysis. 5 × 10^5 RH30 cells expressing HSV-TK under the control of LV-miniMg-Full, LV-miniMg-ΔMEF3/NF1 or LV-ΔminiMg in 100 µl PBS: Matrigel (50:50) were injected subcutaneously into the right flank. Once tumours were visible (from two months post-injection), tumour size was measured with calipers until size reached 300 mm^3 (day 0), with GCV administration started 24 h later. Animals then received IP injections of 1 mg GCV/PBS every 24 h for 8 days (day 1 to day 8), and were sacrificed the day of the last injection. After sacrifice, tumours were weighed before being dissected into pieces for later protein and mRNA extraction, as well as imaging. Samples for protein and mRNA extraction were flash frozen in liquid nitrogen and stored at −80 °C. Samples for staining were washed in PBS, fixed in 4% paraformaldehyde (PFA)/PBS for 48 h, passed through 15 and 30% sucrose/PBS before being embedded and frozen in OCT and stored at −80 °C until further processing.

Animal experiments were performed in two separate batches. The first batch showed successful tumour growth in 4/4/2 animals for LV-miniMg-Full/LV-ΔminiMg/LV-miniMg-ΔMEF3/NF1, and the second batch 4/3 animals for LV-ΔminiMg/LV-miniMg-ΔMEF3/NF1 respectively.

**Cell culture**

RH30 (CVCL_0041) and RH41 (CVCL_2176) were maintained in DMEM GlutaMax (Gibco, 10566016) with 10% foetal calf serum (FBS) and 1% Pen/Strep (Sigma). C25 and 16U myoblasts were maintained in Promocell skeletal muscle foetal calf serum (FBS) and 1% Pen/Strep (Sigma). C25 and 16U myoblasts were maintained in Promocell skeletal muscle foetal calf serum (FBS) and 1% Pen/Strep (Sigma). C25 and 16U myoblasts were maintained in Promocell skeletal muscle foetal calf serum (FBS) and 1% Pen/Strep (Sigma). C25 and 16U myoblasts were maintained in Promocell skeletal muscle foetal calf serum (FBS) and 1% Pen/Strep (Sigma). C25 and 16U myoblasts were maintained in Promocell skeletal muscle foetal calf serum (FBS) and 1% Pen/Strep (Sigma). C25 and 16U myoblasts were maintained in Promocell skeletal muscle foetal calf serum (FBS) and 1% Pen/Strep (Sigma). C25 and 16U myoblasts were maintained in Promocell skeletal muscle foetal calf serum (FBS) and 1% Pen/Strep (Sigma). C25 and 16U myoblasts were maintained in Promocell skeletal muscle foetal calf serum (FBS) and 1% Pen/Strep (Sigma).

**Plasmids**

We designed LV-miniMg-Full and LV-miniMg-Full-HSV (with the MYOGENIN promoter flanked by XbaI and BamHI sites), which was then manufactured by VectorBuilder. Deletions were introduced through site-directed mutagenesis according to manufacturer’s instructions (ThermoFishier, A14604). Mutant promoters were PCR amplified from LV-miniMg constructs and restriction enzyme sites introduced. The MYOGENIN promoter in LV-miniMg-Full-HSV was then exchanged with mutant promoters.

