Soluble Heparin Binding Epidermal Growth Factor-Like Growth Factor Is a Regulator of GALGT2 Expression and GALGT2-Dependent Muscle and Neuromuscular Phenotypes

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ABSTRACT GALGT2 (also B4GALNT2) encodes a glycosyltransferase that is normally confined to the neuromuscular and myotendinous junction in adult skeletal muscle. GALGT2 overexpression in muscle can inhibit muscular dystrophy in mouse models of the disease by inducing the overexpression of surrogate muscle proteins, including utrophin, agrin, laminins, and integrins. Despite its well-documented biological properties, little is known about the endogenous regulation of muscle GALGT2 expression. Here, we demonstrate that epidermal growth factor receptor (EGFR) ligands can activate the human GALGT2 promoter. Overexpression of one such ligand, soluble heparin-binding EGF-like growth factor (sHB-EGF), also stimulated mouse muscle Galgt2 gene expression and expression of GALGT2-inducible surrogate muscle genes. Deletion analysis of the GALGT2 promoter identified a 45-bp region containing a TFAP4-binding site that was required for sHB-EGF activation. sHB-EGF increased TFAP4 binding to this site in muscle cells and increased endogenous Tfap4 gene expression. sHB-EGF also increased muscle EGFR protein expression and activated EGFR-Akt signaling. sHB-EGF expression was concentrated at the neuromuscular junction, and Hbegf deletion reduced Galgt2-dependent synaptic glycosylation. Hbegf deletion also mimicked Galgt2-dependent neuromuscular and muscular dystrophy phenotypes. These data demonstrate that sHB-EGF is an endogenous regulator of muscle Galgt2 gene expression and can mimic Galgt2-dependent muscle phenotypes.

KEYWORDS AAV, EGFR, GALGT2, HB-EGF, agrin, laminin, muscular dystrophy, neuromuscular junction, skeletal muscle

In a variety of neuromuscular disorders, the primary genetic defect can be overcome not only by gene replacement but also by overexpression of surrogate genes that encode proteins homologous in structure or function to the mutated gene. For example, overexpression of utrophin or integrin α7 can inhibit muscular dystrophy (MD) in the dystrophin-deficient mdx model of Duchenne muscular dystrophy (1–5), and overexpression of laminin α1, agrin, or laminin α4 can inhibit MD in the laminin α2-deficient dyw model of congenital muscular dystrophy 1A (6–8). We have studied a surrogate gene therapy called GALGT2 (also called B4GALNT2). When overexpressed in skeletal muscle, GALGT2 induces the ectopic overexpression of many surrogate genes and proteins, including utrophin, plectin 1, agrin, laminin α2, laminin α4, laminin α5, integrin α7, and integrin β1 (9, 10). The GALGT2 gene encodes β1,4-N-acetylgalacosaminyltransferase that creates the cytotoxic T cell (CT) glycan Neu5Aca2-3[GalNAcβ1-4][Galβ1-4GlcNAcβ-R] on glycoproteins, including α dystroglycan (11–14). Dystroglycan, which comprises α and β chains, is a muscle membrane protein that...
serves as an essential transmembrane link between the extracellular matrix (ECM) and the F-actin cytoskeleton (15, 16). GALGT2 overexpression increases expression of ECM proteins, including laminin α4, laminin α5, and agrin, increases ECM protein binding to α dystroglycan, and increases expression of cytoplasmic F-actin binding proteins that link the cytoskeleton to β dystroglycan, including dystrophin, utrophin, and plectin1 (9, 10, 17–20). In changing the molecular nature of the muscle membrane, GALGT2 overexpression protects both wild-type (WT) and dystrophin-deficient (mdx) skeletal myofibers from eccentric contraction-induced injury and can inhibit the development of muscular dystrophy in four different animal models of the disease (mdx, dyw, Sgca, and FKRP448L) (14, 18, 19, 21, 22). Many of the surrogate genes and proteins induced by GALGT2 are, like GALGT2, normally confined in expression to the neuromuscular junction (NMJ) and myotendinous junction (MTJ) in adult muscle (23–27). Similarly, like GALGT2, many of these genes and proteins are highly expressed in extrasynaptic muscle regions in the early postnatal period (23, 25, 26, 28–34). Thus, by drawing such proteins into an extrasynaptic expression pattern, GALGT2 is essentially recreating the molecular environment normally seen in a young muscle. As such, GALGT2 overexpression provides a means of understanding mechanisms controlling the expression of these therapeutic genes and proteins, with regard to not only muscle therapy but also normal muscle development.

Although the potential of GALGT2 gene therapy has been appreciated and successfully applied in neuromuscular disease models, regulation of endogenous Galgt2 gene expression in mouse muscle remains poorly understood. Given its potential as a therapeutic, a better understanding of the endogenous mechanisms controlling muscle Galgt2 expression, as well as the expression of GALGT2-inducible muscle surrogate genes and GALGT2-dependent muscle phenotypes, is highly warranted, and this is the goal of our current study.

RESULTS

EGFR ligands stimulate the human GALGT2 promoter in muscle cells. Using C2C12 myotube cultures stably expressing a bp −5870 GALGT2 promoter firefly luciferase (Luc2) reporter, we performed a screen of trophic factors to assess their ability to induce the GALGT2 promoter (Fig. 1A). Positives were also validated using C2C12 myotubes stably expressing a GALGT2 promoter Renilla luciferase (hRluc) reporter (not shown). Many trophic factors, including brain-derived neurotrophic factor (BDNF), glucagon-like peptide 2, ciliary neurotrophic factor (CNTF), nerve growth factor 2.5S (NGF 2.5S), leukemia inhibitory factor (LIF), human growth hormone (HGH), neurotrophin 3 (NT3), peptide YY, glial cell line-derived neurotrophic factor (GDNF), hepatocyte growth factor (HGF), calcitonin gene-related peptide (CGRP), somatostatin, and neu- regulin 1 (NRG1) did not increase luciferase signal when added for 24h. Concentrations chosen are described in Materials and Methods and exceed or equal the binding affinity of each ligand for their known signaling receptor. Ligands known to activate the epidermal growth factor receptor (EGFR), including soluble heparin-binding EGF-like growth factor (sHB-EGF), epidermal growth factor (EGF), amphiregulin, epiregulin, and transforming growth factor alpha (TGFα), all induced GALGT2 promoter activity, as did insulin and insulin-like growth factor 1 (IGF1) (Fig. 1A). We specifically tested sHB-EGF, as the full-length transmembrane form of HB-EGF does not activate EGFR until it is cleaved to generate sHB-EGF by ADAM proteases (35–38). Of the EGFR agonists, only HB-EGF and TGFα are appreciably expressed in skeletal muscle, with HB-EGF showing more specificity for muscle than TGFα, which is expressed in almost all tissues (39). sHB-EGF was able to activate the GALGT2 promoter in a dose-dependent manner, with saturation occurring at 10 nM (Fig. 1B). sHB-EGF only activated the GALGT2 promoter luciferase reporter in C2C12 cells if they were first differentiated into myotubes (not shown). Furthermore, in differentiated C2C12 cells, sHB-EGF increased the expression of endogenous Galgt2 mRNA levels by 10-fold relative to levels for untreated cells (Fig. 1C).
sHB-EGF activates the human GALGT2 promoter via a TFAP4-responsive element. We next wanted to define the DNA element or elements in the GALGT2 promoter responsible for driving elevated luciferase reporter expression in response to sHB-EGF. To do this, we created a series of deletions in the GALGT2 promoter (Fig. 2A). Deletions up to the last bp, bp −950, of the 5’ end of the promoter had no impact on the ability of sHB-EGF to activate luciferase. Deletions leaving only bp −500 or bp −200, however, significantly diminished promoter reporter activity (Fig. 2B), suggesting the 450-bp sequence between bp −950 and bp −500 responded to sHB-EGF. We next created five smaller deletions, each approximately 50 bp, within this 450-bp sequence. Of these, only the second deletion (950Δ2; deletion of bp −822 to −777) showed a significant decrease in luciferase signal (Fig. 2C). A motif analysis identified only two major transcription factor binding sites in the bp −822 to −777 region (Fig. 2D) (40). One site included two overlapping FOXD3 binding sites at bp −818 to −804 and bp −815 to −801, while the second site contained overlapping binding sites for TFAP4, LMO2, and myogenin (MYOG) (which binds at E boxes) at bp −798 to −781, bp −796 to −785, and bp −793 to −788, respectively.
We next assessed whether the transcription factors predicted to bind to the bp −822 to −777 region could activate the GALGT2 promoter when overexpressed. To do this, we obtained adenovirus (Ad) constructs that overexpress MYOG, FOXD3, LMO2, or TFAP4. Ad-GFP (green fluorescent protein) was used as a negative control. C2C12 myotube cultures stably expressing the bp −950 GALGT2 promoter firefly luciferase reporter were treated with each Ad vector either in the presence or absence of sHB-EGF. Neither Ad-GFP, Ad-MYOG, nor Ad-FOXD3 significantly increased promoter activity (relative to untreated cells), while Ad-LMO2 and Ad-TFAP4 did, with Ad-TFAP4 showing the greatest activation (Fig. 3A). Ad-LMO2 and Ad-TFAP4 also significantly activated the bp −950 GALGT2 promoter in the presence of sHB-EGF relative to sHB-EGF alone (Fig. 3A). In contrast, Ad-LMO2 or Ad-TFAP4 was unable to significantly increase expression in the 950Δ2 construct (Fig. 3B).

