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The potential for gene-editing to increase muscle growth in pigs: experiences with editing myostatin

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Abstract

Gene-editing holds promise as a new technique for growth promotion in livestock, especially in the face of increased opposition to traditional methods of growth promotion like feed additives. However, to date, there has been only limited progress toward models of growth promotion through gene-editing. The vast majority of gene-editing projects have focused on a single gene, myostatin (*MSTN*), with several reports of successful editing events. These attempts have been limited by the low efficiency of successful edits and issues of viability. The use of both microinjection and somatic cell nuclear transfer appear to be susceptible to these viability issues. Herein, we report a successful editing of myostatin in pigs using a zinc-finger nuclease targeted at exon 3 of myostatin. Overall, the successful editing rate was 1% (37 edited cell lines/3616 screened cell lines). Edits included a variety of small indels and larger deletions. One male and one female cell line with a deletion of one thymidine residue (− 1 T) were selected for somatic cell nuclear transfer. Of the ~ 900 embryos transferred, there were 12 live births (1 male, 11 females) but only 5 female pigs survived to sexual maturity. These animals were bred to commercial sires to expand the population and determine potential interactive effects between myostatin mutations and the naturally-occurring g.3072G > A mutation in insulin-like growth factor 2. Even in subsequent generations (F0xWT, F1xF0, F1xF1), viability of piglets continued to be poor and was associated with the progeny's proportion of the original clone genome, even in pigs lacking *MSTN* loss-of-function (LOF) mutations. However, viability of pigs with myostatin LOF ($MSTN^{-/-}$) was especially poor as none of the 37 animals born were viable. Sequencing of cloned pigs indicated that off-target effects did not explain this poor viability. Reducing the percentage of the cloned genome by outcrossing successfully improved viability of $MSTN^{+/+}$ and $MSTN^{\pm}$ pigs, but not of $MSTN^{-/-}$ pigs. Characterization of $MSTN^{\pm}$ pigs did reveal an increase in muscle growth and body weight compared with $MSTN^{+/+}$ pigs. Therefore, in order for the promise of gene-editing of myostatin for growth promotion in livestock to be fulfilled, issues of viability of offspring and efficacy of editing have yet to be overcome. Additionally, gene-editing targets other than myostatin must be explored.

Keywords: Gene-editing, Growth, Livestock, Myostatin, Muscle, Pig, Zinc-finger nuclease

Background

In the next 30 years, the world population is predicted to increase to between 9.5 and 10 billion people, resulting in an increased demand for food. In particular, the demand for animal-derived protein is expected to increase 50–100%. While alternative proteins like those from plant-based meat substitutes or derived from cultured cells may help in meeting this demand, a large part

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of this demand will need to be met by improved production efficiencies. This need for increased production efficiencies and reduced environmental impact is especially important because land availability for agriculture will be in flux due to climate change and population growth (Zhang and Cai 2011).

In the past, increased production efficiencies have been achieved through genetic selection, improved diet formulation and management practices, and by using growth promoting feed additives and technologies. However, consumer resistance, especially towards growth-promoting technologies, is threatening their continued use in many scenarios. Additionally, consumer scrutiny regarding animal housing practices has increased. Giving up these technologies and techniques will threaten the ability of animal agriculture to continue making gains in production efficiencies and sustainability. Therefore, new methods to increase efficiency are needed. To date, only a limited number of genetically engineered animals have been approved for human consumption and commercial availability remains very restricted. However, this technology has the potential to replace other growth promoting technologies and speed improvements in animal efficiency.

Myostatin as a target for gene editing in pigs

Myostatin (*MSTN*) is a powerful regulator of muscle growth, primarily affecting prenatal muscle cell hyperplasia (McPherron et al. 1997). Myostatin increases p21 expression and reduces Cdk2 activity leading to cell cycle arrest and regulation of the number of myoblasts present to form muscle. Myostatin also inhibits myoblast differentiation, decreasing myoblast fusion into myotubes. Naturally occurring loss-of-function (LOF) mutants in cattle exhibit generalized increases in muscle growth and improved feed conversion (Arthur 1995; Grobet et al. 1998). In fact, LOF mutations in *MSTN* cause "double-muscling" in several species including cattle, sheep, mice, humans, and dogs (Grobet et al. 1998; Mosher et al. 2007; McPherron and Lee 1997; Schuelke et al. 2004; Kambadur et al. 1997; Clop et al. 2006). In contrast, myostatin LOF variants in pigs have not been reported although several putative regulatory variants have been associated with *MSTN* gene expression, growth rate, and lean meat yield (Stinckens et al. 2008; Yu et al. 2007; Liu et al. 2011; Tu et al. 2014). Because commercial pig breeding populations are under intense selection for lean yield, the failure to detect spontaneous *MSTN* LOF mutations in pigs that increase muscularity is surprising.

Given the significant impact *MSTN* polymorphism has on growth and development, and the paucity of other genes that have effects of similar magnitude, *MSTN* has become the primary focus for gene editing efforts aimed at improving lean growth efficiency within livestock species (Table 1, end of document). All three gene editing technologies, zinc-finger nucleases (ZFNs), transcription activator-like effector nuclease (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein (Cas) 9 (CRISPR/Cas9) system (Miller et al. 2007, 2011; Wood et al. 2011; Carlson et al. 2012; Le et al. 2013) have been applied to editing of *MSTN* with limited success. Cattle have been targeted the least, presumably due to the high number of existing LOF variants already present in many breeds dispersed throughout the world (Grobet et al. 1998). In addition, due to the persistent inefficiencies associated with gene editing, the long gestation length and typical singleton births in cattle can make the production of live edited animals very costly. Although naturally occurring mutations exist in sheep and goats, the primary aim of editing *MSTN* has been directed at the improvement of breeds previously not selected intensely for meat production. The pig has been the most popular target for editing, likely due to the lack of naturally occurring LOF variants within the species.

Currently, there are two primary approaches for implementing gene editing methods to produce live animals. These are somatic cell nuclear transfer (SCNT), and microinjection (MI) of zygotes derived from natural mating or in vitro fertilization (MI-IVF). The primary advantage of SCNT is the potential to select for specific modifications prior to generating embryos, thus significantly improving the proportion of animals carrying a specific mutation(s). However, SCNT has also been associated with the occurrence of early pregnancy loss and congenital abnormalities that can significantly decrease the number of viable individuals produced. Alternatively, MI is typically associated with fewer congenital defects, but efficacy is directly reliant on the targeting efficiency of the gene editing method being used. Furthermore, MI is associated with the potential to generate mosaicism or multiple editing events within individual offspring, which could be viewed positively as it could potentially provide more allelic variation from which to choose.