**RNA extraction and RT-qPCR analysis**

For whole tumour lysates, 30 mg of tumour tissue was thoroughly homogenized with a TissueRuptor (Qiagen, 9002755) in 700 µl RLT lysis buffer. Cultured cells were lysed directly in 350 µl RLT lysis buffer. mRNA was isolated with the RNeasy kit (Qiagen, 74104) according to manufacturer’s instructions. Reverse transcription was performed with Quantitect Reverse transcription Kit (Qiagen, 205311), SYBR green qPCR was performed (Takyon, UF-NSMT-B0101) on biological replicates [3, 4]. Relative gene expression was normalised to RPLP0, and values are represented as 2−ΔΔCT. Primer sequences: RPLP0, 5'-TG GTCATCAGCAGGTGTCCGA-3'(forward) and 5'-ACA GACACTGGCAACATTGCGG-3'(reverse); eGFP, 5'-GA AGCGGATCATCAGG-3'(forward) and 5'-CCATGCC AGAATGTAC-3'(reverse); mCherry, 5'-GTGACCCGTG ACCAAGAC-3'(forward) and 5'-GCGCAGCTTCCAC TTGTAG-3'(reverse); NF1B, 5'-CAGGAGTCATGATG TGC-3'(forward) and 5'-CCCCAGGCCGATGAT-3'(reverse); MYOGENIN, 5'-CCAGG TGGTCCAGC-3'(forward) and 5'-AGCCGTAG CAGATGTAC-3'(reverse); MYOMAKER, 5'-AAGATG TACCCAAGGAAGG-3'(forward) and 5'-GTAGAGA CCTGTGCGAATA-3'(forward) and 5'-CCCTCAGTA GGATGCATAA-3'(reverse); HSV-TK, 5'-TACCCGAGC CGATGACTTA-3'(forward) and 5'-CCTGGTTTG GTGTAAGAT-3'(reverse); MYOGENIN, 5'-CCAGG TGGTCCAGC-3'(forward) and 5'-AGCCGTAG CAGATGTAC-3'(reverse); MYOMAKER, 5'-AAGATG TACCCAAGGAAGG-3'(forward) and 5'-GTAGAGA CCTGTGCGAATA-3'(forward) and 5'-CCCTCAGTA GGATGCATAA-3'(reverse); HSV-TK, 5'-TACCCGAGC CGATGACTTA-3'(forward) and 5'-CCTGGTTTG GTGTAAGAT-3'(reverse); MYOGENIN, 5'-CCAGG TGGTCCAGC-3'(forward) and 5'-AGCCGTAG CAGATGTAC-3'(reverse); MYOMAKER, 5'-AAGATG TACCCAAGGAAGG-3'(forward) and 5'-GTAGAGA CCTGTGCGAATA-3'(forward) and 5'-CCCTCAGTA GGATGCATAA-3'(reverse); HSV-TK, 5'-TACCCGAGC CGATGACTTA-3'(forward) and 5'-CCTGGTTTG GTGTAAGAT-3'(reverse); MYOGENIN, 5'-CCAGG TGGTCCAGC-3'(forward) and 5'-AGCCGTAG CAGATGTAC-3'(reverse); MYOMAKER, 5'-AAGATG TACCCAAGGAAGG-3'(forward) and 5'-GTAGAGA CCTGTGCGAATA-3'(forward) and 5'-CCCTCAGTA GGATGCATAA-3'(reverse); HSV-TK, 5'-TACCCGAGC CGATGACTTA-3'(forward) and 5'-CCTGGTTTG GTGTAAGAT-3'(reverse); MYOGENIN, 5'-CCAGG TGGTCCAGC-3'(forward) and 5'-AGCCGTAG CAGATGTAC-3'(reverse); MYOMAKER, 5'-AAGATG TACCCAAGGAAGG-3'(forward) and 5'-GTAGAGA CCTGTGCGAATA-3'(forward) and 5'-CCCTCAGTA GGATGCATAA-3'(reverse); HSV-TK, 5'-TACCCGAGC CGATGACTTA-3'(forward) and 5'-CCTGGTTTG GTGTAAGAT-3'(reverse); MYOGENIN, 5'-CCAGG TGGTCCAGC-3'(forward) and 5'-AGCCGTAG CAGATGTAC-3'(reverse); MYOMAKER, 5'-AAGATG TACCCAAGGAAGG-3'(forward) and 5'-GTAGAGA CCTGTGCGAATA-3'(forward) and 5'-CCCTCAGTA GGATGCATAA-3'(reverse); HSV-TK, 5'-TACCCGAGC CGATGACTTA-3'(forward) and 5'-CCTGGTTTG GTGTAAGAT-3'(reverse); MYOGENIN, 5'-CCAGG TGGTCCAGC-3'(forward) and 5'-AGCCGTAG CAGATGTAC-3'(reverse); MYOMAKER, 5'-AAGATG TACCCAAGGAAGG-3'(forward) and 5 '-GTAGAGA CCTGTGCGAATA-3'(forward) and 5'-CCCTCAGTA GGATGCATAA-3'(re...
counterstained with DAPI 1:1000 and mounted in vectashield (VWR, 101098–042). Samples were viewed and imaged on a Leica AxioVert 200 M.

**TUNEL assay**

OCT embedded tumours were acclimatized from −80 °C storage temperature to −21 °C in a cryostat and sectioned at 20 µm. TUNEL assay was performed on cryosections according to manufacturer’s instructions (Abcam, ab66110), nuclei were counterstained with DAPI 1:1000, and mounted in vectashield, samples were imaged on a Leica AxioVert 200 M.

**Luciferase viability assay**

Cells were seeded at 1000 cells/well in white 96-well plates (Merck, M0187-32EA). After 24 h, the medium was changed to a medium containing luciferase reagents (Promega, G9711) and GCV sodium salt (SantaCruz, 107910–75–8). Luciferase signal was measured 1 h later, and then every 24 h until 72 h post-medium change. Signal obtained at 24, 48 and 72 h was displayed as fold change to the one-hour timepoint.

**Luciferase apoptosis assay**

Cells were seeded at 1000 cells/well in white 96-well plates. After 24 h, the medium was changed to medium containing luciferase reagents (Promega, JA1011) and GCV sodium salt. Luciferase signal was measured repeatedly between 1 and 72 h after the medium change. The presence of apoptosis was considered detectable when the signal increased over background levels and the signal is shown for this timepoint (1 h) and 24 h later.

**Cell counting**

Cells were seeded at 5000 (RH30), 1000 (RH41, 16U) or 2000 (C25) cells per well in 24 well plates. After 24 h, the medium was changed to medium containing GCV, and cells were counted using a hematocytometer 24 and 48 h later.

**SiRNA transfection**

80,000 RH30 or C25 cells were transfected with 1.5 nM SiRNA against NF1B (Qiagen FlexiTube GeneSolution siRNA, GS4781) or scrambled control SiRNA according to the manufacturer’s instructions, for 24 h. Proliferating RH30 cells were fixed and immunolabelled 48 h post transfection. C25 myoblasts were seeded 24 h post transfection, switched to differentiation medium after an additional 24 h, maintained in differentiation medium for 48 h before fixation and immunolabelling.

**Chemotherapy regime**

Vincristine sulfate (V8388-1MG, Sigma), Actinomycin D (A1410-5MG, Sigma) and Cyclophosphamide monohydrate (93813–100MG, Sigma) were used at a ratio of 1.5 mg/m²: 0.045 mg/kg: 2200 mg/m², as used for patients >3 years of age [28]. A stock of 96 nM VAC (0.061 mM Vincristine: 0.065 mM Actinomycin D: 96 mM Cyclophosphamide) in PBS was used at 5 fold serial dilutions, starting at a concentration of 9.6 µM (1:10,000) to 0.07 µM VAC (1:1,250,000).