We next used short interfering RNAs (siRNAs) targeting mouse Tfap4 or Lmo2 to knock down mRNA expression and then retest sHB-EGF induction (Fig. 3C). siRNA targeting Tfap4 significantly reduced GALGT2 promoter activity in sHB-EGF-treated cells (P < 0.01), while siRNA targeting Lmo2 and a scrambled Tfap4 control did not (Fig. 3C). The siRNA targeting Tfap4 knocked down endogenous Tfap4 gene expression in both resting and sHB-EGF-treated cells by 58% relative to the relevant control (Fig. 3D). Interestingly, sHB-EGF treatment alone induced Tfap4 gene expression 1.9- ± 0.3-fold (P < 0.001). siRNA targeting Lmo2 reduced Lmo2 gene expression by only 30%, and sHB-EGF did not induce endogenous Lmo2 gene expression (not shown). Interestingly, both Ad-TFAP4 and Ad-FOXD3 increased endogenous Galgt2 gene expression in C2C12 myotubes, while Ad-MYOG, Ad-GFP, and Ad-LMO2 did not (Fig. 3E). Ad-TFAP4, Ad-
LMO2, Ad-MYOG, and Ad-FOXD3 all induced expression for their respective (human) genes by 15- to 20-fold relative to levels for the same endogenous mouse gene, but Ad-FOXD3 also increased endogenous mouse Tfap4 expression by 2.1- ± 0.1-fold and decreased endogenous mouse Lmo2 expression to 0.3 ± 0.1 of normal levels. Thus, FOXD3 overexpression also led to increased expression of Tfap4 in C2C12 cells. As we...
could not detect any expression of Foxd3 in mouse muscle (not shown), we did not pursue this result further.

Lastly, we determined if sHB-EGF would induce binding of LMO2 or TFAP4 to the bp −951 to −726 region by performing chromatin immunoprecipitation (ChIP) of nuclear lysates from C2C12 myotubes before or after sHB-EGF addition (Fig. 3F). sHB-EGF increased TFAP4 occupancy to this site at 24 and 48 h after sHB-EGF addition. LMO2 binding, in contrast, showed the opposite pattern. Thus, sHB-EGF appears to induce a switch in occupancy of transcription factors in the bp −951 to −726 region of the GALGT2 promoter, increasing bound TFAP4 at the expense of bound LMO2.

**sHB-EGF activates an EGFR phosphorylation cascade in muscle cells.** To better characterize the sHB-EGF signaling pathway, we added inhibitors of various signaling pathways to the bp −5870 GALGT2 promoter reporter muscle cells along with sHB-EGF. All inhibitors were tested in a dose-response curve, and concentrations shown reflect maximal levels of inhibition. Inhibitors of EGFR (tyrophostin; AG-1478), a known sHB-EGF receptor, phosphatidylinositol 3 (PI3) kinase (LY294002), Akt kinase (MK-2206), mTOR (rapamycin), Raf kinase (vamurafenib), and extracellular signal-regulated kinase (ERK; SCH772984) all maximally inhibited sHB-EGF activation by more than 50%, while inhibitors of Src (KX2-391), Bcr/Abl (nilotinib), STAT5, c-Jun N-terminal kinase (JNK; SP600125), and c-myc (10058-F4) did not (Fig. 4A).

We next assessed sHB-EGF induction of EGFR, Akt, Erk1/2, MEK1/2, and C-Raf activity by comparing signals on Western blots using phosphospecific and protein-specific antibodies to each component (Fig. 4B to D). sHB-EGF addition caused a 20-fold increase in phosphorylation of EGFR (pEGFR [Tyr1173]/EGFR, 23.9 ± 1.3; P < 0.05) within 15 min compared to levels for untreated cells (Fig. 4B). Similarly, phosphorylation of Akt (pAkt [Ser473]/Akt, 19.7 ± 1.6; P < 0.001) and mTOR (pmTOR [Ser2448]/mTOR, 7.6 ± 1.7; P < 0.05) was increased (Fig. 4B), as was that of C-Raf (pC-Raf [Ser289/296/301]/C-Raf, 34.3 ± 3.8; P < 0.001), MEK1/2 (pMEK1/2 [Ser217/221]/MEK1/2, 6.9 ± 0.1; P < 0.001), and Erk1/2 (pErk1/2 [Thr202/Tyr204]/Erk1/2, 4.0 ± 0.3; P < 0.01) (Fig. 4C). Activation of all proteins was transient, with most protein phosphorylation reverting to near baseline levels by 60 min after treatment (Fig. 4B and C). Phosphorylation of c-Src was not significantly increased following sHB-EGF treatment (pSrc [Tyr416]/Src, 1.8 ± 0.4; P = 0.37) (Fig. 4D), consistent with the results of our inhibitor screen (Fig. 4A). These studies support the notion that sHB-EGF activates an EGFR-Akt/mTOR-Mek/Erk kinase signaling cascade in muscle cells (Fig. 4E).

