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Interactions between genes involved in growth and muscularity in pigs: *IGF-2*, *myostatin*, *ryanodine receptor 1*, and *melanocortin-4 receptor*

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Abstract

In the swine breeding industry, two economical traits are of particular importance in sires, namely, muscle growth and average daily gain (ADG). These traits are quantitative, which implies that they are under the control of multiple genes. Mutations in these genes, associated with either muscularity or growth, are useful quantitative trait nucleotides (QTN) for unraveling genetic variation of these traits and can be used in marker-assisted selection. Until now, QTN involved in muscle growth and/or ADG in pigs were identified in porcine *ryanodine receptor 1* (*RYR1*), *insulin-like growth factor-2 (IGF-2),* and *melanocortin-4 receptor* (*MC4R*). Recently, a fourth possible QTN was found in porcine *myostatin* (*MSTN*). All four QTN have an influence on muscle growth and/or somatic growth, so an influence of one mutation on one or more of the other mutations should not be excluded. However, although the polymorphisms in the *RYR1* and the *MC4R* gene affect the function of the respective protein, the polymorphisms of the *IGF-2* and *MSTN* gene influence the mRNA expression of the respective gene. Therefore, this study investigated possible interactions between the genotypes of *MSTN*, *IGF-2,* and *MC4R* (population 1) or the *RYR1*, *IGF-2,* and *MSTN* QTN (population 2) on *IGF-2* and *MSTN* expression in different muscle types in pigs. In both skeletal muscle and heart muscle growth, the *IGF-2:MSTN* ratio seems to play an important role. Also, the *RYR1* genotype had a significant effect on *IGF-2* expression in m. longissimus dorsi. No effect of the *MC4R* QTN could be seen.

Keywords: Interactions; IGF-2; Myostatin; Melanocortin-4 receptor; Ryanodine receptor 1

1. Introduction

The physiological regulation of muscle growth and average daily gain (ADG) in animals is under the control of multiple genes. Polymorphisms in these candidate genes that show association with specific economically important traits are useful quantitative trait nucleotides (QTN) for marker-assisted selection. Previously, in pigs, three important QTN involved in muscle growth and/or ADG, located in the *ryanodine receptor 1* (*RYR1*), *IGF-2,* and *melanocortin-4 receptor* (*MC4R*) gene, respectively, had been identified [1–3]. Recently, a fourth possible

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QTN was found in the porcine *myostatin* (*MSTN*) gene [4].

The first QTN involved in muscularity in pigs was detected in the *RYR1* gene, the gene encoding the predominant calcium release channel in the sarcoplasmic reticulum of skeletal muscle. A substitution at position 1843 in the porcine *RYR1* gene (*Ryr1* g.1843C>T), leading to an alteration from an arginine residue to a cysteine residue, was found to be associated with greater lean meat content and muscularity in different pig breeds, including the heavily muscled Piétrain breed [1].

In 2003, a second QTN involved in porcine muscle growth was discovered in the paternally expressed porcine *IGF-2* gene [3,5,6]. The IGF-2 protein plays a key role in skeletal muscle growth [7,8]. A substitution in an evolutionary conserved CpG island in intron three of the *IGF-2* gene (*IGF-2* intron3-g.3072G > A) leads to a 3-fold increase in *IGF-2* mRNA expression and a subsequent increase in muscle growth and heart size and a decrease in fat deposition [3].

A third known QTN in pigs is located in the *MC4R* gene. However, different from the two previously discussed QTN, the influence of the mutation in *MC4R* is mainly on somatic growth and to a lesser extent on muscle growth [2]. The MC4R gene is a G-protein coupled receptor with a vital role in mediating the effect of leptin on food intake and energy balance [2,9–11]. Characterization of the porcine *MC4R* gene revealed a guanine-to-adenine substitution at position 893 of the coding sequence of *MC4R* (*MC4R* c.893G > A). This missense mutation replaced an aspartic acid residue (GAU) with an asparagine (AAU) residue and, in the original study, had a clear effect on backfat and ADG [2]. However, in later studies using a different population, these results could not unambigously be confirmed [9–12].