Cells were plated and allowed to attach for 24 h, before being treated with a fresh growth medium containing VAC, GCV and a luciferase substrate (Promega, JA1011) and viability was measured 1 and 24-h post treatment. For counting, cells were plated and allowed to attach for 24 h, then treated with VAC and GCV and counted 24 h later.

**Protein extraction and western blot**

Tumour tissue was homogenized with a TissueRuptor in a total of 600 µl RIPA buffer (Sigma, R0278), supplemented with 1:100 phosphatase inhibitor cocktails 2 (Merck, P5726-1ML) and 3 (Merck, 524627–1 ML), and 1:7 protease inhibitors (Merck, 11836170001). The lysate was agitated at 4 °C for 2 h, centrifuged at max speed at 4 °C for 20 min, 4x Laemmli buffer was added to the supernatant and samples were boiled for 5 min at 95 °C. 50 µg protein and 5 µl precision plus protein standards dual colour ladder (BioRad, #161–0374) were loaded in a 4–20 % precast gel (BioRad, #4561094) and run for 1 h at 60 V, and transfer to nitrocellulose membrane was performed at constant 70 V for 1.5 h. The membrane was stained first for BAX protein (polyclonal rabbit-α-BAX, CST, #2772) 1:1000 over night at 4 °C, followed by incubation with α-rabbit-HRP secondary antibody (Sigma, GENA934–1ML) at 1:25,000 for 1 h at room temperature, and development with clarity ECL substrate. The same membrane was then stained against β-TUBULIN (monoclonal mouse -α-β-TUBULIN, DSHB, E7) 1:4000 over night at 4 °C, followed by incubation with α-mouse-HRP secondary antibody (Sigma, NA931V) for 1 h at room temperature, and development with clarity ECL substrate. Blot was imaged on a ChemiDoc imaging system (BioRad, 17001401).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 8.0. Experiments were performed with an N of at least 3, with detailed N numbers given with each figure. The variance between groups was compared using a Brown Forsythe test and revealed no significant difference. A comparison between two groups was performed using an
unpaired homoscedastic two-tailed student's *t*-test. A comparison of more than two groups was performed using a one-way ANOVA followed by Dunett's post test as different groups were compared with the control group. *P* < 0.05 were considered significantly different.

**Results**

**Generation of an ARMS-specific promoter by modification of the human *MYOGENIN* promoter**

*MYOGENIN* is constitutively expressed in rhabdomyosarcoma, but only transiently during myogenic differentiation in skeletal muscle, and so was selected for modification to enhance ARMS, but reduce skeletal muscle expression. A lentiviral construct was generated where the minimal human *MYOGENIN* promoter [17] drives *eGFP*, while the ubiquitously active CMV promoter drives *mCherry* (termed LV-miniMg-Full—Fig. 1a, b). Evaluation of LV-miniMg-Full was initially performed in the RH30 ARMS cell line [29] and C25 immortalised human skeletal myoblasts [30]. Endogenous *MYOGENIN* expression increased significantly as C25 myoblasts differentiate, with a peak two days after induction of differentiation, as myoblasts fuse and generate immature multinucleated myotubes (Fig. 1c). Conversely, RH30 cells show a moderate constitutive expression of *MYOGENIN* (Fig. 1c). As expected, expression of both *eGFP* and *mCherry* from LV-miniMg-Full was robust in C25 myoblasts undergoing myogenic differentiation and in proliferating RH30 cells (Fig. 1b). LV-miniMg-Full activity, measured by *eGFP* normalised to *mCherry*, showed the same trend as endogenous *MYOGENIN*, with significantly higher expression of *eGFP* in differentiating C25 myoblasts compared to during proliferation, and moderate levels in RH30 cells. Thus, LV-miniMg-Full mimics *MYOGENIN* expression (Fig. 1d).

To generate a *MYOGENIN* promoter with low/negligible activity in proliferating C25 myoblasts, reduced activity in differentiating C25 myoblasts and stable/increased activity in RH30 cells, we deleted each of six conserved and well-studied transcription factor binding site motifs. Using site-directed mutagenesis, we deleted either the half palindromic NF1, MEF2, MEF3, PBX, E-box E1 or E-box E2 site [19] in LV-miniMg-Full (Fig. 1a). RT-qPCR for *eGFP* expression was used as a proxy to assay mutant *MYOGENIN* promoter activity for each construct in proliferating C25 myoblasts, differentiating C25 myoblasts and RH30 cells (Fig. 1e). Identifying a modification that would cause promoter activity to decrease in proliferating C25 myoblasts compared to LV-miniMg-Full eliminated LV-miniMg-ΔE2 and LV-miniMg-ΔMEF2. LV-miniMg-ΔPBX, LV-miniMg-ΔNF1 and LV-miniMg-ΔE1 each showed similar activity to LV-miniMg-Full in differentiating C25 myoblasts and RH30 cells, while LV-miniMg-ΔMEF3 was significantly reduced in both, and LV-miniMg-ΔMEF2 was enhanced in differentiating C25 myoblasts (Fig. 1e).