**sHB-EGF induces endogenous expression of Galgt2 and Galgt2-inducible surrogate genes in skeletal muscle.** We next assessed the ability of sHB-EGF to induce Galgt2 transcription in mouse skeletal muscle tissue. To do this, we created a recombinant, self-complementary [r(sc)] adeno-associated virus (AAV) vector that would express either full-length human HB-EGF or active sHB-EGF. The AAV9 serotype of viral capsid was used, which allows for robust muscle transgene expression (41, 42). r(sc)AAV9.CMV.HB-EGF or r(sc)AAV9.CMV.sHB-EGF (5 × 10^{10} vector genomes [vg]) was injected into the gastrocnemius muscles of 8-week-old C57BL/6J mice, with the contralateral gastrocnemius receiving an equal volume of phosphate-buffered saline (PBS) as a control. Injected muscles showed 20,000 to 50,000 vg per μg of genomic DNA, while contralateral control muscles showed two or more logarithms less transduction (Fig. 5A). Because the rAAV9 serotype efficiently crosses the vascular barrier to transduce muscles throughout the body (43, 44), this modest amount of AAV vector was expected in the contralateral muscles even after a focal intramuscular injection, but the extent of transduction was extremely low (no more than 1 vg per 150 nuclei). Expression of sHB-EGF and HB-EGF was increased between 10- and 20-fold over endogenous mouse Hbegf expression at both 4 and 12 weeks after AAV injection (Fig. 5B). Overexpression of full-length, uncleaved HB-EGF was unable to significantly increase Galgt2 gene expression, while sHB-EGF increased Galgt2 expression more than 6-fold compared to that of mock-injected contralateral muscles (Fig. 5C). In addition to elevating Galgt2, sHB-EGF increased the expression of surrogate muscle genes that can
have a therapeutic impact on muscular dystrophy, including the utrophin (Utrn), agrin (Agrn), laminin α2 (Lama2), laminin α4 (Lama4), and integrin α1 (Itgb1) genes (9, 10), while full-length HB-EGF did not (Fig. 5C). Interestingly, neither shHB-EGF nor HB-EGF significantly upregulated expression of the dystroglycan (Dag1), dystrophin (Dmd), laminin α5 (Lama5), or integrin α7 (Itga7) gene (Fig. 5C), while GALGT2 overexpression can increase expression of these genes in muscle (10). Thus, HB-EGF must be cleaved to its active soluble form in skeletal muscle to increase endogenous Galgt2 transcription.
and can induce the expression of some, but not all, Galgt2-inducible therapeutic surrogate genes.

We next analyzed Galgt2 protein and CT glycan expression in sHB-EGF-injected muscles (Fig. 5D). sHB-EGF was expressed along the sarcolemmal membrane of skeletal myofibers in muscles analyzed at 4 weeks postinjection. CT glycan was highly upregulated along the sarcolemmal membrane in such fibers, as shown by costaining with sHB-EGF protein, while myofibers not overexpressing sHB-EGF did not have elevated CT glycan. As Galgt2 is a trans-Golgi protein (12), it would not be expected to be colocalized with sHB-EGF at the sarcolemmal membrane. Galgt2 protein staining was, however, highly elevated in many sHB-EGF-overexpressing myofibers (Fig. 5D), consistent with increased Galgt2 mRNA expression (Fig. 5C). As in cultured myotubes, sHB-EGF overexpression in skeletal muscle also increased Tfap4 gene expression (1.9 ± 0.1-fold).

sHB-EGF activates an EGFR-Akt pathway in skeletal muscle. Skeletal muscles treated with sHB-EGF for 4 weeks showed a significant increase in Akt phosphorylation (pAkt [Ser473]/Akt, 2.6 ± 0.2; *P < 0.05) but showed no significant change in phosphorylation for EGFR (pEGFR [Tyr1173]/EGFR, 0.5 ± 0.1; *P = 0.501), mTOR (pmTOR [Ser2448]/mTOR, 0.5 ± 0.1; *P = 0.074), C-Raf (pC-Raf [Ser289/296/301]/C-Raf, 1.5 ± 0.4; *P = 0.457), MEK1/2 (pMEK1/2 [Ser217/221]/MEK1/2, 0.7 ± 0.1; *P = 0.251), or Erk1/2 (pErk1/2
Expression of EGFR protein, however, was increased almost 5-fold after HB-EGF treatment (EGFR/GAPDH, 5.7 ± 0.6; P < 0.05) (Fig. 6A), as was mTOR protein (9± ± 5-fold). Thus, while the pEGFR/EGFR and pmTOR/mTOR ratios did not significantly change, elevated EGFR and mTOR protein expression meant pEGFR and pTOR levels also increased. Mouse Egfr gene expression was also significantly increased at both 4 and 12 weeks by sHB-EGF compared to that of the PBS-injected control (P < 0.001 for each) (Fig. 6B). Overexpression of full-length transmembrane HB-EGF, in contrast, did not significantly increase Egfr mRNA (Fig. 6B). sHB-EGF overexpression also caused a 2-fold increase in Erbb2 mRNA expression at 4 and 12 weeks postinjection, although this did not reach statistical significance (4 weeks, 2.2 ± 0.5; P = 0.13; 12 weeks, 2.2 ± 0.3; P = 0.13), while expression of Erbb3, Erbb4, and Akt1 did not change (Fig. 6B). We also immunostained untreated and sHB-EGF-treated muscles with antibodies to EGFR protein. In control muscle, EGFR staining was concentrated at the neuromuscular junction (NMJ), but staining was increased along extrasynaptic regions of the myofiber membrane after sHB-EGF treatment (Fig. 6C). Thus, some facets of EGFR activation and Akt signaling found in muscle cells were present after sHB-EGF overexpression in muscle tissue, but others (MEK1/2, Erk1/2, and C-Raf) were not.

sHB-EGF is required for Galgt2-dependent NMJ glycosylation. To stain for sHB-EGF in muscle, we used a function-blocking sHB-EGF-specific monoclonal antibody (MAb 2591). We confirmed the specificity of MAb 2591 by staining Chinese hamster ovary (CHO) cells transfected with a cDNA encoding the full-length transmembrane form of HB-EGF (208 amino acids [aa]) or with a cDNA encoding only sHB-EGF (148 aa). MAb 2591 stained sHB-EGF-transfected cells but not HB-EGF-transfected cells, while polyclonal antibody (PAb) 259 NA, an HB-EGF polyclonal antibody, recognized both

[Thr202/Tyr204]/Erk1/2, 0.9 ± 0.1; P = 0.669) (Fig. 6A). Expression of EGFR protein, however, was increased almost 5-fold after sHB-EGF treatment (EGFR/GAPDH, 5.7 ± 0.6; P < 0.05) (Fig. 6A), as was mTOR protein (9± ± 5-fold). Thus, while the pEGFR/EGFR and pmTOR/mTOR ratios did not significantly change, elevated EGFR and mTOR protein expression meant pEGFR and pTOR levels also increased. Mouse Egfr gene expression was also significantly increased at both 4 and 12 weeks by sHB-EGF compared to that of the PBS-injected control (P < 0.001 for each) (Fig. 6B). Overexpression of full-length transmembrane HB-EGF, in contrast, did not significantly increase Egfr mRNA (Fig. 6B). sHB-EGF overexpression also caused a 2-fold increase in Erbb2 mRNA expression at 4 and 12 weeks postinjection, although this did not reach statistical significance (4 weeks, 2.2 ± 0.5; P = 0.13; 12 weeks, 2.2 ± 0.3; P = 0.13), while expression of Erbb3, Erbb4, and Akt1 did not change (Fig. 6B). We also immunostained untreated and sHB-EGF-treated muscles with antibodies to EGFR protein. In control muscle, EGFR staining was concentrated at the neuromuscular junction (NMJ), but staining was increased along extrasynaptic regions of the myofiber membrane after sHB-EGF treatment (Fig. 6C). Thus, some facets of EGFR activation and Akt signaling found in muscle cells were present after sHB-EGF overexpression in muscle tissue, but others (MEK1/2, Erk1/2, and C-Raf) were not.