The fourth and last QTN included in the study is a possible QTN located in the promoter of the porcine *MSTN* gene. Myostatin is a negative regulator of growth and development of muscle mass. Since 1997, naturally occurring mutations leading to the so-called double-muscling phenotype were described in the *MSTN* gene of cattle, dogs, humans, sheep, and mice [13–19]. In pigs, one particular breed, the Belgian Piétrain, also shows a heavily muscled phenotype. The similarity of muscular phenotypes between double-muscled cattle and Piétrain pigs suggested *MSTN* as a candidate gene for muscular hypertrophy in pigs. Very recently, an adenine-to-guanine substitution was found in the promoter of the porcine *MSTN* gene (*MSTN* g.447A > G). This mutation led to an abrogation of a myocyte enhancer factor three binding site and a subsequent decrease in *MSTN* expression at the age of four week [4].

Since the mutations in *RYR1, IGF-2, MC4R,* and *MSTN* all have an influence on muscle and/or somatic growth, there is a possibility that an influence of one QTN on one or more of the other QTN exists. To test this hypothesis, the effect of each of the QTN on the *IGF-2* and *MSTN* expression was investigated, along with the effects of each combination of two of the four QTN studied. No expression of *RYR1* or *MC4R* was measured, since the polymorphisms in these two genes are missense mutations and lead to an inactive protein instead of leading to differential expression of the genes [1,2]. Since our populations were originally composed for research on the effect of the *IGF-2* genotype or the combination of the *IGF-2* and the *RYR1* genotype, our populations were too limited to test all polymorphisms at once. Therefore, the analysis was split into 2; in a first population, the interaction between the *IGF-2, MC4R,* and *MSTN* polymorphisms was tested, whereas in a second population, the interaction between the *RYR1, IGF-2,* and *MSTN* QTN was tested.

2. Materials and methods

2.1. Animals

This experiment was conducted under the supervision of the ethical committee of the University of Leuven.

Population 1 consisted of animals originating from 2 damlines selected for high prolificacy from Rattlerow-Seghers (Lokeren, Belgium). Line A is a Landrace line and line B is a pure Large White line; both lines are homozygous wildtype (CC) for the *Ryr1* g.1843C > T mutation. The sires were heterozygous for the *IGF-2* intron3-g.3072G > A mutation to obtain offspring of both genotypes. Intact male piglets of the 2 damlines were genotyped for the *IGF-2* intron3-g.3072G > A (wildtype [G^{pat}] or mutant [A^{pat}]), the *MSTN* g.447A > G (homozygous wildtype [AA^{M}], heterozygous [AG^{M}], or homozygous mutant [GG^{M}]), and the *MC4R* c.893G > A (homozygous wildtype [GG^{R}], heterozygous [AG^{R}], or homozygous mutant [AA^{R}]) mutation. Piglets were selected based on their combined genotypes. Numbers of animals per group varied from three to eight.

For population 2, 13 sows, heterozygous for the *RYR1* g.1843C > T mutation, were mated to two sires that were heterozygous for the mutation in *IGF-2* and *RYR1*. These animals were selected to obtain all combined genotypes for *RYR1* and *IGF-2*. Piglets were genotyped for the QTN in *IGF-2* (A^{pat} or G^{pat}), *Ryr1* (CC, heterozygous [CT] or homozygous mutant [TT]), and *MSTN* (AA^{M}, AG^{M} or GG^{M}). Pigs were selected based on their combined genotypes for the markers. Numbers of ani-

mals per group are given in Table 3. Both populations were previously described in [20] and [4]; however, in this study additional animals were selected and additional genotypings were performed.

In both populations, tails were collected at birth or blood samples were taken at 2-4 wk of age by puncture of the jugular vein. The piglets were weaned at four week of age and then kept in the rearing unit until eight week. They were fattened in the finishing unit at a different site until 26 wk (population 1) or approximately 109 kg (population 2). In the first population, pigs were slaughtered at 26 wk and samples were taken from m. masseter (M) and m. longissimus dorsi (LD). These skeletal muscles were selected because of large differences in oxido-glycolytic metabolism. In population 2, pigs were slaughtered at an average live weight of 109 kg and samples were taken of m. triceps brachii (TB), LD, and heart (H). In both populations, the animals were slaughtered in compliance with the current ethical guidelines for animal welfare. Carcasses were chilled at 4 °C. The entire muscle was removed from the carcass, after which the epimysium was carefully dissected. Muscle samples were taken within an hour after slaughtering. A sample of about 2 g was taken and snap-frozen in liquid nitrogen. The samples were stored at -80 °C until further analysis.

