

Research article

## Development of a porcine skeletal muscle cDNA microarray: analysis of differential transcript expression in phenotypically distinct muscles

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### Abstract

**Background:** Microarray profiling has the potential to illuminate the molecular processes that govern the phenotypic characteristics of porcine skeletal muscles, such as hypertrophy or atrophy, and the expression of specific fibre types. This information is not only important for understanding basic muscle biology but also provides underpinning knowledge for enhancing the efficiency of livestock production.

**Results:** We report on the *de novo* development of a composite skeletal muscle cDNA microarray, comprising 5500 clones from two developmentally distinct cDNA libraries (*longissimus dorsi* of a 50-day porcine foetus and the *gastrocnemius* of a 3-day-old pig). Clones selected for the microarray assembly were of low to moderate abundance, as indicated by colony hybridisation. We profiled the differential expression of genes between the *psaos* (red muscle) and the *longissimus dorsi* (white muscle), by co-hybridisation of Cy3 and Cy5 labelled cDNA derived from these two muscles. Results from seven microarray slides (replicates) correctly identified genes that were expected to be differentially expressed, as well as a number of novel candidate regulatory genes. Quantitative real-time RT-PCR on selected genes was used to confirm the results from the microarray.

**Conclusion:** We have developed a porcine skeletal muscle cDNA microarray and have identified a number of candidate genes that could be involved in muscle phenotype determination, including several members of the casein kinase 2 signalling pathway.

### Background

Skeletal muscle is a heterogeneous tissue that has the ability to rapidly undergo biochemical and physical changes in response to external stimuli, such as appropriate nervous and hormonal stimulations, to adapt to the accompa-

nying functional demands imposed on it. There is wide variation in phenotype between anatomical muscles in the body. Postural muscles, often described as red muscles, such as the *soleus* and *psaos* in the pig, are under continuous but modest activation. They are adapted to

undertake chronic contractile activity without fatigue, under aerobic respiratory conditions. On the other hand, white muscles, such as the *gastrocnemius* and *longissimus dorsi*, are recruited sporadically during brief periods of intense muscular activity, like running. They are susceptible to fatigue as their main source of energy is derived from anaerobic glycolysis. Red muscles are better endowed with capillaries, myoglobin, lipids and mitochondria than white muscles [1,2]. Red and white muscles also differ in their fibre type composition. Postnatal mammalian muscles (e.g. in pigs and rodents) have 4 major fibre types characterised by the expression of the slow/ $\beta$ , 2a, 2x and 2b myosin heavy chain (MyHC) gene isoforms [2-4]. Based on MyHC isoform expression, postnatal muscle fibres in the pig have recently been resolved by the combination of immunocytochemistry and *in situ* hybridisation into 4 major types [5-7]. Metabolic, biochemical and biophysical characteristics, such as oxidative and glycolytic capacities, fibre size, colour, and glycogen and lipid contents, vary between MyHC fibre types [2,8,9]. The slow/ $\beta$  and fast 2b fibres, also known as slow oxidative and fast glycolytic respectively, represent two extreme metabolic profiles. The fast 2a and fast 2x fibres are intermediate fast oxidative-glycolytic fibres. Fast 2a fibres are more closely related to slow/I fibres, and fast 2x are more similar to fast 2b fibres. Hence the composition of fibre types in a muscle is a major determinant of its phenotypic properties.

Understanding the molecular processes that govern the phenotypic characteristics of muscles, such as hypertrophy/atrophy, and expression specific fibre types, is of agricultural and medical importance [9-12]. Microarray technology can simultaneously measure the differential expression of a large number of genes in a given tissue and may identify the genes responsible for the relevant phenotype [13]. We report here on the *de novo* development of a porcine skeletal muscle cDNA microarray, comprising 5500 clones from two cDNA muscle libraries. Its functional integrity was assessed by profiling the differences in gene expression between red (*psaos*) and white (*longissimus dorsi*, LD) muscles. Among the differentially expressed genes, a number of novel candidate genes were found that could determine the phenotypic differences among different muscle types.