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sHB-EGF and HB-EGF (Fig. 7A). C18, an antibody to the C-terminal domain of HB-EGF (that only exists in the full-length HB-EGF), recognized HB-EGF but not sHB-EGF (Fig. 51A). In adult skeletal muscle, MAb 2591 stained NMJs (Fig. 7B). sHB-EGF staining coincided with H9251 staining, which stains nicotinic acetylcholine receptors (AChRs) in the postsynaptic muscle membrane (Fig. 7B) (45). sHB-EGF muscle staining was eliminated in mice lacking HB-EGF (Hbegf /−/−) (Fig. 7B). Staining for Galgt2 protein (Fig. 7C) and the CT glycan (Fig. 7D) was reduced, and sometimes eliminated, at NMJs in Hbegf /−/− muscles, suggesting a requirement, or partial requirement, of HB-EGF. Staining of Galgt2 protein and CT glycan remained present in intramuscular capillaries in Hbegf /−/− mice, providing an internal staining control (Fig. 7C and D).

Absence of sHB-EGF mimics Galgt2 /−/− neuromuscular junction and muscle phenotypes. Because Hbegf −/− muscles had reduced Galgt2 and CT glycan expression, we next determined if loss of Hbegf would mimic NMJ or muscle phenotypes found in Galgt2 /−/− or mdx:Galgt2 /−/− mice. Galgt2 /−/− muscles show increased fragmentation of postsynaptic AChRs and an increased localization of AChRs in clathrin-coated endosomes (46). The number of AChR microaggregates in the vicinity of the NMJ was also increased by an order of magnitude in 7-month-old Hbegf /−/− muscles (Fig. 8A and B). This was equivalent to the pattern and extent of subsynaptic AChR alterations found in 3-month-old Galgt2 /−/− muscles (46). Galgt2 is also a modulator of skeletal muscle disease severity in mdx mice (31). mdx:Galgt2 /−/− muscles show increased size and decreased strength compared to mdx muscles. Eight- to 12-month-old mdx:Hbegf −/− mice had increased skeletal muscle mass relative to mdx mice (Fig. 8C), much like mdx:Galgt2 /−/− muscles (31). Muscles in Hbegf −/− mice, in contrast, were not significantly different from those of the WT. Forelimb (Fig. 8D) and hindlimb (Fig. 8E) grip strength also trended lower in mdx:Hbegf −/− mice relative to that of mdx mice, although these changes did not reach statistical significance. Hbegf −/− forelimb grip strength was unchanged from that of the WT, while hindlimb grip strength was reduced. When run on a treadmill, Hbegf −/− mice were indistinguishable from WT mice (P > 0.999), while mdx mice showed reduced ambulation (P < 0.01 and P < 0.05 versus WT and Hbegf −/−, respectively) and mdx:Hbegf −/− mice were further significantly
**FIG 8** Hbegf<sup>-/-</sup> mice exhibit Galgt2<sup>-/-</sup> neuromuscular junction and muscle phenotypes. (A) Examples of increased AChR microaggregates at neuromuscular junctions in Galgt2<sup>-/-</sup> (2 panels) and Hbegf<sup>-/-</sup> (4 panels) muscles compared to levels for the wild type (2 panels). Tibialis anterior muscle is shown at 3 months of age for Galgt2<sup>-/-</sup> muscle and at 7 months of age for Hbegf<sup>-/-</sup> and WT muscle. Scale bar is 10 μm. (B) Quantification of AChR microaggregates in WT, Galgt2<sup>-/-</sup>, and Hbegf<sup>-/-</sup> muscle. WT (n = 45) and Hbegf<sup>-/-</sup> (n = 75) TA muscles were from 7-month-old mice, while Galgt2<sup>-/-</sup> (n = 37) TA muscles were from 3-month-old mice. Data are means ± SEM. ***, P < 0.001 by one-way ANOVA. (C) Mouse skeletal muscle weights were normalized to total body mass. Abbreviations: TA, tibialis anterior; Gastroc, gastrocnemius; Quad, quadriceps. (D to F) Mice participated in a week-long test of strength and endurance via forelimb grip strength (D), hindlimb grip strength (E), and total run time on a treadmill (F). For panels D and E, force is normalized to body weight in each animal. For panels D to F, data are means ± SEM for n = 6 (WT), n = 4 (mdx and mdx:Hbegf<sup>-/-</sup>), and n = 3 (Hbegf<sup>-/-</sup>) measures. *, P < 0.05; **, P < 0.01; ***, P < 0.001 by one-way ANOVA.
diminished relative to mdx mice (P < 0.001 versus mdx mice) (Fig. 8F). Thus, deletion of Hbegf in mdx mice mimicked dystrophic phenotypes of Galgt2 deletion. sHB-EGF overexpression, however, was very different than GALGT2 overexpression in mdx muscles under certain circumstances. For example, while GALGT2 overexpression can prevent muscle damage, can reduce fibrosis, can prevent loss of muscle force in skeletal muscles, and has an excellent safety profile at high doses (22, 47–49), systemic overexpression of sHB-EGF at postnatal day 1 in mdx and wild-type mice led to uniform lethality within 3 weeks of treatment (5 out of 5 each).

**DISCUSSION**

The induction of therapeutic surrogate genes and proteins to inhibit muscle damage associated with muscular dystrophy represents a major opportunity for researchers trying to improve muscle disease outcomes. GALGT2 gene overexpression is an attractive approach to inducing surrogate therapeutic genes and proteins because it can alter expression of multiple muscular dystrophy modifiers. Such genes include those encoding utrophin (9, 10, 21), which can inhibit muscular dystrophy in the mdx mouse model of Duchenne muscular dystrophy (DMD) (1–3), agrin (9, 10, 18), which can inhibit muscular dystrophy in the dy/md mouse model of laminin α2-dependent congenital muscular dystrophy (or MDC1A) (8), plectin 1 (9, 19), which when deleted increases the severity of muscular dystrophy in mdx mice (50) and itself causes muscular dystrophy (with epidermolysis bullosa) (51), laminin α4 and α5 (9, 10), which can diminish muscular dystrophy caused by deficits in laminin α2 (7, 52), and integrin α7, which can inhibit muscular dystrophy in mdx mice (4, 53). Endogenous regulation of these therapeutic genes and proteins in skeletal muscle, however, remains poorly understood, which is why we undertook the current studies focused on regulation of endogenous muscle Galgt2 gene expression.

While studies of nerve-derived signals that induce expression of synaptic muscle genes have been fairly extensive, little is known about how muscle-derived factors might induce synaptic gene expression, particularly in the postnatal period when Galgt2 expression becomes confined to the synapse. Neuregulin 1 (NRG1), for example, is a nerve-derived signal that induces synaptic gene expression in embryonic skeletal muscle, including expression of nicotinic acetylcholine receptor genes (54). Galgt2, along with other synaptic proteins, such as utrophin, agrin, laminin α4, and laminin α5, becomes localized to the NMJ in skeletal muscle 2 to 3 weeks after birth (23, 25, 26, 29–31, 34), a later developmental epoch than is seen for many NRG1-regulated genes (55, 56). We identified a group of EGFR ligands (57, 58), including EGF, sHB-EGF, amphiregulin, epiregulin, and TGFα, that stimulated muscle Galgt2 gene expression. NRG1, which does not bind the EGFR but instead binds EGFR family members ErbB3 and ErbB4 (57, 58), in contrast, did not stimulate muscle Galgt2 expression. Similar to NRG1 receptors ErbB3 and ErbB4 (59), EGFR is localized to the NMJ in adult skeletal muscle, a finding first described by Sanes and colleagues (60) and confirmed in this study. Thus, EGFR ligands and EGFR may be involved in the localization of genes (and proteins) to the NMJ in the postnatal period, akin to NRG1’s role in embryonic muscle.