2.2. Extraction of DNA and RNA

From whole blood and semen, DNA was extracted according to the method described by Sambrook and Russell [21]. Total RNA was extracted from muscle samples using TriReagent (Sigma-Aldrich). The amount of extracted DNA and RNA was quantified by measuring the absorbance at 260 nm with a UV spectrophotometer.

2.3. Genotyping

An allelic discrimination assay was performed for the *IGF-2* intron3-g.3072G > A mutation using the ABI Prism 7700 sequence detection system (Applied Biosystems), as described by Stinckens et al [20]. The g.1843C > T substitution in *RYR1* found by Fujii et al [1] was also analyzed as described by Stinckens et al [20]. For analysis of the c.893G > A substitution in *MC4R* found by Kim et al [2], a 589-bp sequence was amplified using forward primer 5'-TTACTCGCCTCAATT-TGCAGC-3' and reverse primer 5'-ACAAATCACAGAGGCCACC-3'. Digestion of the resulting PCR product with *Taq*I was carried out using standard conditions. Genotyping of *MSTN* was done as described in Stinckens et al [4].

2.4. Measurement of IGF-2 and MSTN expression using quantitative real-time polymerase chain reaction

A standard protocol for the reverse transcription step was followed [21]. Real-time polymerase chain reaction (PCR) was performed in triplicate using the ABI Prism 7700 sequence detection system. For measurement of the *IGF-2* expression, PCR primers and specific Taqman probes were according to Van Laere et al [3]. Measurement of *MSTN* expression was done as described by Stinckens et al [4]. For both genes, the expression levels of each group of animals were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* expression. The expression of the *GAPDH* gene was analyzed using a modification of the comparative Ct method ($2^{-\Delta\Delta Ct}$ method), the $2^{-\Delta Ct}$ method. In this method, the expression of the housekeeping gene is presented as $2^{-\Delta Ct}$, where ΔCt ($Ct_{group\ x} - Ct_{reference\ group}$) and reference group represents the group with the lowest expression of the housekeeping gene [22,23]. Glyceraldehyde-3-phosphate dehydrogenase was found to be consistent in all groups studied and therefore was found to be a good internal control for this experiment.

2.5. Statistical analysis

The two most commonly used methods for the relative quantification of real-time data are the comparative Ct method and the standard curve method. Both methods are perfectly compatible. The standard curve method can be used in every real-time assay with good PCR efficiencies for both the target and the reference genes but has the disadvantage that in every real-time assay, a standard curve has to be run. This is not the case when using the comparative Ct method. However, to use the comparative Ct method, the PCR efficiencies of the target and the reference genes should be approximately equal. A sensitive method to analyze if two amplicons have the same PCR efficiency is to observe the ΔCt variation with dilution. If the absolute slope of this graph is close to zero, the assumption of equal PCR efficiencies is met [23]. Based on the slope of the dilution curve of *IGF-2/GAPDH* and *MSTN/GAPDH* real-time data, it was concluded that the *IGF-2* expression data could be analyzed using the comparative Ct method [23]. Because of the calculation method of the comparative Ct method, the confidence interval is asymmetrically distributed relative to the average value. Therefore, the relative *IGF-2* expression results are displayed as the fold change relative to the experimental group with the lowest expression of *IGF-2,* and confidence intervals are given. The *MSTN*

expression data, however, could not be analyzed with the comparative Ct method and were analyzed using the standard curve method, in which standard errors are symmetrically distributed, and so relative *MSTN* expression results are displayed as the fold change relative to the experimental group with the lowest expression of *MSTN* ± standard error.

In population 1, data were analyzed with a univariate general linear model (GLM) that included the *IGF-2* paternal allele, *MSTN* g.447A > G genotype, *MC4R* c.893G > A, boarline, damline, and muscle as fixed factors and their interactions.