The application of gene editing platforms has certainly evolved over the past decade. However, even as proficiencies have increased, particularly with the accessibility of tools like CRISPR/Cas9, success has been limited (Table 1). To date, among the four attempts targeting *MSTN* in cattle, only seven live edited calves have been reported (Ge et al. 2021; Luo et al. 2014; Gim et al. 2021; Proudfoot et al. 2015). All three editing platforms were used with similar targeting efficiencies. As would be expected from SCNT, approximately 8% of the embryos transplanted resulted in live born calves with all matching the biallelic edits of the donor cells (Luo et al. 2014). In

Table 1 Gene editing to improve lean growth and efficiency in livestock species

References	Gene	Method	Species	Outcome	Phenotypic traits	Gene expression	Histology
Zou et al. (2018)	*FBXO40*	CRISPR/Cas9 SCNT	Pig	12 Live born/5424 embryos 10 Homozygous edited 2 Biallelic edited	9% ↑ Muscle mass	↑ *IRS1* ↑ IGF1/Akt activation	Hypertrophy
Xiang et al. (2018)	*IGF2*	CRISPR/Cas9 MI	Pig	8 Live born/19 embryos 2 Wild-type 2 Mosaic edited 6 Biallelic edited	↑ Average daily gain 33% ↑ Carcass weight	2 to sixfold ↑ *IGF2*	Hypertrophy
Ge et al. (2021)	*MSTN*	CRISPR/Cas9 SCNT	Cattle	No calves reported	–	–	–
Luo et al. (2014)	*MSTN*	ZFN SCNT	Cattle	3 Live born/35 pregnancies 3 Biallelic edited	↑ Muscularity	↑ *MYOG* ↓ *CDKN1A* ↓ *MYF5*	Hypertrophy
Gim et al. (2021)	*MSTN*	CRISPR/Cas9 MI-IVF	Cattle	17 Live born/19 embryos 3/17 Calves edited 0.1 to 89.5% mosaicism	↑ Muscularity	–	–
Proudfoot et al. (2015)	*MSTN*	TALEN MI-IVF	Cattle	2 Live born/20 embryos 1 Wild-type/1 edited Multiple alleles/mosaicism	↑ Muscularity	–	–
Zhang et al. (2019)	*MSTN*	CRISPR/Cas9 SCNT	Sheep	4 Live born/440 embryos 3 Edited	–	–	Hypertrophy
Crispo et al. (2015)	*MSTN*	CRISPR/Cas9 MI-IVF	Sheep	22 Live born/53 embryos 2 Monoallelic edited 8 Biallelic edited	20–30% ↑ Body weight	–	–
Han et al. (2014)	*MSTN*	CRISPR/Cas9 MI	Sheep	35 Live born/213 embryos 2 Biallelic edited Multiple allele mosaicism	–	–	–
Proudfoot et al. (2015)	*MSTN*	TALEN MI-IVF	Sheep	9 Live born/26 embryos 1 Monoallelic edited	–	–	–
Wang et al. (2015a)	*MSTN*	CRISPR/Cas9 MI	Sheep	36 Live born/578 embryos 10 Edited Multiple alleles	↑ Birth weight ↑ Average daily gain 29% ↑ Body weight @ 240 days	–	Hypertrophy
Ding et al. (2020)	*MSTN*	CRISPR/Cas9 MI	Sheep	35 Live born/640 embryos 10 Monoallelic edited 3 w/ mosaicism	↑ Average daily gain ↑ Body weight ↑ Muscularity	–	–
Ni et al. (2014)	*MSTN*	CRISPR/Cas9 SCNT	Goat	3 Live born/7 pregnancies 1/3 Died due to SCNT associated phenotypes	–	–	–
Zhang et al. (2018)	*MSTN*	CRISPR/Cas9 SCNT	Goat	1 Live born/134 embryos Monoallelic edited	↑ Birth weight ↑ Growth rate	–	Hypertrophy
Yu et al. (2016)	*MSTN*	TALEN SCNT	Goat	1 Live born/403 embryos Monoallelic edited	–	–	Hypertrophy
Guo et al. (2016)	*MSTN*	CRISPR/Cas9 MI	Goat	4 Live born/18 embryos 1/4 Edited 33% Mosaicism	–	–	–
He et al. (2018)	*MSTN*	CRISPR/Cas9 MI	Goat	6 Edited/8 live born 4 Monoallelic 2 Biallelic	↑ Growth rate ↑ Muscularity	–	Hyperplasia & hypertrophy
Wang et al. (2015a)	*MSTN*	CRISPR/Cas9 MI	Goat	79 Live born/416 embryos 15 Edited	–	–	–
Guo et al. (2016)	*MSTN*	CRISPR/Cas9 MI	Rabbit	34 Born/315 embryos 24 Biallelic edited Multiple allele mosaicism 0 to 88.9% mosaicism	14/34 Born with large-tongue syndrome ↑ Birth weight ↑ Muscularity	↑ *MYOG*	–

Table 1 (continued)

References	Gene	Method	Species	Outcome	Phenotypic traits	Gene expression	Histology
Bi et al. (2016)	*MSTN*	CRISPR/Cas9 SCNT	Pig	2 Live born/685 embryos 2 Monoallelic edited	12% ↑ Body weight 72% ↑ *L.dorsi* muscle 65% ↓ Backfat	↑ *MYOD1* ↑ *MYF5* ↑ *MYOG*	Hyperplasia
Kang et al. (2017)	*MSTN*	TALEN SCNT	Pig	18 Live born/646 embryos 100% Biallelic edited	10% ↑ Dressing percent 30% ↑ *L.dorsi* area 60% ↓ Backfat 56% ↑ Type II myofibers 80% ↓ Adipocyte size	–––	Hypertrophy
Kang et al. (2014)	*MSTN*	–	Pig	Heterozygous edited piglets viable Homozygous edited piglets lethal < 10 days of age	Heterozygotes ↑ Lean proportion ↓ Decrease backfat	Homozygotes ↓ *TNMD* ↓ *TNC* ↓ *COL1A1*	Hypertrophy
Li et al. (2020)	*MSTN*	CRISPR/Cas9 SCNT	Pig	16 Live born/955 embryos 1 Monoallelic edited 2 Biallelic edited 13 Died < 3 weeks	↑ Muscularity	↑ *IGF2* ↑ *MYOD1* ↑ *MYF5* ↑ *MYOG* ↑ IGF1/Akt activation	Hyperplasia
Rao et al. (2016)	*MSTN*	TALEN SCNT	Pig	4 Live born/55 embryos 4 Biallelic edited 3/4 Died in first 24 h 1 Euthanized at 28 days	70% ↑ *L.dorsi* muscle ↑ Type II myofibers	–	Hyperplasia
Qian et al. (2015)	*MSTN*	TALEN SCNT	Pig	19 Live born/2631 embryos 9 Survived to adulthood Mendelian segregation in 2nd generation	15% ↑ Body weight 24% ↑ Carcass weight 12% ↑ Lean percent 7% ↓ Body fat ↑ Vertebrae number	–	Hyperplasia
Wang et al. (2015b)	*MSTN*	CRISPR/Cas9 SCNT	Pig	12 Live born/955 embryos 8 Biallelic edited 8/8 Edited piglets died within 1 week	–	↑ *MYOG* ↓ *MYOD1* ↓ *MYF5*	Hyperplasia
Wang et al. (2016b)	*MSTN*	CRISPR/Cas9 SCNT	Pig	1 Stillborn/930 embryos Monoallelic edited	–	–	–
Wang et al. (2017)	*MSTN*	CRISPR/Cas9 SCNT	Pig	26 Live born/2290 embryos 23 Biallelic edited	↑ Birth weight ↑ Muscularity	–	–
Zou et al. (2019)	*MSTN*	CRISPR/Cas9 SCNT	Pig	2 Live born/1143 embryos 2 Monoallelic edited	–	–	–