Our studies suggest that sHB-EGF controls important postnatal muscle gene expression programs and has important roles in muscle biology. Overexpression of sHB-EGF in skeletal muscle not only induced expression of mouse muscle Galgt2 expression but also upregulated the expression of utrophin, laminin α2, laminin α4, and integrin β1. Overexpression of full-length HB-EGF, in contrast, did not produce these changes, confirming the importance of HB-EGF cleavage to its soluble sHB-EGF form to mediate EGFR signaling (35, 36, 61). Interestingly, Engvall and colleagues have shown that transgenic overexpression of ADAM12, which can cleave transmembrane HB-EGF protein to create active sHB-EGF, is therapeutic in mdx mice and can increase integrin α7 and utrophin expression (62, 63). Those results may be related to the mechanism we have described here. sHB-EGF was concentrated at the NMJ in adult muscle, and deletion of mouse Hbegf decreased synaptic expression of muscle Galgt2 protein and the CT glycan, which is made by Galgt2 (12, 46, 64). Similarly, deletion of Hbegf led to
increased fragmentation of postsynaptic acetylcholine receptors to a degree seen with deletion of Galgt2 (46), albeit on a slightly different time scale. Moreover, in mdx mice, Hbegf deletion modulated muscle disease phenotypes in a manner similar to deletion of Galgt2. Such phenotypes included increases in muscle hypertrophy and muscle weakness with reduced ambulation. Not all phenotypes, however, were matched between Hbegf and Galgt2. For example, perinatal systemic overexpression of shHB-EGF was lethal in mdx mice, a finding not found with GALGT2 (14). While this may be due to the use of different promoters in these experiments (cytomegalovirus [CMV] for shHB-EGF and MCK for GALGT2), HB-EGF has additional well-described functions not reported with GALGT2. For example, Hbegf-deficient mice have extensive phenotypes related to cardiac development (61, 65, 66), cardiac hypertrophy (66, 67), tumor cell growth and angiogenesis (57, 68), embryo implantation and placental development (69–71), cognitive function and synaptic plasticity (72, 73), tissue fibrosis (74–77), and intestinal health and regeneration after injury (78–82). Some of these phenotypes have been not reported in Galgt2−/− mice, although Galgt2 deletion can give rise to blood, cardiac, pregnancy, and tumor-related phenotypes (14, 31, 83–85). Similarly, GALGT2 overexpression in skeletal muscle induced the expression of some surrogate genes not induced by shHB-EGF, including Lama5 and Itga7, while other surrogate genes were induced by both.

Most of the functions described for shHB-EGF correlate with its ability to activate EGFR and downstream pathways, including PI3 kinase, Akt, mTOR, ERK, and MEK, as well as cross talk with IGF receptor 1 (IGFR1) signaling (58, 86). While we have not delineated the entire signaling pathway in muscle, we have shown that shHB-EGF activates the muscle EGFR signaling and increases Akt phosphorylation. shHB-EGF also increases muscle EGFR gene and protein expression, thereby causing the overexpression of its own receptor. This no doubt facilitates the ability of shHB-EGF to induce overexpression of other normally synaptic therapeutic genes in the extrasynaptic muscle membrane. Importantly, while shHB-EGF induces increased Mek1/2 and Erk1/2 phosphorylation in muscle cells, it did not do so in muscle tissue. This suggests that additional regulatory factors or mechanisms are involved in shHB-EGF signaling in vivo that are not involved in shHB-EGF signaling in cultured muscle cells.

We have also shown that addition of shHB-EGF to muscle cells stimulates increased occupancy of the GALGT2 promoter by TFAP4. TFAP4 overexpression is sufficient to activate the GALGT2 promoter and requires a specific binding site to do so. LMO2 overexpression could also stimulate the same site on the GALGT2 promoter, but it was less potent than TFAP4. Intriguingly, addition of shHB-EGF to muscle cells decreased LMO2 occupancy at the same time that it increased TFAP4 occupancy in ChIP experiments. As such, shHB-EGF may increase GALGT2 expression by activating a switch from a less active transcription factor (LMO2) to a more active one (TFAP4). shHB-EGF also increased endogenous Tfap4 gene expression, which may further facilitate increased TFAP4 occupancy on the GALGT2 promoter. Like shHB-EGF, TFAP4 overexpression induced mouse Galgt2 gene expression in muscle cells. Surprisingly, FOXD3 overexpression also did this. FOXD3, however, may act via TFAP4, as FOXD3 overexpression also increased endogenous mouse Tfap4 gene expression (while decreasing Lmo2). Also, unlike that of Tfap4, FoxD3 expression was not detectable in adult mouse skeletal muscle. The fact that there is also an E-box within the TFAP4/LMO2 binding site suggests that myogenin (MYOG) also activates GALGT2 expression, but we found no inductive effects from myogenin overexpression. Goldman, Yao, Schaeffer, and others have previously shown that myogenin protein is downregulated in skeletal muscle by muscle activity in a calcium- and histone deacetylase (HDAC) 4- and/or 9-dependent manner (87–92). As such, one would not expect appreciable myogenin to be present in extrasynaptic regions of a functioning muscle cell, where shHB-EGF was overexpressed in our experiments. The activation of TFAP4, however, may provide a mechanism for late-developing synaptic genes to maintain gene expression even in the presence of muscle activity by activating transcription from sites that might normally be occupied by myogenin. TFAP4 may also offset suppression of GALGT2 expression by promoter
methylations, as is known to occur in gastrointestinal cancers and cancer cell lines (93, 94).

These experiments provide the first evidence that endogenous muscle proteins can activate Galgt2 gene expression in a manner that mimics its own ability to induce downstream therapeutic genes that can inhibit forms of muscular dystrophy. This work should provide avenues to understanding how to exploit endogenous muscle signaling pathways to induce expression of therapeutic muscle genes for these disorders.

MATERIALS AND METHODS

Mice. All mice were kept and used in accordance with protocols approved by the Institutional Animal Care and Use Committee at The Research Institute at Nationwide Children’s Hospital (Columbus, OH). C57BL/6J and mdx mice were purchased from Jackson Laboratories (Bar Harbor, ME). Hbegf−/− mice were kindly provided by Gail Besner (The Research Institute at Nationwide Children’s Hospital, Columbus OH). Hbegf−/− mice were bred with mdx mice to create stable double knockout mice (mdx:Hbegf−/− mice). Galgt2−/− mice were provided by the Consortium for Functional Glycomics and originally generated by John Lowe (Genentech, South San Francisco, CA).

Cell lines. The mouse C2C12 cell line was grown in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Manassas, VA) containing 20% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA) and differentiated in DMEM containing 2% horse serum (Thermo Fisher Scientific). The human endothelial kidney (HEK293) cell line was maintained in DMEM containing 10% FBS. The CHO cell line was maintained in DMEM:F-12 medium (Gibco, Gaithersburg, MD) containing 10% FBS. All cell lines were cultured in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin (Pen/Strep; Mediatech).