In the second population, data were analyzed with a univariate GLM with the *IGF-2* paternal allele, *RYR1* genotype, *MSTN* genotype, muscle, and sex as fixed factors and their interactions. When a variable was significant, factor level means were compared with the Tukey comparison of mean test. For significant 2-way interactions, means for one factor were compared within the other factor. *P* values < 0.05 were considered as significant. The statistical analyses were done using SAS, version 9.1.3 for Windows.

The first population was originally set up to test the effect of the *IGF-2* genotype on *IGF-2* expression, whereas the second population was set up to test the 2-way interactions between the *IGF-2* and the *RYR1* genotype. Afterward, the animals were also genotyped for the *MSTN* g.447A > G and *MC4R* c.893G > A mutation or solely the *MSTN* g.447A > G mutation, respectively, and groups for the combined genotype of *IGF-2, MSTN,* and *MC4R* or *IGF-2*, *RYR1,* and *MSTN*, respectively, are small. Because of the limited number of animals per group, it was impossible to interpret the 3-way interaction between the three genotypes, and it was moved to the error term.

3. Results

3.1. Myostatin expression

3.1.1. Population 1

In population 1, relative expression of *MSTN* in LD in animals at 26 wk of age was determined. No significant effects were found for any of the factors included in the model (*IGF-2* paternal allele, *MSTN* genotype, *MC4R* genotype, boarline, sowline, and their interactions).

3.1.2. Population 2

In population 2, relative *MSTN* expression in three muscle types (LD, TB, and H) was determined. The model with *MSTN* genotype ($P < 0.05$) and muscle ($P < 0.0001$) as fixed factors, and their 2-way interaction ($P < 0.0001$) explained 55% of the variation ($P < 0.0001$).

Since muscle types differed ($P < 0.0001$), all three muscles were analyzed seperately. In LD, a model with *MSTN* genotype ($P < 0.001$) as fixed factor explained 50% of the variation ($P < 0.001$). Animals that were homozygous for the mutation in *MSTN* (AA^M and GG^M) had a significantly lower *MSTN* expression compared to heterozygous animals (Table 2).

In TB and H, the model with *IGF-2* paternal allele, *RYR1* genotype, *MSTN* genotype, muscle, and sex as fixed factors and their interactions was not significant (Table 2).

3.2. IGF-2 expression

3.2.1. Population 1

In population 1, relative *IGF-2* expression in two muscles (M and LD) was determined. The model that included the *IGF-2* paternal allele ($P < 0.0001$) and muscle ($P < 0.0001$) as fixed factors explained 84% of the variation ($P < 0.0001$).

Since *IGF-2* expression over muscle types differed ($P < 0.0001$), both muscles were analyzed seperately. In LD and M, a model with the *IGF-2* paternal allele (LD: $P < 0.005$; M: $P < 0.0001$) as a fixed factor explained 32% and 49% of the variation, respectively (LD: $P < 0.005$; M: $P < 0.0001$). In both muscle types, there was greater *IGF-2* expression in animals that inherited the mutant allele from their sire (A^{pat}) compared with those animals that received the wildtype allele from their sire (G^{pat}).

Also, both *IGF-2* genotypes were analyzed seperately. In A_{pat} and G_{pat}, a model with muscle (A_{pat}: $P < 0.0001$; G_{pat}: $P < 0.0001$) as fixed factor explained 82% and 70% of the variation, respectively (A_{pat}: $P < 0.0001$; G_{pat}: $P < 0.0001$). In both genotypes, the *IGF-2* expression in M was greater than the expression of *IGF-2* in LD.

3.2.2. Population 2

In population 2, relative *IGF-2* expression in three muscle types (H, LD, and TB) was determined. The model with the *IGF-2* paternal allele ($P < 0.0001$), *MSTN* genotype ($P > 0.05$) and muscle ($P < 0.0001$) as fixed factors and the 2-way interaction between *IGF-2* and *MSTN* ($P < 0.005$) explained 77% of the variation ($P < 0.0001$).

Since *IGF-2* expression over muscle types differed, all three muscles were analyzed seperately. In different muscles, different factors and/or interactions were significant (Table 2). Relative $2^{-\Delta\Delta Ct}$ values are given in Table 3.

Table 1
Relative expression of myostatin (*MSTN)* in population 2.