contrast, the MI-IVF embryos yielded more pregnancies and live calves yet produced similar numbers of edited progeny due to targeting efficiency and direct transfer of embryos with no opportunity for selection (Gim et al. 2021; Proudfoot et al. 2015). Both studies using MI-IVF, calves had a high degree of mosaicism or multiple alleles. All edited calves displayed increased muscularity similar to existing naturally occurring LOF *MSTN* alleles. In contrast to the hyperplasia generally associated with *MSTN* LOF, hypertrophy was reported as the mechanism underlying increased muscularity (Luo et al. 2014). In sheep, the production of live, edited offspring is higher (n = 33) (Proudfoot et al. 2015; Zhang et al. 2019; Crispo et al. 2015; Wang et al. 2016a; Ding et al. 2020; Han et al. 2014), although the number of embryos produced for transfer is many times higher than in cattle, still indicating a relatively low yield. The litter bearing nature of sheep allows the transfer of multiple embryos to recipient females potentially leading to increased pregnancies, and subsequently the opportunity to observe more edited progeny. Most reports have been based on CRISPR/Cas9 and MI where the yield of edited offspring ranged from 6% (2 of 35) to 45% (10 of 22). Multiple alleles within individuals and mosaicism were also reported in more than half of these studies (Wang et al. 2016a; Ding et al. 2020; Han et al. 2014). The detailed phenotypic characterization of *MSTN*-edited sheep is limited but the general characteristics of increased growth rate and muscling are reported (Crispo et al. 2015; Wang et al. 2016a; Ding et al. 2020). Similar to edited cattle, two studies have reported muscle

fiber hypertrophy as the basis of the increased muscularity (Zhang et al. 2019; Wang et al. 2016a). Of the six reported outcomes in goats, three efforts used SCNT resulting in four live offspring, two of which were heterozygous (Ni et al. 2014; Zhang et al. 2018; Yu et al. 2016). MI was used in the remainder of the reports resulting in 22 edited animals (Guo et al. 2016; He et al. 2018; Wang et al. 2015a). This again suggests that although almost 100% of animals derived from SCNT will be modified, MI, either using natural embryos or IVF, may be a more efficient approach for producing edited animals where the species is amenable to the production and transfer of many single cell embryos. As in sheep, the detailed phenotypic characterization of edited animals is lacking, but increased growth and muscularity are also described (Zhang et al. 2018; He et al. 2018). The underlying basis of the increased muscle development was reported as hypertrophy in two studies (Zhang et al. 2018; Yu et al. 2016) and both hyperplasia and hypertrophy in a third (He et al. 2018). There is a single report of *MSTN* editing in rabbits using CRISPR/Cas9 and MI (Guo et al. 2016). Although the targeting efficiency was relatively high, yielding 24 biallelic edited offspring out of 34 live born kittens, 14 succumbed to large tongue syndrome, a condition more often associated with SCNT (Kurome et al. 2013). In addition, a high degree of mosaicism and multiple alleles was also present. Knockout of *MSTN* resulted in a significant increase in birth weight [~ 20%) and weights of the quadricep and bicep muscles of 50% and 98%, respectively.

There have been nearly twice the number of attempts to edit *MSTN* in pigs as there have been in any other livestock species. Although all three editing platforms have been used, only SCNT has been implemented for the generation of live animals (Bi et al. 2016; Li et al. 2020; Rao et al. 2016; Qian et al. 2015; Wang et al. 2015b, 2016b, 2017; Zou et al. 2019; Kang et al. 2014, 2017). As indicated previously, the primary impetus for targeting of *MSTN* is due to the lack of naturally occurring LOF mutations within the species although four of the current attempts were performed in Chinese indigenous breeds to rapidly increase lean yield (Bi et al. 2016; Li et al. 2020; Qian et al. 2015; Wang et al. 2017). The use of SCNT is best suited to the pig where large numbers of reconstructed embryos can be transferred to a single female, thus increasing the probability of successful pregnancies. Somewhat unexpectedly, the results have been highly variable although perhaps not due to the same issues as in the other livestock species. The earliest attempt did not specify either the editing or reproduction method (Kang et al. 2014), yet successfully produced live piglets with both monoallelic and biallelic editing events. Interestingly, all biallelic offspring died < 10 days after birth, while monoallelic-edited piglets were viable. Two additional efforts observed the same viability phenomenon between genotypes (Rao et al. 2016; Wang et al. 2015b). In contrast, three attempts generated modest numbers of biallelic edited piglets that were viable and raised to harvest age or adulthood (Qian et al. 2015; Wang et al. 2017; Kang et al. 2017). Notably, two of the three studies that reported high viability of biallelic edited animals were conducted in Chinese breeds where the *IGF2* g.3072G > A polymorphism is not segregating (Qian et al. 2015; Wang et al. 2017). This suggests the combination of *MSTN* LOF and the *IGF2* g.3072A allele may influence viability. However, the third study was conducted using modern commercial germplasm were *IGF2* g.3072A is assumed to be present (Kang et al. 2017). Three other studies only produced monoallelic edited progeny at very low efficiency having only one or two offspring generated from hundreds of reconstructed embryos (Bi et al. 2016; Wang et al. 2016b; Zou et al. 2019). Most recently, Li et al. (2020) reported the production of 16 liveborn piglets, however only three were edited, one monoallelic and two biallelic. Unfortunately, of the three edited alleles identified, two allowed production of mature myostatin protein although at reduced rates (Li et al. 2020). Phenotypic analyses of both monoallelic and biallelic edited animals shows the tremendous potential of *MSTN* LOF mutations with significant increases in lean muscle and reduction of adiposity for both genotypes (Ni et al. 2014; Rao et al. 2016; Qian et al. 2015; Kang et al. 2017). More consistent with the known function of *MSTN*, most reports defined the underlying mechanism of increased muscularity to be from hyperplasia (Bi et al. 2016; Li et al. 2020; Rao et al. 2016; Qian et al. 2015; Wang et al. 2015b) rather than hypertrophy (Kang et al. 2014, 2017).