To confirm promoter activity at the protein level, we also measured *eGFP* fluorescence normalised to *mCherry* fluorescence (Fig. 1f). The LV-miniMg-ΔE1 promoter was excluded due to significantly increased expression in proliferating C25 myoblasts and unchanged activity in differentiating C25 myoblasts. LV-miniMg-ΔMEF3 and LV-miniMg-ΔNF1 promoters each showed significantly reduced expression in differentiating C25 myoblasts, while the LV-miniMg-ΔNF1 promoter had increased activity in RH30 cells. The effect of each mutation is summarised in Fig. 1g.

The reduction of *MYOGENIN* promoter activity after deletion of the MEF3 motif was expected [31], but the role of NF1 in ARMS is little known. NF1B is the only NF1 isofrom identified as a putative target gene of PAX3-FOX01 [32]. We performed SiRNA-mediated knockdown of NF1B to test if this would affect MYOGENIN expression. To test the trend that we see in our LV-miniMg reporter experiments. Indeed, when NF1B was knocked down, there was a significant increase in the proportion of RH30 cells containing MYOGENIN (Fig. 1h), while NF1B knockdown significantly reduced the proportion of MYOGENIN positive differentiating C25 myoblasts (Fig. 1i). This confirms that NF1B is differently involved in the upstream regulation of MYOGENIN expression in healthy myoblasts and RH30 cells.

Thus, removal of the NF1 or MEF3 DNA binding motif in the minimal human *MYOGENIN* promoter generates promoters with enhanced specificity for RH30 ARMS cells over healthy human C25 myogenic cells, with minimal activity in proliferating C25 myoblasts.

**Custom promoter driving HSV-TK decreases cell viability more in ARMS cells than myogenic cells**

Since deletion of the NF1 or MEF3 DNA binding domain in the minimal human *MYOGENIN* promoter enhanced specificity for RH30, we also generated a promoter version with both the NF1 and MEF3 sites removed (LV-miniMg-ΔMEF3/NF1). To test if those modified human *MYOGENIN* promoters could drive HSV-TK expression efficiently in RH30 cells, we generated new constructs with the promoters driving HSV-TK in cis with a CMV promoter driving *eGFP* (Fig. 2a). We then assessed mean viability of RH30 expressing LV-miniMg-Full-HSV, LV-miniMg-ΔNF1-HSV, LV-miniMg-ΔMEF3-HSV, LV-miniMg-ΔMEF3/NF1-HSV or LV-ΔminiMg-HSV (LV backbone lacking a promoter).

To first identify the optimal concentration of GCV, RH30 cells transduced with LV-miniMg-Full-HSV were treated with GCV (10 to 0.01 μg/ml) for 72 h and cell
viability measured with a RealTime MT Glo cell viability assay every 24 h (Fig. 2b). This identified 10 µg/ml GCV as the lowest concentration inducing a significant decrease in cell viability.

The effect of all HSV-TK constructs on cell viability was then analysed in parallel using 10 µg/ml GCV for 72 h. Mean cell viability was significantly reduced as early as 24 h after GCV treatment by most promoters except the LV-ΔminiMg-HSV control. After 72 h, LV-ΔminiMg-ΔMEF3/NF1-HSV was found the most effective at reducing cell viability, suggesting that the deletion of both transcription binding motifs rendered the promoter highly active in RH30 cells (Fig. 2c). In C25 myoblasts, LV-ΔminiMg-ΔMEF3 or LV-ΔminiMg-ΔNF1 significantly decreased viability after 24 h (Fig. 2d). Seventy-two hours after GCV treatment, although myoblast viability was reduced in all groups, LV-ΔminiMg-ΔMEF3/NF1-HSV showed the least effect, showing that deletion of these two DNA binding motifs further reduced promoter activity in skeletal myoblasts.

HSV-TK induces apoptosis in response to GCV, and to confirm that reduced cell viability was indeed due to apoptosis, we quantified Annexin 5 exposed on the cell membrane.

Fig. 1 ARMS promoter created by deletion of the MEF3 or NF1 DNA binding site. a Schematic of the lentiviral construct where the minimal human MYOGENIN promoter (miniMg) drives eGFP while CMV drives mCHERRY. Transcription factor binding motifs in the MYOGENIN promoter, together with the deletion constructs, are shown. b Representative images of LV-miniMg-transduced C25 myoblasts and RH30 cells. c, d RT-qPCR for endogenous MYOGENIN and eGFP as proxy for minimal MYOGENIN promoter activity in C25 myoblasts, myocytes and myotubes, and proliferating RH30 cells. N = 3, with significant differences, assessed using a student’s t-test as indicated by bars. e RT-qPCR for eGFP to measure activity of the various mutant MYOGENIN promoters with motif deletion in proliferating and differentiating C25 and proliferating RH30, N = 3, statistical difference assessed using a One-Way ANOVA with a Dunnett’s post hoc test comparing all samples to control LV-miniMg. f Fluorescence measurement of eGFP protein to measure mutant promoter activity in proliferating and differentiating C25 and proliferating RH30, N = 3–4, statistical difference assessed using a one-way ANOVA with a Dunnett’s post hoc test comparing all samples to control LV-miniMg-Full: ‘+’ indicates a significant increase in promoter activity, ‘−’ a significant decrease, ‘=’ no observed change, ‘n.a.’ is not assessed. Red indicates the exclusion of the promoter from further study, while green indicates inclusion. h Immunolabelling for MYOGENIN in RH30 cells after SiRNA-mediated knockdown of NF1B (SiNF1B), with quantification of the proportion of MYOGENIN positive nuclei/total nuclei per unit area. i Immunolabelling for MYOGENIN in two day differentiated C25 myoblasts after SiRNA-mediated knockdown of NF1B, with quantification of the proportion of MYOGENIN positive nuclei/total nuclei per unit area. N = 3, statistical significance assessed using a student’s t-test comparing SiNF1B to SiCtrl groups. Data expressed as mean ± SD. Scale bar equals 100 µm.
membrane via a RealTime-Glo™ Annexin V Apoptosis assay. We measured the samples regularly until the observed apoptotic signal increased over background threshold values, and then measured 1 and 24 h after this time-point. LV-miniMg-Full-HSV and LV-miniMg-ΔMEF3/NF1-HSV caused a significant increase in apoptosis between 1 and 24 h in RH30 cells. Importantly, LV-miniMg-ΔMEF3/NF1 did not change apoptosis in C25 myoblasts, while control LV-miniMg-Full-HSV induced high levels (Fig. 2e). Thus, our ΔMEF3/NF1 MYOGENIN promoter is more efficient at inducing apoptosis in RH30 cells than in C25 myoblasts, and can deliver HSV-TK differentially between ARMS and myoblast cells, causing apoptosis in ARMS.