AAV vectors. The DNA region encoding the human sHB-EGF protein and the cDNA encoding the full-length transmembrane human H8-EGF were subcloned into a self-complementary (sc) AAV vector designed by Douglas McCarty (Pfizer, Raleigh, NC) (95). Both AAV vectors contained a cytomegalovirus (CMV) promoter and a simian virus 40 (SV40) polyadenylation sequence. r(sc)AAV9.CMV.sHB-EGF and r(sc)AAV9.CMV.H8-EGF vectors were made by the Viral Vector Core at Nationwide Children’s Hospital using the triple transfection method in HEK293 cells (96) and were purified using iodixanol gradients and anion-exchange chromatography (97). For sHB-EGF, the human HBEGC sequence contains only the first 148 amino acids (soluble form). For H8-EGF, the human HBEFG sequence contains all 208 amino acids (full-length transmembrane form) (98).

GALGT2 promoter luciferase reporter plasmids. All constructs are depicted in Fig. 2A. The bp −5870 human GALGT2 promoter was isolated by PCR from genomic DNA and ligated into the pGL4.14(luc2/Hyg) vector (Promega, Madison, WI) containing the firefly luciferase gene at the Xhol and BglII restriction sites in the poly linker region. bp −950, −500, and −200 deletion constructs were amplified and purified from the bp −5870 plasmid by PCR. Generation of serial deletions in the bp −950 construct were also made by PCR using the ultrahigh-fidelity PfuUltra II fusion HS DNA polymerase (Agilent Technologies, Santa Clara, CA) for DNA amplification by methods previously described (99). All plasmids were verified by DNA sequencing. Plasmids were transfected into C2C12 myoblasts using Effectene (Qiagen, Germantown, MD) and selected with 200 μg/ml hygromycin B (Mediatech). Control cells were transfected with a promoterless firefly luciferase plasmid as a negative control. Positive results were also confirmed using the human GALGT2 promoter cloned into a pGL4.76[hLuc/Hygro] vector (Promega) and selected with hygromycin B, with promoterless controls also generated as described above. Successfully transfected C2C12 cells were differentiated into myotubes following 6 days in differentiation media. All lines formed healthy myotubes under these conditions.

GALGT2 promoter luciferase reporter trophic factor screen. Reporter assays were performed in C2C12 myotube cultures stably expressing the bp −5870 GALGT2 promoter firefly luciferase reporter. These cells were plated in 96-well plates and became confluent 24 to 48 h after plating. When cells had reached confluence, fusion medium (DMEM containing 2% horse serum and Pen/Strep) was added to cells for 4 days, followed by treatment with trophic factors in serum-free DMEM for 24 h. Trophic factors screened included recombinant human epidermal growth factor (EGF; 100 ng/ml) (R&D Systems, Minneapolis, MN), recombinant soluble human heparin-binding EGF-like growth factor (sHB-EGF; 100 ng/ml) (R&D Systems), recombinant human amphiregulin (50 ng/ml) (Sigma-Aldrich, St. Louis, MO), recombinant mouse epiregulin (100 ng/ml) (Sigma-Aldrich), recombinant human transforming growth factor-α (TGFα; 100 ng/ml) (Sigma-Aldrich), recombinant human insulin-like growth factor 1 (IGF1; 100 ng/ml) (Sigma-Aldrich), insulin (solution from bovine pancreas; 3 μg/ml) (Sigma-Aldrich), recombinant human brain-derived neurotrophic factor (BDNF; 100 ng/ml) (EMD Millipore, Burlington, MA), recombinant human ciliary neurotrophic factor (CNTF; 100 ng/ml) (EMD Millipore), mouse nerve growth factor 2.5S (NGF 2.5S; 100 ng/ml) (EMD Millipore), recombinant mouse leukemia inhibitory factor (LIF; 50 ng/ml) (EMD Millipore), synthetic glucagon-like peptide 2 (10 μM) (Abbiotec, San Diego, CA), human growth hormone (HGH; 100 ng/ml; from pituitary gland) (EMD Millipore), recombinant human neurotrophin-3 (NT-3; 100 ng/ml) (R&D Systems), synthetic human peptide YY (100 nM) (Sigma-Aldrich), recombinant human glial cell line-derived neurotrophic factor (GDNF; 50 ng/ml) (Sigma-Aldrich), recombinant human hepatocyte growth factor (HGF; 100 ng/ml) (Sigma-Aldrich), human calcitonin gene-related peptide (CGRP; 1 μg/ml) (Sigma-Aldrich), synthetic human somatostatin (1 μM) (AbD Serotec, Raleigh, NC), and human neuregulin 1 (NRG1; 10 nM) (R&D Systems). Firefly luciferase activity was determined by following the manufacturer’s instructions using the Bright-Glo luciferase assay system (Promega) in a GloMax 96
microplate luminometer (Promega). Relative light units (RLU) in trophic factor-treated cells were normalized to levels for untreated cells.

**Transcription factor binding motif analysis.** Potential transcription factor binding sites in the human GALGT2 promoter were analyzed using TFBind gene tool software (http://tfbind.hgc.jp/) with a cutoff value of 0.85 (40).

**Ad-mediated gene overexpression.** Ads containing human FOXD3 (NM_012183), LMO2 (BC034041), TFFAP4 (BC010576), MYOG (BC035899), or GFP were purchased from Applied Biological Materials (Richmond, BC, Canada). Ad was amplified in HEK293 cells per the manufacturer’s instructions. Expression of Ad-GFP in C2C12 myotubes was used to determine the necessary multiplicity of infection (MOI) to attain >80% infection efficiency without cell death and to serve as a negative control in reporter assays. Following 4 days of differentiation, Ad was added to GALGT2 promoter luciferase reporter C2C12 cultures stably expressing either the bp −950 or the −950Δ22 GALGT2 promoter construct in serum-free medium at MOIs of 2, 4, 8, 16, 32, and 64 for 48 h in the presence or absence of shHB-EGF. Firefly luciferase activity was determined by following the manufacturer’s instructions using Steady-Glo luciferase assay systems (Promega, Madison, WI) in a GloMax 96 microplate luminometer. An MOI of 32 was required by all Ad constructs, except Ad-FOXO3 (MOI of 16), to generate maximal firefly luciferase activity without toxicity.

**ChIP analysis.** C2C12 myoblasts expressing the bp −5870 GALGT2 promoter firefly luciferase reporter were plated in 10-cm dishes and differentiated in fusion medium for 6 days as described above. Once differentiated into myotubes, cells were treated with human recombinant shHB-EGF protein for 24 h or 48 h. Cross-linking and harvesting of cells was performed using the Magna ChIP A/G kit from EMD Millipore per the manufacturer’s instructions. Chromatin was sheared using 8 cycles of 3 s on/15 s off at power setting 0.5, repeated 7 times on a Misonix sonicator 3000 (Misonic, Farmingdale, NY), to generate DNA fragments with a mean size between 200 and 1,000 bp. Sonicated DNA was precleared and then incubated with biotinylated polyclonal antibodies (Sigma-Aldrich), or IgG control (12-371; EMD Millipore) overnight at 4°C by following Magna ChIP A/G protocol instructions (EMD Millipore). Quantitative PCR (qPCR) in a 7500 real-time PCR system (Applied Biosciences, Foster City, CA) with SsoAdvanced universal SYBR green supermix (Bio-Rad, Hercules, CA) was used to quantify DNA fragments pulled down with the antibodies compared to evaluation of input DNA, which was not incubated with antibodies. Primers were created to stably include a single location along the GALGT2 promoter that was 150 to 250 bp in length: bp −950 to −726 (forward, 5’TTAAAGATTCTTGCCGTGCTG; reverse, 5’CGCTCZAATGATTCTCCACCT).