Two additional genes have also been targeted in pigs, F-box protein 40 (FBXO40) and insulin-like growth factor 2 (*IGF2*) (Zou et al. 2018; Xiang et al. 2018) (Table 1). Both reports demonstrated modest success generating 20 live edited offspring between them. One of these introduced LOF mutations in the FBXO40 gene based on prior observations in mice (Shi et al. 2011). The knockout of FXBO40 resulted in muscular hypertrophy that significantly increased lean yield with no apparent negative effects on animal health (Zou et al. 2018). The targeting of *IGF2* was aimed at mimicking the previously identified g.3072G > A mutation within the ZBED6 repressor binding site in intron 3 of the gene (Laere et al. 2003). The *IGF2* editing was performed in the miniature Chinese Bama breed, which only has the ancestral *IGF2* g.3072G allele. Successful editing generated increases in *IGF2* expression and subsequently significant differences in growth rate between edited and wild type pigs (Xiang et al. 2018). These differences are consistent with the

impact of this mutation in the vast majority of commercial germplasm used in modern pork production.

Impact of *IGF2* on lean muscle growth in pigs

Both insulin-like growth factor 1 (IGF1) and *IGF2* are key regulators of myogenesis. Specifically, *IGF2* promotes differentiation of myoblasts in both cell culture (Florini et al. 1991) and somatic (Pirskanen et al. 2000) from chicks. The expression of *IGF2* is elevated during embryonic and fetal development but is down-regulated after birth (Rotwein et al. 1987), which is different from the expression of IGF1 that remains relatively high postnatally (Clark et al. 2014; Beck et al. 1987; Bondy et al. 1990). In swine, *IGF2* is of particular interest as a quantitative trait locus (QTL) controlling muscle growth and fat deposition. The quantitative trait nucleotide (QTN) has been identified as a single base pair substitution in intron 3 of *IGF2* (g.3072G > A). The substitution alters a conserved CpG island that is hypomethylated in skeletal muscle and has been identified as a binding site for the transcriptional repressor, zinc-finger BED-containing 6 (ZBED6) (Laere et al. 2003; Markljung et al. 2009). This mutation results in a fourfold increase in *IGF2* expression in postnatal muscle (Laere et al. 2003), and a tendency to increase late prenatal *IGF2* expression (Clark et al. 2015 May 1), resulting in increased muscle hyperplasia and muscle weight. Most of the germplasm used for commercial pork production has this *IGF2* mutation due to the intensive selection for lean growth. The g.3072A allele increases lean meat yield through increased muscle accretion and reduced subcutaneous adipose tissue deposition (Jeon et al. 1999; Nezer et al. 1999).

If the mechanism by which increased postnatal *IGF2* expression increases lean meat yield overlaps with that of LOF *MSTN* mutations, it is possible that the near fixation of the *IGF2* g.3072A allele in commercial pigs may mask the appearance of naturally-occurring myostatin mutants, thus precluding them from selection. Alternatively, potential epistasis between such alleles may produce phenotypes that are less desirable, or potentially even subject to negative selection pressure. We have partially characterized the mechanism by which the *IGF2* mutation results in increased muscle accretion (Clark et al. 2014; Clark et al. 2015 May 1). *IGF2* is imprinted resulting in expression of only the paternal allele (DeChiara et al. 1991). As such, experimental animals were generated by mating heterozygous A/G boars to A/A sows. As expected, *IGF2* expression was increased in longissimus muscle of A^{pat} (i.e., inheriting the paternally expressed A allele) pigs compared with G^{pat} pigs beginning at birth and continuing to market age (176 d, 127 kg). Surprisingly, despite the increase in *IGF2* expression of approximately fourfold at market age, there was no increase in muscle fiber cross-sectional area in A^{pat} pigs. However, there was a tendency for *IGF2* expression to also be increased at d90 of gestation, and expression of IGF1 receptor was increased in A^{pat} pigs at d60 of gestation. This increase in receptor expression and the tendency for increased *IGF2* expression prenatally likely contributed to the 11% increase in muscle fiber number of A^{pat} pigs (Clark et al. 2015). Therefore, we concluded that the increase in muscle weight of A^{pat} pigs at market age was solely from muscle fiber hyperplasia, suggesting increased prenatal muscle fiber formation (Clark et al. 2015). In mice, we have demonstrated that the complete lack of functional myostatin increased *IGF2* expression in muscles but did not alter IGF1 expression (Clark et al. 2015). However, in *IGF2* A^{pat} pigs, myostatin expression was unaltered compared with G^{pat} pigs (Clark et al. 2015). This suggests that while *MSTN* LOF mutations may exert some growth-promoting effects through the *IGF2* signaling pathway, *IGF2* does not reciprocally regulate *MSTN*.

Generation of *MSTN* edited pigs

Given this potential overlap of mechanisms between increased muscling from increased *IGF2* and myostatin LOF, we considered *IGF2* status when generating myostatin LOF mutations in pigs. Two commercial crossbred sows homozygous for the *IGF2* g.3072A allele (A/A) were artificially inseminated with semen from a heterozygous (A/G) purebred Berkshire boar. Pregnancies were terminated at day 60 of gestation and 25 fetuses were harvested and used to create fetal fibroblast cell lines. Each fetus was genotyped for the *IGF2* QTN and only those cell lines with an A/G genotype were used for editing. One male and one female cell line were selected for nucleofection with a ZFN (Sigma) targeted at exon 3 of the porcine *MSTN* gene. Approximately 2×10^6 cells were nucleofected (Nucleofector™, Amaxa) with 5 ug of ZFN mRNA and 2 ug of pmaxGFP vector using program U-023. Alternatively, for homology directed repair (HDR), 0.1 nmol of synthetic single-stranded oligo (5'-TTCACACAGAAT CCCTTTTTAGAAGTCAAGGTAACAGACACACCA AAAAGATCTAGATGAGATTTGGTCTTGACTGTG ATGAGCACTCAACAGAATCTCGATGCTGTCGTT ACCCTCTAA-3') was added to the nucleofection reaction. Following nucleofection, cells were recovered for five days and were sorted by positive fluorescence using a BD FACS Aria III flow cytometer. Approximately four cells were sorted into each well of multiple 96-well tissue culture plates and cultured for 14 days with media replacement every three days. Surviving sublines were consolidated into 96-well plates and a sample taken for DNA isolation. PCR was performed to amplify the region flanking the ZFN target site. Amplicons were sequenced directly to determine potential editing events. Amplicons