**LV-miniMg-ΔMEF3/NF1-HSV driven suicide therapy specifically reduces cell number in multiple ARMS cell lines**

Having identified LV-miniMg-ΔMEF3/NF1-HSV as an effective promoter, we next tested in further cell lines: namely the immortalized RH41 ARMS line [33] and human 16U myoblasts [34], in parallel with RH30 and C25 cells. Lines were transduced with LV-ΔaminMg-HSV (negative control), LV-miniMg-Full-HSV (positive control) and LV-miniMg-ΔMEF3/NF1-HSV, then incubated with 10 µg/ml GCV and counted 24 and 72 h post-treatment (Fig. 3a). LV-ΔaminMg-HSV did not affect cell proliferation between 24 and 72 h in any cell line, while LV-miniMg-Full-HSV...
Fig. 3 LV-miniMg-A MEF3/NF1-HSV reduces cell number in ARMS lines but not in human myoblasts. a Schematic depicting experimental regime where ARMS (RH30, RH41) and myoblasts (C25, 16U) were transduced with LV-ΔminiMg-HSV, LV-miniMg-Full-HSV or LV-miniMg-ΔMEF3/NF1-HSV, then treated with 10 µg/ml GCV and counted 24 and 72 h post treatment. b Most cell lines (except RH41) increased the mean cell number from 24 to 72 h post-treatment with control LV-ΔminiMg-HSV. Cell numbers were unchanged where HSV-TK was under control of the miniMgfull-HSV promoter (except C25). Cell numbers were unchanged in RH30 and RH41 lines if the miniMg-ΔMEF3/NF1 promoter drove HSV-TK expression, while cell numbers increased in both myoblast lines C25 and 16U. Dashed line shows mean cell number of the control group at 24 and 72 h. N = 4, statistical significance assessed using a student’s t-Test comparing cell numbers between 24 and 72 h. c RH30 transduced with the LV-miniMg-ΔMEF3/NF1-HSV, were treated with 10 µg/ml GCV or vehicle control and gene expression analysed 72 h later. d Mean expression of HSV-TK, and cell cycle regulators CCND1 and CDKN1A (encodes P21) and (e) expression of myogenesis markers MYOGENIN, MYOMAKER and MYHC. N = 3, statistical differences were assessed using a student’s t-Test, comparing expression values between vehicle control and samples treated with 10 µg/ml GCV. All data are expressed as mean ± SD.
induced either no change in cell numbers from 24 to 72 h (RH30, RH41, 16U) or even a significant reduction (C25). Crucially, LV-miniMg-ΔMEF3/NF1-HSV differentially affected human cancer cells and myoblasts, with reduced proliferation in the RH30 and RH41 ARMS cell lines (Fig. 3b). However, there were no changes in cell numbers in the C25 and 16U myoblast lines between 24 and 72 h (Fig. 3b). Thus, the introduced modifications in the LV-miniMg-ΔMEF3/NF1 promoter reduced toxicity of the suicide gene therapy for human skeletal muscle myoblasts (Fig. 3b).

To directly measure changes in gene expression due to GCV treatment, RH30 cells were transduced with LV-miniMg-ΔMEF3/NF1-HSV, treated with either 10 µg/ml GCV or vehicle control, and gene expression quantified by RT-qPCR after 72 h (Fig. 3c). Mean expression of HSV-TK remained robust after treatment with GCV, suggesting that an HSV-TK positive population still exists after 72 h and so a longer treatment period could see a further reduction of cell viability (Fig. 3d). There was a significant upregulation of CCND1 and a trend towards higher CDKN1A (encodes P21) mean expression in response to GCV treatment, indicating that treated cells are arrested in the G1 phase before induction of apoptosis (Fig. 3d). High levels of P21 and arrest in G1 phase are also signs of myogenic differentiation, so we quantified gene expression for markers of myogenic differentiation: MYOGENIN to assess early stages of differentiation, MYOMAKER to evaluate cell fusion and MYHC to measure terminal differentiation. MYOGENIN, MYOMAKER and MYHC were all unchanged between treated and untreated cells (Fig. 3e). Thus, our proposed therapy causes a reduction in cell viability without induction of a more differentiated phenotype in remaining cells.