	LD	TB	H
AA^M	$1.00^A \pm 0.23$ (8)	$1.00^A \pm 0.17$ (8)	$3.00^A \pm 1.05$ (5)
AG^M	$2.27^B \pm 0.29$ (8)	$1.29^A \pm 0.23$ (7)	$1.00^A \pm 0.13$ (10)
GG^M	$1.06^A \pm 0.11$ (8)	$1.11^A \pm 0.20$ (8)	$2.59^A \pm 0.58$ (13)

Abbreviations: LD: m.longissimus dorsi; TB: m. triceps brachii; H: heart, ^{A,B}: means within the muscle lacking a common superscript differ ($P < 0.05$).
Note: Real-time data were analyzed with the standard curve method and therefore presented as *[MSTN]/[GAPDH]* values. In this analysis, GAPDH serves as a housekeeping gene. Within a muscle, expression values are displayed as the fold change relative to the experimental group with the lowest expression of MSTN (mean ± SEM). The number of animals per group is given in parentheses.

In all three muscle types, an effect of the *IGF-2* paternal allele was seen (LD: $P < 0.0001$; H: $P < 0.005$; TB: $P < 0.005$). In each of the examined muscles, a greater *IGF-2* expression was found in animals that inherited the mutant allele from their father (A^{pat}) compared with those animals that received the wildtype allele from their sire (G^{pat}).

In LD, an effect of the *RYR1* genotype was also found ($P < 0.05$). In this muscle, *IGF-2* expression in animals with the A_{pat}/CC genotype was higher than expression in animals that had the A_{pat}/TT genotype.

In TB, the 2-way interaction between *IGF-2* and *MSTN* was found to be significant ($P < 0.005$).

4. Discussion

In this study, the influence of four known QTN for muscle and somatic growth in pigs on *MSTN* and *IGF-2* expression in different muscle types was examined. In double-muscled, *MSTN* knockout mice, it was found that a decrease in *MSTN* expression led subsequently to a greater expression of *IGF-2* [24]. However, the same effect in the opposite direction was observed in rats that suffered a loss of skeletal muscle mass owing to the microgravity environment of a space shuttle flight [25]. Based on these results, it was hypothesized that the ratio of mRNA and protein concentrations between *IGF-2* and *MSTN* may serve as indicators of muscle mass. In the muscle of animals with normal function of both growth factors, a homeostatic balance between anabolic and catabolic factors exists. However, a mutation in one or both of the growth factors with an influence on the mRNA or protein abundance might disturb this balance and lead to an increase (high ratio) or decrease (low ratio) in muscle mass [24,25]. In this study, both *MSTN* and *IGF-2* expression were measured; therefore, the *IGF-2/MSTN* ratio might be a useful tool for interpreting the results described in this paper. Since it was found that there was a difference in influence of the QTN on growth in skeletal versus heart muscle, both muscle types will be discussed separately.

4.1. Skeletal muscle growth

In skeletal muscle, the effect of the *IGF-2* intron 3-g.3072G > A mutation on *IGF-2* mRNA levels was noticeable throughout the entire postnatal period [3,20] (Tables 1–3). Also, in the LD of animals during the 26th wk of population 2, a significant effect of the *MSTN* QTN on *MSTN* expression was found. However, in the LD of animals of population 1, no such effect was seen. Previously, in the same populations, it was suggested that this difference might be the effect of background line development rather than the effect of the *MSTN* QTN. This hypothesis was supported by the fact that, in total, four different populations of pigs at slaughter weight gave three different outcomes concerning the effect of the *MSTN* mutation [4,26].

Combining the data gathered on the effect of the mutation in both the *IGF-2* and *MSTN* genes on *IGF-2* and *MSTN* expression in this study, it could be summarized that at the age of 26 wk, no influence on *MSTN* expression was found, but a significantly greater *IGF-2* expression in animals with the mutant paternal *IGF-2* allele compared to wildtype animals could be witnessed. Therefore, animals that inherited the mutant allele from the sire have a greater *IGF-2:MSTN* ratio compared to wildtype animals, which leads to an increase in muscle mass [24,25]. This increase in muscle mass could be explained by the fact that the QTN in *IGF-2* at this

Table 2
P values (and R^2) resulting from the analysis of IGF-2 expression in H, LD, and TB with the univariate general linear model with the *IGF-2* paternal allele, *RYR1* genotype, *MSTN* genotype, muscle, and sex as fixed factors and their interactions (Population 2).