Table 2 Summary of editing rate

	Male	Female	Total
No. of sublines	1184	2432	3616
Edited sublines	20	17	37
% Mutants	1.7%	0.7%	1.0%

Table 3 Summary of editing events

Mutation	Male	Female	Total
+ 2 T	1	0	1
+ 1 T	4	3	7
+ GA	0	1	1
+ GATTT	0	1	1
− 1 T	4	8	12
− 1G	1	1	2
− 1A	1	0	1
− GA	0	1	1
− ATT	0	1	1
− GAGA	2	0	2
− GATT	2	0	2
− 4/9 bp	1	0	1
− 7 bp	0	1	1
− 11 bp	1	0	1
− 50 bp	1	0	1
Allele replacement	2	0	2
Total	20	17	37

of edited sublines were subcloned into plasmid vectors and 96 plasmids were sequenced for each cell line to determine clonal status and define alleles in biallelic edited cell lines. From 3,616 cell lines screened, 37 edited lines were identified (Table 2) with various mutations (Table 3).

One male and one female cell line containing a deletion of one thymidine residue (− 1 T) were selected as donor cells for SCNT. SCNT was performed by Trans Ova Genetics (Sioux Center, IA). Single-cell embryos were shipped to the University of Illinois and approximately 150 reconstructed embryos were surgically transferred to pre-synchronized gilts.

From the male cell line, four SCNT/embryo transfers (ET) were conducted with only one producing a pregnancy that resulted in three piglets; two were stillborn and a third piglet died within 24 h of birth. All three piglets displayed congenital abnormalities common in SCNT, including growth retardation and oversize tongues (Kurome et al. 2013). Evidence suggests that epigenetic changes may be one cause of these various phenotypic abnormalities (Shi et al. 2003). Cloned swine have differential methylation patterns in the imprinted genes *H19*, *IGF2R*, *INS*, and *IGF2* (Shen et al. 2012; Jiang et al. 2007 Mar 26; Kang et al. 2001 Oct 26) that may result from incomplete reprogramming of somatic cells during the SCNT process. Therefore, we attempted to produce a viable gene-edited male individual via the "recloning" of the live born male piglet by generation of a new fibroblast cell line following its death. Following SCNT, two ET using this new cell line resulted in a single pregnancy that produced two stillborn piglets with the same abnormal phenotypes. This suggests that specific cell lines and their derivatives may be permanently refractory to SCNT. Furthermore, this phenomenon may be due to improper epigenetic reprogramming of somatic cells that is stable throughout many mitotic divisions.

The transfers using the female cell line resulted in a 100% pregnancy rate producing 14 piglets. Of these piglets, three were stillborn with similar defects to the male line and three more died or were euthanized within 24 h of birth. One of these piglets was alive and healthy but was laid on by the sow. Genomic DNA was isolated from one viable female piglet and one abnormal stillborn female littermate to examine methylation patterns of the *H19*, *IGF2R*, *INS*, and *IGF2* genes. Three individual sites located in the *INS* and *IGF2*_differentially methylated region 2 (DMR2) products exhibited significant differences between the two samples ($P < 0.01$). The entire amplified product of *IGF2*_DMR2 contained 33 CpG sites and was also shown to have significant differences when all sites were considered jointly (Fig. 1). Although only two animals were analyzed, this suggests that the epigenetic variation of these regions may have some causal effect on the resulting abnormal phenotypes, thus leading to the reduced viability. Three more piglets were euthanized before breeding due to various complications (unthrifty, injured, and unable to conceive). A summary of SCNT results is presented in Table 4.

Viability of gene edited piglets is related to the percentage of clone genome and genotype

The five-remaining edited, female clones (F0) were mated to several commercially available Berkshire boars that were heterozygous for the *IGF2* g.3072 mutation (A/G) with the goal of expanding the breeding population and eventually producing myostatin knockout (MKO) pigs. These initial matings (wild type (WT) x F0) produced F1 piglets that were subsequently bred to both WT and F1 individuals. The WT individuals included randomly selected commercial gilts in the case of F1 x WT matings, and unrelated Berkshire boars that were heterozygous at the *IGF2* locus in WT X F1 matings. In both mating types, the segregation ratios were consistent with Mendelian inheritance producing roughly a 1:1 ratio of homozygous ($MSTN^{+/+}$) and