**RH30-derived mouse xenograft tumour size is reduced by the LV-miniMg-ΔMEF3/NF1 suicide gene**

To evaluate the LV-miniMg-ΔMEF3/NF1-HSV suicide gene in vivo we xenografted RH30 cells stably expressing LV-ΔminiMg-HSV, LV-miniMg-Full-HSV or LV-miniMg-ΔMEF3/NF1-HSV subcutaneously into the right flank of Swiss Nude mice. Once tumours reached ~300 mm³, the mouse was treated with daily intraperitoneal injections of 1 mg of GCV from the next day for 8 days and the tumour excised one day after the final GCV dose (day 9) and measured and weighed (Fig. 4a).

RH30 cells formed visible and palpable tumours in most mice, but over a variable timescale for all groups. At the end of the GCV treatment, tumour size was significantly increased in the control group (LV-ΔminiMg-HSV), while there was no measurable difference in size in either LV-miniMg-Full-HSV or LV-miniMg-ΔMEF3/NF1-HSV groups (Fig. 4b, c). The weight of excised tumours was also significantly reduced by LV-miniMg-Full-HSV or LV-miniMg-ΔMEF3/NF1-HSV, compared to control LV-ΔminiMg-HSV (Fig. 4d).

While the mean weight of mice in each group was unchanged during the administration of GCV (Fig. 4e), there was a significant difference in the mean change of weight during this period (Fig. 4f). Mice where the GCV administration reduced mean tumour weight (LV-miniMg-Full-HSV and LV-miniMg-ΔMEF3/NF1-HSV) also showed a net reduction in mean body weight during the treatment period, while control mice with LV-ΔminiMg-HSV demonstrated a mean gain in body weight (Fig. 4f).

To confirm that apoptosis occurs at a higher rate in the LV-miniMg-ΔMEF3/NF1-HSV sample group compared with the LV-ΔminiMg-HSV control, we performed TUNEL staining on cryosections from excised tumours. Accumulation of DNA fragmentation was strongly visible in the LV-miniMg-ΔMEF3/NF1-HSV group, while very little was detected in the LV-ΔminiMg-HSV control group. TUNEL staining in the LV-miniMg-ΔMEF3/NF1-HSV samples was often localized to specific areas (Fig. 4g and Supplementary Fig. 1). RT-qPCR of mRNA isolated from six independent tumour lysates revealed approximately twofold more BAX protein in LV-miniMg-ΔMEF3/NF1-HSV tumours compared to LV-ΔminiMg-HSV controls (Fig. 4h and Supplementary Fig. 1). RT-qPCR of mRNA isolated from six independent tumour lysates showed significantly higher expression of BAX too (Fig. 4i).

To investigate whether tumour cells were present after the 8 day treatment protocol that could still respond to GCV, we assayed HSV-TK expression, which was significantly increased in LV-miniMg-ΔMEF3/NF1-HSV tumour samples compared with LV-ΔminiMg-HSV controls (Fig. 4i). In summary, the LV-miniMg-ΔMEF3/NF1 promoter can drive HSV-TK expression in vivo to slow/prevent tumour growth.

**The LV-miniMg-ΔMEF3/NF1-HSV/GCV regime allows lowering of chemotherapy dose**

Treating any solid tumour remains a challenge due to low drug penetrance and tumour cell heterogeneity that allows a population of resistant tumour cells to survive treatment and re-initiate tumour growth [36]. The HSV-TK/GCV system has the benefit of the bystander effect, where cells in close proximity to HSV-TK expressing cells are also targeted through the movement of phosphorylated GCV through gap junctions into non-expressing cells, so increasing
Fig. 4 LV-miniMg-ΔMEF3/NF1-HSV reduces ARMS tumour volume and weight via apoptosis in a xenograft mouse model. a Schematic depicting experimental regime where RH30 stably transfected with LV-ΔminiMg-HSV, LV-miniMg-HSV or LV-miniMg-ΔMEF3/NF1 were subcutaneously xenografted in the right flank of Swiss Nude mice to generate tumours. Once tumours reached 300 mm³, GCV was administered to the mice via IP injection for 8 days and tumours excised. b Representative images of mice just before tumours were excised and the tumours recovered. c Tumour volume measured with callipers a day before GCV administration and after 8 days of treatment. N = 4–8, statistical differences were assessed using a student’s t-test comparing values at days 0 and 10 in each group. d Excised xenografted tumour weight after 8 days of daily GCV administration. N = 4–8, significant differences were assessed using a one-way ANOVA with Dunnett’s post hoc test comparing each group to the LV-ΔminiMg-HSV control. All data are expressed as mean ± SD. g Representative images of TUNEL assays on tumour cryosections derived from LV-miniMg-ΔMEF3/NF1-HSV and LV-ΔminiMg-HSV samples. TUNEL+ cells are in red; nuclei are counterstained with DAPI. N = 3 mice per group. h Western blot for BAX and β-TUBULIN of tumour lysates from 3 LV-miniMg-ΔMEF3/NF1-HSV mice and 3 LV-ΔminiMg-HSV mice, with quantification of band intensity of BAX relative to β-TUBULIN, expressed as fold change of LV-miniMg-ΔMEF3/NF1-HSV to LV-ΔminiMg-HSV. Statistically significant differences were assessed with an unpaired student’s t-test. i RT-qPCR of BAX and HSV-TK mRNA expression in LV-miniMg-ΔMEF3/NF1-HSV and LV-ΔminiMg-HSV tumours relative to RPLP0. N = 3 for each group, statistical differences were assessed with an unpaired student’s t-test. Scale bar equals 1 cm (b) or 100 µm (g).
motifs had enhanced speci-
MYOGENIN
speci-
MYOGENIN
promoter lacking both the NF1 and MEF3 binding
GENIN
myoblasts, and not in other cell types. The minimal
MYOGENIN
promoter was selected, since
the need for novel therapeutic approaches.