Muscle	R^2	P_{model}	IGF-2 genotype	RYR1 genotype	IGF-2 * MSTN
m. longissimus dorsi	0.80	<0.0001	<0.0001	< 0.05	NS
heart	0.39	< 0.005	< 0.005	NS	NS
m. triceps brachii	0.76	< 0.0005	< 0.005	NS	< 0.005

Table 3
Relative expression of *IGF-2* in population 2 expressed in comparative Ct ($2^{-\Delta\Delta Ct}$) values.

Gene 1	Gene 2	IGF-2 Expression								
IGF-2	MSTN	LD			TB			H		
A^{pat}	AA^{M}	5.22^{AC}	[3.88-7.04]	(4)	8.86^{AC}	[5.97-13.16]	(4)	2.29B	[1.71-3.05]	(4)
	AG^{M}	2.31^{ACD}	[0.92-5.81]	(3)	3.71^{BC}	[3.35-4.10]	(3)	3.93^{AB}	[3.08-5.01]	(3)
	GG^{M}	7.53^{A}	[6.39-8.87]	(4)	17.06^{A}	[14.01-20.78]	(4)	6.57^{A}	[4.84-8.91]	(4)
G^{pat}	AG^{M}	1.00^{BD}	[0.84-1.19]	(4)	2.89^{BC}	[2.30-3.65]	(5)	1.92^{B}	[1.37-2.70]	(5)
	GG^{M}	1.14^{BD}	[0.97-1.34]	(3)	1.00^{B}	[0.63-1.59]	(4)	1.00^{B}	[0.77-1.31]	(4)
IGF-2	**RYR1**									
A^{pat}	CC	8.01^{A}	[7.10-9.05]	(4)	5.29^{AC}	[4.03-6.94]	(6)	3.94^{AC}	[3.46-4.49]	(4)
	CT	4.21^{AC}	[3.49-5.07]	(6)	6.91^{A}	[4.77-10.02]	(6)	2.57^{ABC}	[1.94-3.40]	(5)
	TT	2.29^{BC}	[1.53-3.44]	(6)	8.06^{A}	[5.38-12.06]	(6)	6.34^{C}	[4.92-8.18]	(4)
G^{pat}	CC	1.15^{B}	[1.06-1.25]	(5)	1.65^{CD}	[1.21-2.24]	(6)	1.57^{AB}	[1.15-2.16]	(6)
	CT	1.00^{B}	[0.87-1.15]	(5)	1.00^{BD}	[0.71-1.41]	(6)	1.00^{B}	[0.79-1.26]	(6)
RYR1	**MSTN**									
CC	AA^{M}	3.31^{A}	[2.09-5.23]	(4)	2.20^{A}	[1.79-2.71]	(4)	1.55^{B}	[1.06-2.27]	(4)
	AG^{M}	1.95^{A}	[1.06-3.62]	(3)	1.38^{A}	[1.16-1.64]	(4)	2.88^{A}	[2.30-3.60]	(4)
	GG^{M}	2.29^{A}	[1.22-4.32]	(4)	1.53^{A}	[0.68-3.42]	(4)	2.25^{AB}	[1.14-4.46]	(4)
CT	AG^{M}	1.00^{A}	[0.58-1.71]	(3)	1.00^{A}	[0.74-1.36]	(3)	1.00^{AB}	[0.68-1.47]	(3)
	GG^{M}	3.17^{A}	[1.89-5.32]	(3)	1.76^{A}	[0.67-4.66]	(4)	1.76^{AB}	[1.04-2.99]	(4)

Abbreviations: H, heart; LD, m. longissimus dorsi; TB, m.triceps brachii.
Note: Confidence intervals are given in square brackets. Expression levels are displayed as the fold change relative to the experimental group with the lowest expression of *IGF-2*. Comparative Ct and significance values should be considered within one group (*IGF-2/MSTN, IGF-2/RYR1 and RYR1/MSTN*) and muscle (vertically).
^{A,B,C,D}: means within the same group lacking a common superscript differ ($P < 0.05$).
Number of animals per combined genotype is given in parentheses.