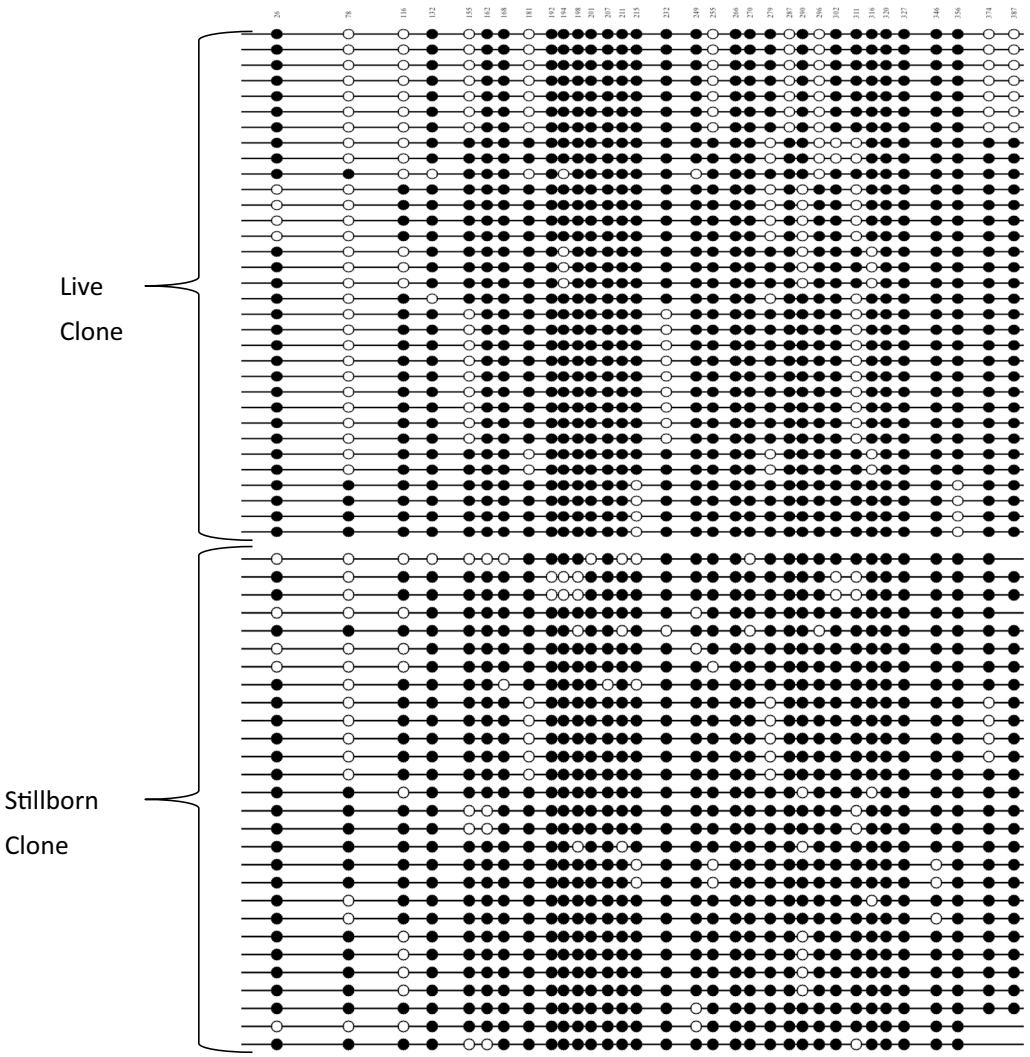

Fig. 1 Lollipop diagram of CpG sites in the *IGF2* DMR2 product. Each circle represents 1 of 33 CpG sites. Filled circles represent methylated sites and open circles represent unmethylated sites. The position of each site in the product is indicated at the top

Table 4 Summary of SCNT success

	Male	Female	Total
Embryo transfers	6	2	8
Pregnancies	2	2	4
Piglets born	5	14	19
Stillborn	4	3	7
Died within 24 h	1	3	4
Died prior to breeding	0	3	3
Bred adult animals	0	5	5

heterozygous ($MSTN^{\pm}$) offspring (Table 5). Following these matings, the F1 individuals were mated to other heterozygous animals including both F1 and F0 pigs. Matings were designed to limit inbreeding as much as possible given the limitations of the population structure (i.e., all animals carrying the edited *MSTN* allele had the same founder clones in the pedigree). Segregation was also consistent with Mendelian ratios for both *MSTN* (Table 5) and *IGF2* (data not shown). Although the edited *MSTN* allele segregated appropriately, and without any evidence of an interaction between paternal *IGF2* allele, two characteristics readily emerged from these matings. Firstly, the viability of pigs in several of these mating types was extremely low (Table 5). Secondly, all homozygous edited piglets ($MSTN^{-/-}$) were non-viable with approximately 25% being stillborn and the remainder dying or requiring euthanasia within 72 h of birth (Tables 5 and 6). $MSTN^{-/-}$ piglets

Table 5 Survival of offspring by generational mating type

Mating	Litters (piglets)	Offspring genotype		
		***MSTN*$^{+/+}$(live)[a]**	***MSTN*$^{\pm}$(live)**	***MSTN*$^{-/-}$(live)**
WT X F0	10 (99)	41 (26) 63.4% live	58 (28) 48.3% live	NA
F1 X WT or WT X F1	18 (169)	80 (59) 73.8% live	89 (68) 76.4% live	NA
F1 X F1	7 (63)	17 (9) 52.9% live	28 (14) 50.0% live	18 (0) 0% live
F1 X F0	7 (82)	23 (8) 34.8% live	40 (16) 40.0% live	19 (0) 0% live

[a] Number of living piglets 72 h after parturition

Table 6 Survival of offspring by parental genotype

Mating	Total litters	Total piglets	Live	Stillborn	Postnatal death (< 72 h)	***MSTN*+/+ (live) (% live)**	***MSTN*± (live) (% live)**	***MSTN*−/− (live) (% live)**
MSTN+/+ X *MSTN*±	34	317	220 (69.4%)	57 (18.0%)	40 (12.6%)	149 (106) (71.1%)	168 (114) (67.9%)	NA
MSTN± X *MSTN*±	46	362	186 (51.4%)	73 (20.2%)	103 (28.4%)	106 (69) (65.1%)	179 (115) (64.2%)	77 (2) (2.6%)

were typically smaller than their littermates at birth and those born alive appeared to be weak and unthrifty (data not shown).

Whole genome sequencing of the primary cell line and a single live clone (approximately 35X genome coverage each) revealed no evidence of deleterious off-target modifications although 4911 variants were detected between the two genomes using the most conservative variant calling pipeline (data not shown). Of these high-quality variant calls, only four were identified in coding regions, all of which were benign. Thus, we feel it is unlikely the reduced viability is due to deleterious mutations caused by off-target editing events. The differences between the genomes of the primary cell line and live clone are most likely the result of somatic mosaicism accumulated during cell culture or during development. Although the accumulation of these mutations could have an effect on the viability of the cells or the cloned animals (Youssoufian and Pyeritz 2002), it would be difficult to demonstrate.

Instead, we suggest there is a relationship between F0 genome proportion and viability (Table 5, Fig. 2). Furthermore, the underlying basis of this relationship can potentially be explained by genome-wide epigenetic variation that is stably inherited from the F0 ancestor, a potential example of transgenerational epigenetic inheritance. However, F0 genome proportion and its hypothesized epigenetic variation alone does not account for the reduced viability of F1 × F1 piglets compared to WT × F0 piglets. Although piglets derived from these matings would be expected to have similar genomic contributions from the F0 individuals (i.e., 50%), viability is reduced in F1 × F1 matings due to the production of *MSTN*$^{-/-}$ progeny in these matings. If overall viability was only associated with changes in genome-wide epigenetic variation, it would be expected that these losses would be randomly distributed among myostatin genotypes (Table 6).