**siRNA knockdown of transcription factors.** C2C12 myoblasts expressing the bp −5870 GALGT2 promoter firefly luciferase reporter were plated in 96-well or 6-well dishes and differentiated in fusion medium for 6 days as described above. Once differentiated, cells were treated with recombinant human shHB-EGF and/or siRNA targeting transcription factors in serum-free DMEM for 48 h. siRNA was added 24 h prior to the addition of shHB-EGF at doses of 5 nM, 10 nM, or 20 nM and included siRNA against mouse LMO2 (SR403448), TFFAP4 (SR411368), or a universal scrambled negative control (OriGene, Rockville, MD). Treated cells were monitored closely to obtain maximal knockdown of targeted transcription factor without resulting in cell death. GALGT2 promoter activation was measured by reporter firefly luciferase activity by following the manufacturer’s instructions using Steady-Glo luciferase assay systems (Promega, Madison, WI) in a GloMax 96 microplate luminometer. Cell lysates were also collected to measure the resulting mRNA expression of targeted transcription factors to ensure siRNA selectivity and activity by semi-quantitative reverse transcription-qPCR (qRT-qPCR).

**shHB-EGF inhibitor screen.** C2C12 myoblasts expressing the bp −5870 GALGT2 promoter firefly luciferase reporter were plated in 96-well plates, becoming confluent after 24 to 48 h. At this time, cells were switched to fusion medium for 2 days, followed by 2 days in fusion medium that had been precleared with heparin-agarose beads (Sigma-Aldrich) to remove endogenous heparin-binding proteins. Cells were then treated with recombinant human shHB-EGF and/or inhibitors of signaling proteins in serum-free DMEM for 24 to 48 h. Inhibitors were added 1 h prior to the addition of shHB-EGF at a range of pharmacologically relevant doses and included EGF receptor (EGFR) inhibitor (tyrphostin AG-1478; 200 nM) (Cell Signaling, Danvers, MA), phosphoinositide 3-kinase (PI3K) inhibitor (LY294002; 5 μM) (EMD Millipore), protein kinase B (Akt) inhibitor (MK-2206; 5 μM) (Selleck Chemicals, Houston, TX), mammalian target of rapamycin (mTOR) inhibitor (rapamycin; 100 nM) (EMD Millipore), Raf kinase inhibitor (vemurafenib/PLX4032/RS7204; 3 μM) (Selleck Chemicals), extracellular signal-regulated kinase (ERK) inhibitor (SCH772984; 10 μM) (Selleck Chemicals), Src inhibitor (RX2-391; 5 nM) (Selleck Chemicals), Bcr/Abi inhibitor (nilotinib/AMN-107; 1 μM) (Selleck Chemicals), STATS inhibitor (25 μM) (EMD Millipore), JNK inhibitor (SP600125; 25 μM) (EMD Millipore), or c-myc inhibitor (10058-F4/CAS 403811-55-2; 10 μM) (EMD Millipore). Drug concentrations shown provided maximum levels of inhibition without causing cell death.

Firefly luciferase activity was determined by following the manufacturer’s instructions using Steady-Glo luciferase assay systems (Promega, Madison, WI) in a GloMax 96 microplate luminometer.

**Western blot analysis.** (i) Muscle cells. C2C12 myoblasts were plated in 12-well plates and differentiated as described above. Cells were then either left untreated or treated with 10 nM recombinant human shHB-EGF in serum-free DMEM for 15, 30, or 60 min. Medium was then removed and cells were rinsed briefly with cold PBS. Cells were incubated for 30 min at 4°C in lysis buffer containing 1% NP-40, 50 mM Tris, 140 mM NaCl, 1 mM EDTA, complete EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN), and PhosSTOP phosphatase inhibitors (Roche). Cell lysates were then collected using cell scrapers, rocked gently at 4°C for 45 min, and centrifuged at 21,000 × g at 4°C for 10 min. Supernatant was collected, and the resulting protein concentration was measured by bicinchoninic acid assay (Thermo Fisher Scientific). Protein (25 μg) was boiled at 100°C for 10 min in NuPAGE LDS sample buffer (Thermo Fisher Scientific) containing 0.1 M β-mercaptoethanol (BME; Fisher Scientific), separated on a
Bolt 4 to 12% Bis-Tris plus gel (Thermo Fisher Scientific), and transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl) containing 0.05% Tween 20 and then probed overnight with antibodies to EGFR (2232; Cell Signaling), phosphorylated EGFR (Tyr1173) (3343;4407; Cell Signaling), Akt (9272; Cell Signaling), phosphorylated Akt (Ser473) (9272; Cell Signaling), mTOR (2972; Cell Signaling), phosphorylated mTOR (Ser2448) (2971; Cell Signaling), MEK1/2 (9122; Cell Signaling), phosphorylated MEK1/2 (Ser217/221) (41G9) (9154; Cell Signaling), Erk1/2 (p44/42 mitogen-activated protein kinase [MAPK]) (137F5) (4695; Cell Signaling), phosphorylated Erk1/2 (p44/42 MAPK) (Thr202/Tyr204) (9101; Cell Signaling), C-Raf (9422; Cell Signaling), phosphorylated C-Raf (Ser289/296/301) (9431; Cell Signaling), Src (36D10) (2109; Cell Signaling), phosphorylated Src (Tyr416) (D949) (6943; Cell Signaling), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; G9545; Sigma-Aldrich, St. Louis, MO). After washing, blots were probed with horseradish peroxidase-coupled secondary antibody (Jackson ImmunoResearch Laboratories), washed again, and developed using an enhanced chemiluminescence (ECL; Lumigen, Southfield, MI) method according to the manufacturer’s instructions.

(ii) Muscle tissue. Frozen skeletal muscle blocks were cut on a cryostat (20 μm/section), and muscle tissue was digested in lysis buffer (mentioned above), also using metal lysis beads with shaking for four times at 30 Hz for 30 s each time (TissueLyser II; Qiagen). Samples were then rocked gently at 4°C for 3 h.

(iii) Band quantification. ImageJ 1.46r software (National Institutes of Health, Bethesda, MA) was used to analyze relative band density in both cell culture and skeletal muscle samples using methods we have previously described (100). Phosphorylated protein expression was normalized to total protein expression and then compared to levels for untreated control lyses. Total protein expression was normalized to the loading control (GAPDH) and again compared to untreated control lyses.