age could be associated with the proliferation and differentiation of the muscle satellite cells. At the age of 26 wk, most of the differentiation of the tertiary myofibers has finished, and the proliferation and differentiation of satellite cells plays an important role in muscle growth [27,28]. This finding is supported by the fact that Van den Maagdenberg et al [29] found that the activity of calpastatin was greater and the m-calpain:calpastatin ratio was significantly lower in animals that inherited the mutant paternal *IGF-2* allele compared to wildtype pigs. Calpastatin is a specific inhibitor of the calpains, a family of Ca^{2+}-sensitive muscle endopeptidases. Greater activity of calpastatin will therefore lead to a suppression of muscle protein degradation. The m-calpain:calpastatin ratio is an expression of the proteolytic potential of a muscle and has been shown to be related to myoblast fusion [30,31]. Clearly, the ratio of m-calpain over calpastatin is very important in a normal muscle cell fusion process and can therefore have an effect on muscle growth by modulating satellite cell activity [32].

Another important result found in this study is an effect of the *RYR1* genotype on *IGF-2* expression. This effect was seen in LD but not in TB, and it has been discussed previously by Stinckens et al [20] in the same population as used here. The explanation for this influence proposed in this paper was the altered Ca^{2+} dependence of the mutant ryanodine receptor, which leads to a higher Ca^{2+} efflux out of the ryanodine receptor 1 [20]. Since it is known that cellular Ca^{2+} levels have an important role in the mRNA stability and decay of several genes, this increased Ca^{2+} level could have a negative effect on the stability of the *IGF-2* mRNA [33]. In different genes, specific regions (*cis*-acting elements) in the 3′ untranslated region of RNA, together with specialized proteins (*trans*-acting factors), determine the mRNA stability and hence the level of expression of these proteins. The interactions between these *cis*- and *trans*-acting factors may be modulated by Ca^{2+} levels, either directly or via the control of the phosphorylation status of the *trans*-acting factors [33]. The explanation for why the *RYR1* genotype in LD has an effect on *IGF-2* expression, whereas in TB no effect could be seen, should be sought in the differences in fiber typing and capillary network between both muscles. Muscles with the combination of a large number of type IIb fibers and a low vascular network, like LD, are shown to be very sensitive to the mutation in *RYR1*. However, muscles like TB, with a low number of type IIb fibers and a good capillary system, are much less prone to this mutation [34].