After 24 h of treatment, viability of LV-miniMg-Full-
HSV expressing RH30 cells was assessed and compared to
low treatment control (0 µM VAC and 1 µg/ml GCV) (Fig. 5a).
Cell viability was significantly decreased at nearly all con-
centrations of VAC (0, 0.4, 1.9 µM) if combined with 20 µg/
ml GCV, compared to 1 µg/ml GCV (Fig. 5b). The
decreased cell viability of LV-miniMg-Full-HSV express-
ing RH30 cells was identical when treated with either
9.2 µM VAC or with 0.4 µM VAC and 20 µg/ml GCV
(34.8% decrease vs 31.9%).

We next compared 9.6 µM VAC with no treatment and
the concentrations with the highest additive effect (0.4 µM
VAC + 20 µg/ml GCV) on RH30 cells expressing HSV-TK
under control of three different promoters. Quantification
revealed that in LV-miniMg-Full-HSV or LV-miniMg-
ΔMEF3/NF1-HSV groups, RH30 cell numbers dropped significantly if cells were treated with 9.6 µM VAC or
0.4 µM VAC and 20 µg/ml GCV, compared to no treatment
control (Fig. 5c–e). RH30 cells expressing HSV-TK under
the LV-ΔminiMg-HSV showed significantly reduced cell
numbers after treatment with 9.6 µM VAC compared to the
no treatment control, but treatment with 0.4 µM VAC and
20 µg/ml GCV had no effect (Fig. 5f). In conclusion, che-
motherapy concentration can be lowered by a factor of 24
from 9.2 to 0.4 µM when supplemented with 20 µg/ml GCV
in RH30 cells expressing LV-miniMg-Full-HSV or LV-
miniMg-ΔMEF3/NF1-HSV. Thus the concentration of che-
motherapy can be significantly lowered to reduce the burden
to the patient, if paired with our suicide gene therapy.

Discussion

Treatment options and survival rates have recently
increased for rhabdomyosarcoma, however survival rate for
patients affected with the fusion-positive variant of ARMS
is still <30% five years after diagnosis [38]. This highlights
the need for novel therapeutic approaches.

To target ARMS tumour cells, the human minimal
MYOGENIN
promoter was selected, since MYOGENIN
is constitutively expressed in rhabdomyosarcoma cells, but
only during myogenic differentiation in skeletal muscle
myoblasts, and not in other cell types. The minimal MYO-
GENIN
promoter lacking both the NF1 and MEF3 binding
motifs had enhanced specificity for ARMS over skeletal
muscle myoblasts/myotubes.

The NF1 and MEF3 motifs are implicated in the activ-
ation of the skeletal muscle-specific human aldolase A
alternative promoter pM [39]. The NF1B isoform is a
putative downstream target of PAX3-FOXO1 [32], and we
found that NF1B knockdown had opposite effects on
MYOGENIN expression in C25 myoblasts and RH30 cells.
While the role of NF1B in skeletal myogenesis or rhabdo-
myosarcoma is undescribed, it is highly expressed in mul-
tiple cancer types, including small cell lung cancer [40],
melanoma [41], squamous cell carcinoma [42] and neu-
roendocrine carcinoma [43]. NF1B acts as a transcriptional
activator or repressor, dependent on cellular context or reg-
ulatory region [44], and has divergent roles during the
development, maintenance and differentiation of stem cells. It
is not unsurprising then that it plays divergent roles in the
regulation of gene expression in myoblasts versus RH30 cells.

The importance of the MEF3 motif in the regulation of
MYOGENIN is well-described, as it is bound by SIX1 and
SIX4, which together with MYOD, facilitate MYOGENIN
expression during skeletal myogenesis [31]. Removal of the
MEF3 site from the MYOGENIN reporter reduced reporter
activity significantly in differentiating myoblasts. Interest-
ingly removal of either the NF1B or MEF3 binding motif
alone from the minimal MYOGENIN promoter was insuf-
ficient to abrogate HSV-TK activity in C25 human myo-
blasts, but the deletion of both motifs together enabled this.
Effects could be indirect, as promoter shortening could reduce
the accessibility of the DNA to other regulatory proteins.
However, reduced toxicity in C25 myoblasts compared to
RH30 cells suggests that regulation of this mutant human
MYOGENIN promoter differs between cell types. Additional
fine-tuning of the promoter sequence might reduce the low
levels of activation in healthy myoblasts even further.