AAV-induced overexpression of HB-EGF and shHB-EGF. The gastrocnemius muscle on the left side of 5-week-old male C57BL/6j mice was injected with 5 x 10^6 vector genomes of r(sc)AAV9.CMV.hHB-EGF or r(sc)AAV9.CMV.shHB-EGF in a volume of 50 μl sterile PBS using a 0.3-ml insulin syringe near the midpoint of the muscle. Muscles on the contralateral (right) side of the mouse were mock injected with an identical volume of sterile PBS. At 4 or 12 weeks postinjection, mice were sacrificed and dissected. Gastrocnemius muscles were embedded in optimal cutting temperature (O.C.T.) compound (Fisher Scientific, Pittsburgh, PA) and snap-frozen in liquid nitrogen-cooled isopentane.

qPCR of AAV vector genomes. TaqMan qPCR was used to quantify AAV vector genome copies in treated gastrocnemius muscles and saline-treated control muscles. Frozen skeletal muscle blocks were cut on a cryostat (20 μm/section), and DNA was extracted from these shavings using the Qiagen DNeasy blood and tissue kit (Germantown, MD). DNA purity and quantity were measured using an ND-1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific). qPCR was performed in a 7500 real-time PCR system with TaqMan gene expression master mix (Thermo Fisher Scientific). A vector-specific primer/probe set was designed (forward, 5’ATCCGGTGGTGGTGCAAAT; probe, 5’/56-FAM/CTCTCTATGTTTGGT/3IABkFQ/; reverse, 5’GTGCTCCTCCTCGTGTGTT). Samples underwent qRT-PCR in a 7500 real-time PCR system with TaqMan gene expression master mix (Thermo Fisher Scientific) as an internal control. Primer/probe sets were designed (PrimerQuest DNA software) and synthesized (Integrated DNA Technologies, Coralville, IA) to recognize HomoSapiens HBEGF (forward, 5’GTGAC TTTCAAGAGGCAGAT; probe, 5’/56-FAM/TATCCTCCA/56-IABkFQ/; reverse, 5’GGTCGTCCCTCCTCGTGTGTT). Mus musculus Hbegf (forward, 5’AGGACTGGAAGGACAGACA; probe, 5’/56-FAM/AGTGGCTTT/56-IABkFQ/; reverse, 5’CCACCTCTTCTTGGTCTGG). Mus musculus Galgt2 (B4Galnt2) (forward, 5’GCCACGGAAGAGTCTCTTATT; probe, 5’/56-FAM/AT AATTACA/ZEN/CCCGGGCAAGAGCCC/3IABkFQ/; reverse, 5’CTCTTGGGTGTTCTCTTACCT). Premade primer/probe sets were designed for Mus musculus Egfr (Mm.PT.28853978), Erbb2 (Mm.PT.29549739), Erbb3 (Mm.PT.30061803), Erbb4 (Mm.PT.16591892), and Akt1 (Mm.PT.8333433) were purchased from Integrated DNA Technologies. Mus musculus primers for Lmo2, Lmo4, Lama5, Itga7, Itgb1, Dmd, Dag1, Utrn, and Agm were purchased and used as previously described (10). Probes for human transcription factor genes TFP4 (Hs00135342; m1), LMO2 (Hs0015342-m1), and MYOG (Hs01072232; m1) were purchased from Applied Biosystems. Mouse Myog probes (Mm00461494; m1) were purchased from Applied Biosystems. Mouse Tfp4 (Mm.PT.58.5559446) and Lmo2 (Mm.PT.82159087) probes were purchased from IDT. Premade primer pairs for Mus musculus Lmo2 (MP207520), Tfp4 (MP217129), and Foxd3 (MP204962) were purchased from OriGene (Rockville, MD).
MD). Relative mRNA levels were averaged for each cohort and compared to levels for PBS-injected muscle.

**Immunofluorescence staining of CHO cells and skeletal muscle.** (i) CHO cells. CHO cells transfected with expression vectors for sHB-EGF or HB-EGF were grown on gelatin-coated glass coverslips using previously described methods (102). Cells were washed in PBS, fixed in 4% paraformaldehyde (PFA) for 10 min, and then fixed further with 4% PFA and 0.1% Triton X-100 for 5 min. After washing, cells were incubated with antibodies that recognize sHB-EGF (MAb 2591; R&D Systems), sHB-EGF and HB-EGF (PAb 259 NA; R&D Systems), or the intracellular C-terminal domain of HB-EGF protein (C18; Santa Cruz Biotechnology, Dallas, TX). After washing, cells were incubated with appropriate fluorophore-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), washed again, and then mounted in ProLong Gold antifade mountant with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific).

(ii) Skeletal muscle. Unfixed frozen muscle blocks were cut on a cryostat into 8- to 10-µm sections. To probe for HB-EGF, staining was performed as described above with MAb 2591 and co-stained with rhodamine-conjugated α-bungarotoxin (B35451; Invitrogen, Carlsbad, CA). To probe for Galgt2 and CT glycan, slides were blocked in 3% bovine serum albumin (BSA) and then stained with a monoclonal antibody to CT glycan (CT2) or a polyclonal antibody to Galgt2 (PAB20841; Abnova, Walnut, CA). After washing in PBS, sections were incubated with appropriate fluorophore-conjugated secondary antibodies and rhodamine-conjugated α-bungarotoxin. After staining, all sections were mounted as described above. For EGFR staining, sections were fixed as described above, blocked with Mouse-On-Mouse (M.O.M.) blocking reagent (Vector Labs, Burlingame, CA) followed by a second block with 3% BSA, incubated with a monoclonal antibody to EGFR (A-10) (sc-373746; Santa Cruz Biotechnology), washed, and incubated with fluorescein isothiocyanate-isothiocyanate-conjugated secondary antibody along with rhodamine-conjugated α-bungarotoxin.

All immunofluorescence staining was visualized with a Zeiss Axioskop2 plus epifluorescence microscope using fluorophore-specific filters, and representative images were captured with a Zeiss AxioCam MRCS camera (Carl Zeiss Microscopy, Jena, Germany). All images comparing individual stains were time matched using identical exposure settings across different experimental conditions.

**Quantification of AChR microaggregates in skeletal muscle.** Quantification of AChR microaggregates based on α-bungarotoxin staining of NMJs was done as previously described (46), using 8-µm-thick cryostat cut sections of the tibialis anterior muscle. A total of 45, 37, or 75 synapses were analyzed for C37BL/6J, Galgt2−/−, and Hbegf−/− muscles, respectively.

**Grip strength and ambulation treadmill.** Forelimb and hindlimb grip strength were assessed in mice using a grip strength meter (Columbus Instruments, Columbus, OH). Measurements were averaged from 5 forelimb repetitions and 10 hindlimb repetitions each day; mice were tested once per day for a week, and the daily measurements were averaged to produce a final forelimb and hindlimb measurement. To measure ambulation, mice were run on a treadmill with a 10° decline (Treadmill Simplex II; Columbus Instruments). The mice ran for 1 min at 5 m/min, which increased by 1 m/min each minute until a maximum of 15 m/min and then remained at that speed for an additional 20 min. The time that the mice remained on the treadmill, up to a total time of 30 min, was recorded. After 2 days of training, mice were tested once per day for 5 days, and the daily measurements were averaged to produce a final measurement of treadmill running time before exhaustion.

**Statistics.** The significance in variance between cohorts was determined by one- or two-way analysis of variance (ANOVA), where appropriate, followed by Bonferroni’s multiple-comparison test. Kaplan-Meier survival plots were analyzed via the log-rank test.

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**REFERENCES**


3. Rafael JA, Tinsley JM, Potter AC, Deconinck AE, Davies KE. 1998. Skeletal muscle-specific expression of a utrophin transgene rescues utrophin-
dystrophin deficient mice. Nat Genet 19:79–82. https://doi.org/10.1038/ng0598-79.


54. Heller KN, Montgomery CL, Janssen PM, Clark KR, Mendell JR, Rodino-Klapac LR. 2013. AAV-mediated overexpression of human alpha7 in-