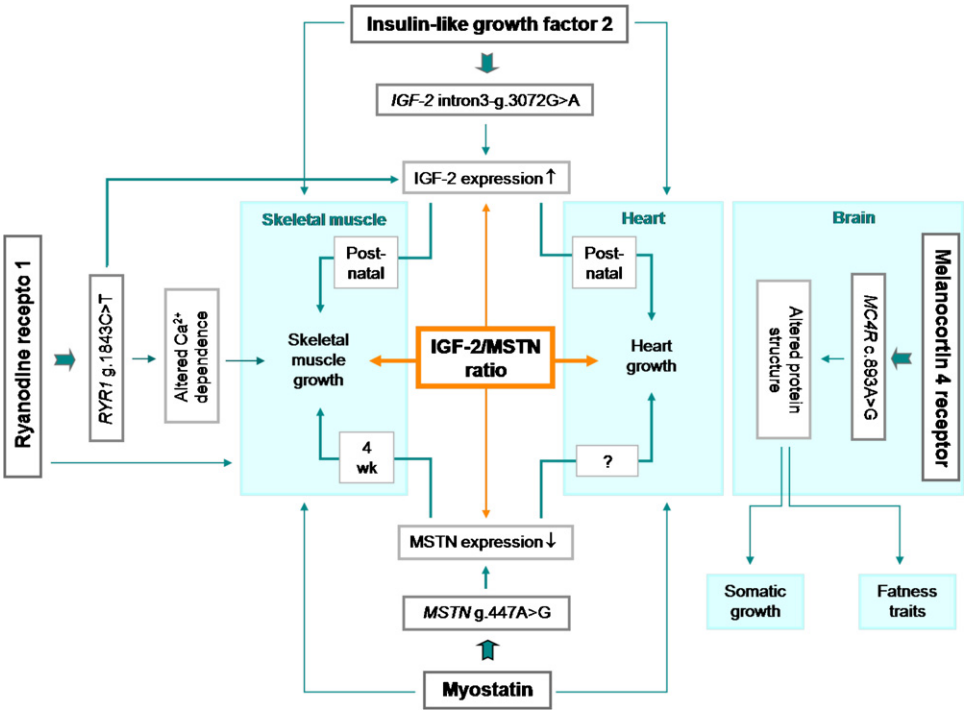

Fig. 1. Summary of the hypothesized interactions between *IGF-2*, *Myostatin (MSTN)*, *Ryanodine receptor 1 (RYR1)*, and *Melanocortin-4 receptor (MC4R)* and their respective QTN. The recurring aspect in this schema is the influence of the *IGF-2/MSTN* ratio on both skeletal and heart muscle growth. Furthermore, in skeletal muscle, an effect of *RYR1* genotype on *IGF-2* expression can be seen. In both skeletal and heart muscle, no effect of the mutation in *MC4R* on *IGF-2* or *MSTN* expression could be found. Block arrows indicate a mutation found in a particular gene. Normal arrows indicate proposed actions. The localization within the body of the pig of the expression of the genes and the effects of the mutations is grouped in three blue areas: skeletal muscle, heart muscle, and brain.

4.2. Heart muscle growth

Although a lot of information exists on the effect of both *IGF-2* and *MSTN* in skeletal muscle growth, the information on their function in heart muscle growth is significantly less abundant. In this study, expression of the *MSTN* gene was detected in the cardiac muscle of pigs of population 2. These data coincide with the findings of Sharma et al [35], who found that *MSTN* expression was also apparent in the heart muscle of both fetal and adult sheep and that the primary structure of heart *MSTN* was well conserved in mammals. More recently, it was found that *MSTN* levels were dynamically regulated in the heart and, as in skeletal muscle, act to modulate cardiomyocyte hyperthrophic and hyperplastic growth, depending on the physiological and growth status of the cardiomyocytes [36,37].

A role for the *IGF-2* gene in fetal rat myocyte proliferation was established in 1996 [38]. However, it was not until the finding of a QTL with an effect on skeletal and cardiac muscle mass in pigs, that an effect of IGF-2 on postnatal heart muscle growth was also postulated [5]. In heart muscle, *IGF-2* expression and the effect of the mutation in intron three of the *IGF-2* gene were found to occur throughout the entire postnatal life, that is, 3 wk until 26 wk [3,20] (Tables 2 and 3).

Therefore, since clear evidence exists for the role of both *MSTN* and *IGF-2* in pre- and postnatal cardiac growth, and since the developmentally regulated expression in both genes appears to be parallel, it could also be stated that in cardiac muscle, the ratio of mRNA and protein expression levels between *IGF-2* and *MSTN* may serve as indicators of muscle mass. Therefore, as in skeletal muscle growth, mutations in one or both genes could lead to increased (high ratio) or decreased (low ratio) myocyte growth [24,25].

In the present study, at the age of 26 wk, only an effect of the *IGF-2* genotype on *IGF-2* expression in the porcine heart was witnessed. No differential *MSTN* expression could be seen. As a consequence, *MSTN* expression has no effect on the *IGF-2/MSTN* ratio, and animals that inherited the mutant allele from the sire have a higher *IGF-2* over *MSTN* ratio compared to wildtype animals. Postnatal cardiac growth is mainly a result of hypertrophy of the existing cardiomyocytes. Especially later in adult life, no effect of proliferation

of cardiomyocytes could be witnessed [37]. Therefore, the increased *IGF-2/MSTN* ratio in postnatal cardiac muscle will predominantly increase hypertrophic cardiomyocyte growth and thus increase cardiac muscle mass.

4.3. Conclusion

Based on the results of this study, combined with those in the published literature, a schema was drawn summarizing the hypothesis presented in this paper (Fig. 1). The recurring aspect in this schema is the influence of the *IGF-2/MSTN* ratio on both skeletal and heart muscle growth. Furthermore, in skeletal muscle, an effect of the *RYR1* genotype on *IGF-2* expression can be seen. In both skeletal and heart muscle, no effect of the mutation in *MC4R* on *IGF-2* or *MSTN* expression could be found.

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