There are two potential explanations for this interaction between proportion of F0 genome contribution and myostatin genotype. Firstly, heritable epigenetic changes produced during SCNT specifically may affect gene networks involved in growth and development, and these networks require functional myostatin. Epigenetic regulation (i.e., methylation) allows for programming of gene expression by the environment. The classic example of this epigenetic programming is in response to maternal nutrient deficiencies where gene expression of the fetus is programmed to adapt to a nutrient-scarce environment (Funston et al. 2010; Du et al. 2010). Changes in metabolism, growth and efficiency then tend to conserve nutrients. Cell culture, where edited fetal fibroblast cells used for SCNT and embryo transfer were held for several weeks, can also alter methylation patterns in cells, and these changes are heritable (Nilsson et al. 2005; Choi et al. 2012). Cells dividing in culture are provided

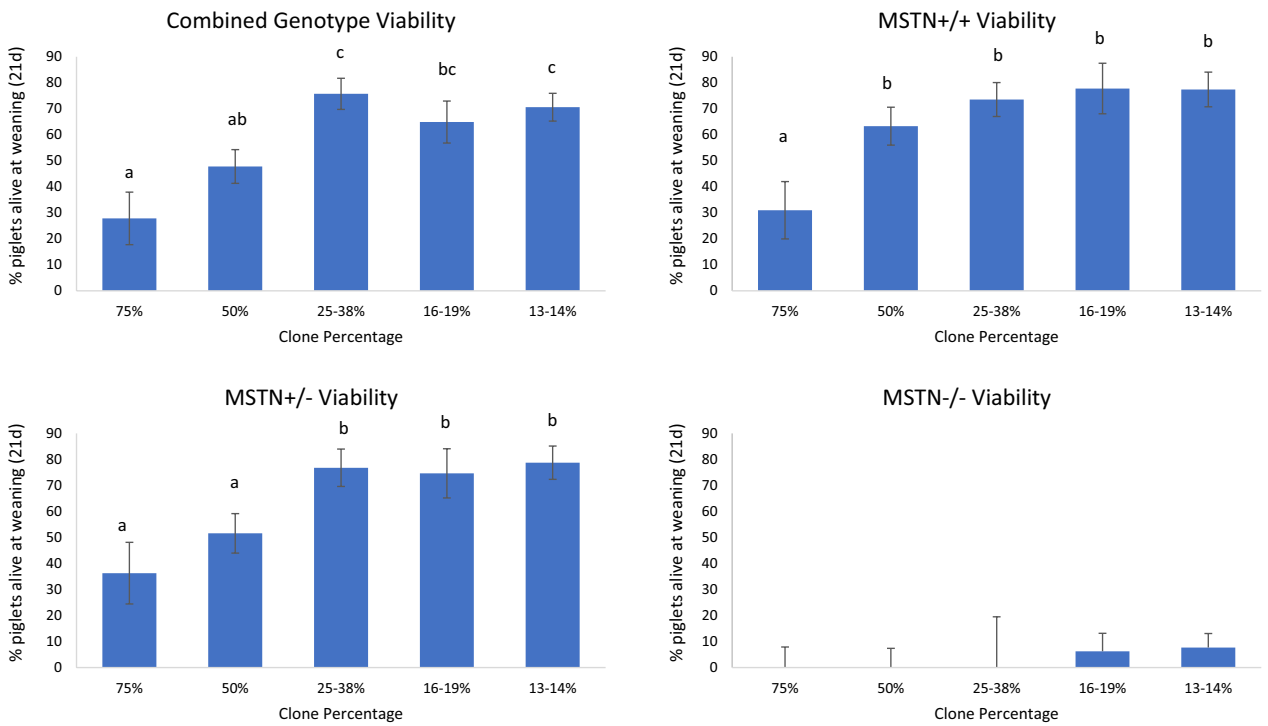

Fig. 2 Viability of Gene-edited Piglets based on Percentage of Clone Genome. The percentage of clone genome was assigned to offspring by averaging the contribution of each parent. For example, mating of a cloned female (100% clone genome) with a commercial male (0% clone genome) resulted in offspring that were 50% clone genome. When those offspring were mated to a cloned female, the resulting progeny were 75% clone genome. Bars represent means ± standard errors. Bars with different superscripts are different ($P < 0.05$)

with an optimal environment in terms of nutrients, temperature, and space. Thus, cells may have responded to this environment with epigenetic changes that adapted their "offspring" to survive in a nutrient-rich environment. For example, global demethylation of genes regulating growth would increase their expression and allow cells to proliferate rapidly. When those cells then underwent SCNT, epigenetic marks appear to be maintained in cloned (F0) pigs and inherited in future (F1) generations. In general, myostatin inhibits cell proliferation. Therefore, myostatin in piglets with inherited epigenetic marks to accelerate proliferation (increased F0 genome) may aid in holding accelerated proliferation in check; this allows for some viable $MSTN^{+/+}$ and $MSTN^{\pm}$ piglets to be produced. Without myostatin, however, piglets epigenetically programmed for rapid proliferation during development are not viable.

Secondly, the mechanism may involve heritable genetic or epigenetic variation that has been selected for due to the presence of the *IGF2* g.3072A allele, though not result directly from the interaction of the *IGF2* and myostatin alleles. With the near fixation of the *IGF2* g.3072A allele in commercial swine, it can be hypothesized that other genetic polymorphism or stable epigenetic modifications have been selected for and incorporated in the "context" of *IGF2* g.3072A, thus changing the genetic architechure of muscle development to be incompatible with MSTN LOF. Such polymorphisms or epigenetic modifications would not be genetically linked to *IGF2* and therefore, would not be detected as an epistatic interaction between *IGF2* and myostatin genotypes. This mechanism may be supported by results of other groups that have also successfully edited myostatin in pigs where MKO offspring have also been 100% non-viable using commercial germplasm (Rao et al. 2016; Wang et al. 2015b; Kang et al. 2014). In contrast, MKO piglets derived from both Meishan and Erhualian germplasm are viable (Qian et al. 2015; Wang et al. 2017) These germplasms differ significantly in *IGF2* g.3072A allele frequency and selection pressure for lean growth. Interestingly, one study has reported biallelic editing in improved germplasm, but the allele frequencies of the *IGF2* g.3072 locus were not disclosed (Kang et al. 2017).

We have tentatively shown that developmentally important genes such as *IGF2* have distinctly different epigenetic profiles in viable and non-viable piglets (Fig. 2), which is regulated by myostatin. Epigenetic changes in growth-regulating genes like IGF2 receptor

have been noted to reduce viability in cattle (Chen et al. 2015). Thus, we hypothesize that the aberrant epigenetic variation that leads to the observed non-viability of MKO piglets will map to specific gene networks where myostatin is essential for proper development.

Characterization of pigs with one edited myostatin allele

Despite the viability issues of MKO piglets, we were able to successfully characterize the phenotype of pigs carrying one edited allele. In total, 44 pigs were slaughtered at the University of Illinois Meat Science Laboratory. These pigs represented 4 experimental genotypes: 1) wild-type *MSTN* ($MSTN^{+/+}$) with *IGF2* G^{pat} (n = 13); 2) $MSTN^{+/+}$ with *IGF2* A^{pat} (n = 11); 3) one edited *MSTN* allele ($MSTN^{\pm}$) with *IGF2* G^{pat} (n = 10); and 4) $MSTN^{\pm}$ with *IGF2* A^{pat} (n = 10). Both barrows and gilts were represented in each genotype. Pigs were approximately 175d of age at the time of slaughter. Carcass characteristics and muscle weights were collected from these animals and the effects of sex, myostatin genotype, *IGF2* genotype and their interactions were analyzed with the PROC MIXED procedure of SAS.