Forcible conversion of cells from proliferation to post-
mitotic differentiation is a viable strategy for rhabdomyo-
sarcoma treatment. As RH30 cells share characteristics with
skeletal myoblasts, our suicide gene could also potentially
induce differentiation, as well as apoptosis. However, dif-
ferentiation markers including MYOGENIN, MYOMAKER
and MYHC were unchanged in response to treatment of
RH30-LV-miniMg-ΔMEF3/NF1-HSV with GCV. Increased
CCND1 transcripts, possibly due to the accumu-
lation of cells in G1, together with increased P21 transcript,
suggests that cells are inducing apoptosis. Together with the
increase of Annexin 5 exposed on the cell surface, this
strongly suggests that our suicide gene therapy is killing
tumour cells.

Apoptosis was also detected in vivo after treating RH30-
LV-miniMg-ΔMEF3/NF1-HSV with GCV, confirming that
it drives HSV-TK expression strongly enough, as also
shown by the significantly smaller tumours observed.
HSV-TK causes apoptosis through generating unrepairable
DNA double-strand breaks [45], and in accordance with
such DNA damage-induced apoptosis, a significant increase of BAX expression was observed in the LV-miniMg-ΔMEF3/NF1 samples. HSV-TK was still strongly expressed after the treatment period, and it would be of interest to understand if that indicates a higher success is achievable with longer treatment, or that a resistant cell population
remains after the initial treatment, as happens in response to various chemotherapeutic treatment regimens [46].

A major goal was to design a promoter specific for rhabdomyosarcoma cells over myogenic cells, and while we increased rhabdomyosarcoma specificity with our ΔMEF3/NF1 MYOGENIN promoter, there remains residual toxicity in myoblasts. Normally, a limitation of the HSV-TK1/GCV system is its ability to only affect dividing cells, but this is an advantage for muscle tissue, which is composed of mature, terminally differentiated post-mitotic skeletal muscle fibres, so refractory to the suicide gene. ARMS usually affects pediatric patients at an age when satellite cell-derived myoblasts will be required for muscle fibre growth/hypertrophy. The minimal activity of our mutant human MYOGENIN promoter in myoblasts may be a concern, but any corresponding toxic effect should only be exerted on myoblasts as they enter myogenic differentiation, leaving mature muscle unaffected. Chemo/radiotherapy also affects dividing cells though, but are used in children/adolescents where there are no alternatives. Targeting the therapy to tumours and adjusting dosing regimens could mitigate off-target effects on skeletal muscle.

Both viral and non-viral targeting options exist for gene delivery. Liposomal nanoparticles can deliver SiRNA against PAX3-FOXO1 efficiently into in vivo ARMS models, delaying tumour initiation/growth, but failed to induce apoptosis [47]. Targeting peptides have been developed that show specificity to RH30 [48], and these could modify liposomal carriers to enhance specificity. Among other factors, effective delivery of plasmid DNA into the nucleus is dependent on the size of the pDNA. We could reduce size by removing LV sequences to only retain the modified MYOGENIN promoter driving HSV-TK. An NLS could further enhance nuclear import [49]. Adeno-associated viruses (AAVs) with high tropism for muscle such as AAV2 or AAV9 could also be promising candidates for delivery. Inclusion of a tumour targeting sequence (e.g. NGRAHA, containing the NGR motif facilitating integrin binding) increases uptake by RD cells 10–20× compared to wildtype AAV2 [50]. Tumour cell selective killing by HSV-TK delivered by an AAV was successful for hepatocellular carcinoma [51] and oral squamous cell carcinoma [52] in mice, suggesting that AAV delivery is viable.

Another general challenge with cancer treatment is the low drug penetrance of tumour tissue [53]. The HSV-TK/GCV system benefits from a strong bystander effect, mediated through GAP-junctions of tightly connected cells, which extends treatment beyond primary transduced cells. HSV-TK shows a strong affinity towards thymidine though, requiring high concentrations of GCV to be administered. This may explain why we required a GCV concentration of ≥10 µg/ml to induce apoptosis, in the upper range used in vitro. Higher affinity substrates exist for HSV-TK, such as acyclovir [54]. Furthermore, HSV-TK efficiency differs between cell types [55], and HSV-TK mutants show varying efficiencies towards their substrate [56]. Fine-tuning HSV-TK variant and substrate should generate a combination to reduce potential side-effects of the HSV-TK/GCV system.

It is unlikely that any therapy could kill 100% of tumour cells, even with full tumour penetrance, as intra-tumoral heterogeneity often renders sub-populations of cells resistant. However, synergistic effects of treatments with HSV-TK/GCV occurs in murine and human colon carcinoma cells [57], where survival of nude mice was higher if GCV was used with the topoisomerase inhibitor topotecan. In addition, HSV-TK/GCV suppresses cell growth in chemo resistant K562 and THP-1 cells (leukaemia) [58]. Evaluating our suicide gene therapy with a standard chemotherapy regime (VAC) showed identical effects even if we reduced the chemotherapy concentration by >24× from 9.6 to 0.4 µM if supplemented with 20 µg/ml GCV.

In conclusion, we generated a modified human MYOGENIN promoter by deleting MEF3 and NF1 binding sites that has enhanced specificity for rhabdomyosarcoma over skeletal myogenic cells. Our LV-miniMg-ΔMEF3/NF1 promoter is capable of driving HSV-TK to levels sufficient to induce apoptosis in ARMS cells, but not myoblasts. Such a LV-miniMg-ΔMEF3/NF1 directed suicide gene therapy could also lower doses of chemotherapeutic agents if used in combination.

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**Author contributions** JP, PSZ, MG and MTM designed the research study and wrote the manuscript. JP, IH and PH conducted experiments and acquired and analysed data.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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