Though ending live weights were not different between $MSTN^{\pm}$ pigs and $MSTN^{+/+}$ pigs, dressing percentage was greater in $MSTN^{\pm}$ pigs compared to $MSTN^{+/+}$ pigs. In previous cattle studies, it is suggested that organ weights are decreased in double muscled cattle (Fiems 2012), which would lead to increased dressing percentages. Loin muscle area, an indication of whole-body muscle deposition, was increased by approximately 20% in $MSTN^{\pm}$ pigs compared with $MSTN^{+/+}$. Additionally, the interaction of *IGF2* and myostatin genotype tended to be significant for loin muscle area (P = 0.09]. Loin muscle area increased 14% in $MSTN^{\pm}$ A^{pat} pigs compared to $MSTN^{+/+}$ A^{pat} pigs, but increased 28% in $MSTN^{\pm}$ G^{pat} pigs compared to $MSTN^{+/+}$ G^{pat} pigs (Table 7).

Individual muscle weights (Table 8) including the longissimus dorsi, psoas major, and semitendinosus were also increased in $MSTN^{\pm}$ pigs compared with $MSTN^{+/+}$ pigs by 7–21%. When expressed as a percentage of chilled side weight, these increases were maintained and the

Table 7 Carcass characteristics of myostatin heterozygous and wild type pigs

Item	$MSTN^{+/+}$[1]		$MSTN^{\pm}$[1]		SEM	P-value		
	IGF2 G.[2]	*IGF2* A.[2]	*IGF2* G	*IGF2* A		*MSTN*	*IGF2*	*MSTN* x *IGF2*
Pigs, n	13	11	10	10				
Ending live weight, kg	124.28	127.25	127.84	128.73	5.674	0.50	0.62	0.78
Dressing percentage, %	76.1	75.7	76.8	76.9	0.39	< 0.01	0.64	0.43
Hot carcass weight, kg	93.9	96.0	97.6	98.5	4.47	0.33	0.64	0.85
Loin muscle area, cm^2	49.49	54.14	63.45	62.21	1.786	< 0.01	0.33	0.09
10th rib back fat depth, cm	2.25	2.10	2.03	1.98	0.211	0.41	0.63	0.82
Estimated carcass lean, %	52.88	54.43	56.94	56.21	1.207	0.02	0.73	0.33

[1] $MSTN^{+/+}$ = homozygous wild-type myostatin, $MSTN^{\pm}$ = one edited myostatin allele

[2] *IGF2* paternal G allele (*IGF2* G) or paternal A allele (*IGF2* A)

Table 8 Muscle weights of myostatin heterozygous and wild type pigs

	$MSTN^{+/+}$[1]		$MSTN^{\pm}$[1]		SEM	P-value		
	IGF2 G^2	*IGF2* A^2	*IGF2* G	*IGF2* A		*MSTN*	*IGF2*	*MSTN* x *IGF2*
Longissimus dorsi, kg	2.95	3.35	3.72	3.92	0.115	< 0.01	< 0.01	0.36
% Chilled side wt	6.83	7.41	8.16	8.28	0.206	< 0.01	0.08	0.26
Psoas major, kg	0.40	0.45	0.47	0.51	0.023	< 0.01	0.03	0.75
% Chilled side wt	0.89	0.96	0.99	1.04	0.068	0.07	0.26	0.83
Semitendinosus, kg	0.55	0.58	0.57	0.64	0.028	0.07	0.02	0.24
% Chilled side wt	1.21	1.22	1.17	1.30	0.053	0.55	0.06	0.11
Triceps brachii, kg	1.43	1.44	1.48	1.52	0.108	0.37	0.81	0.72
% Chilled side wt	1.63	1.95	2.33	2.89	0.401	0.01	0.18	0.71

[1] $MSTN^{+/+}$ = homozygous wild-type myostatin, $MSTN^{\pm}$ = one edited myostatin allele

[2] *IGF2* paternal G allele (*IGF2* G) or paternal A allele (*IGF2* A)

percentage of chilled side weight as triceps brachii weight was also increased in $MSTN^{\pm}$ compared with $MSTN^{+/+}$ pigs.

These data suggest that while the complete loss of myostatin function may be lethal in much of commercial pig germplasm, a partial loss increased muscle deposition similar to observations in other species. Though we previously reported that myostatin expression was not altered in *IGF2* A^{pat} pigs compared with G^{pat} pigs (Clark et al. 2014), the increase in loin muscle area was doubled in $MSTN^{\pm}$ G^{pat} pigs compared with $MSTN^{\pm}$ A^{pat} pigs suggesting that the pathways by which *IGF2* and the loss of myostatin increase loin muscle deposition may overlap.

Conclusions

To date, there has been limited success generating viable gene-edited animals for growth promotion. This lack of success may be attributed to several reasons, all which warrant future research and improvement. First, only a limited scope of targets have been edited. The vast majority of reported edits have been for *MSTN*. Therefore, additional targets need to be identified. Second, efficacy of generating successful edits remains low, though there continue to be improvements in techniques to improve targeting efficiency. For example, the CRISPR/Cas9 system holds great promise for targeting efficiency. Third, there appears to be significant issues with both the use of SCNT and MI for the production of viable offspring. We have demonstrated substantial challenges in piglet viability following SCNT that appear unrelated to the viability issues related to *MSTN* genotype in pigs. Overcoming the issue of low viability after gene-editing may prove to be the most difficult task. Therefore, while gene-editing holds great promise to accelerate growth-promotion in livestock in the future, several technical challenges must be overcome before its true potential can be reached.

Abbreviations

DMR: Differentially methylated region; ET: Embryo transfer; FBXO40: F-Box protein 40; *IGF1*: Insulin-like growth factor 1; *IGF2*: Insulin-like growth factor 2; LOF: Loss of function; MI: Microinjection; MI-IVF: Microinjection during in vitro fertilization; *MSTN*: Myostatin; MKO: Myostatin knock-out; QTL: Quantitative trait loci; QTN: Quantitative trait nucleotide; SCNT: Somatic cell nuclear transfer; WT: Wild type.

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Author contributions

LH, BM, and XC collected and analyzed the data. AD and JE wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed and during the current study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate

Approval for animal use was obtained from the University of Illinois Institutional Animal Care and Use committee (protocols 13332, 17021, 19262).

Consent for publication

All authors have consented to this submission.

Competing interests

The funders listed above had no role in the composition or writing of the manuscript, or in the decision to publish.

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