## Results

### Construction of a composite porcine skeletal muscle cDNA microarray

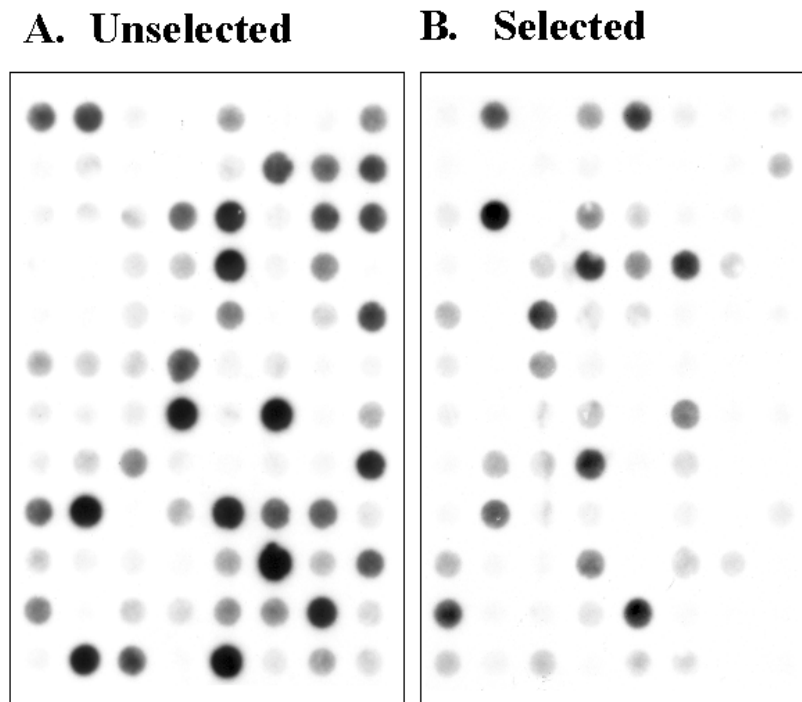
The *de novo* development of a porcine skeletal muscle cDNA microarray was based on the use of two representative, directionally cloned  $\lambda$ ZAP-Express cDNA expression libraries; one derived from the back muscle of a 50-day-old foetus and the other from the *gastrocnemius* of a 3-day-old pig. Genes that are expressed in skeletal muscle are likely to be present in one or both libraries. In our porcine

skeletal muscle cDNA libraries, about a third of the clones were found to be from highly expressed genes, based on signal intensity of hybridisation (Fig. 1A). Weakly to moderately expressed genes were selected for microarray assembly; highly expressed clones were avoided (Fig. 1B). On average, between a quarter and a half of the randomly picked colonies from each square agar plate were eventually selected for microarray assembly. A total of 5,500 plasmid clones were selected, of which 3,500 clones were taken from the 3-day-old muscle library and 2,000 clones were taken from the 50-day foetal muscle library. After each production step of miniprep plasmid preparation, insert amplification, and PCR product purification, about a quarter of the selected clones were checked by gel electrophoresis or spectrophotometry. Approximately 10% of all selected clones were found to be without any cDNA insert, a figure expected in a typical cDNA library (data not shown). Each selected clone was printed twice on the microarray (Fig. 2A).

### Functional assessment of the porcine cDNA microarray: red-white muscle analysis

To evaluate the performance of our composite porcine skeletal muscle cDNA microarray, a profiling experiment was conducted to determine the differential expression of genes between red (*psaos*) and white (*longissimus dorsi*, LD) muscle. Dual-colour hybridisation (Fig. 2A) was performed on seven replicate microarray slides. An intensity-dependent (LOWESS) step was used to normalise data. Fig. 2B plots the median expression of each clone in the *psaos* against the median expression of the same clone in the LD muscle. Each point is the median of 14 values (2 replicates per slide, 7 slides per muscle). Most points cluster around the middle line indicating similar levels of expression in both muscles. There were, however, a number of clones falling substantially below the line, indicating consistently lower levels of expression in the *psaos* compared with the LD muscle. Low signal readings for both dyes may indicate the absence of a cDNA insert. About 12% of the printed clones were found to fall below 50 units for both dyes and were considered to be without cDNA inserts. This value was consistent with the earlier estimate of insertless clones in the cDNA libraries.

A normalised *psaos*/LD ratio of 2.0 or more was used to identify genes that were more highly expressed in *psaos* than in LD muscle. This ratio represents the top 5% of differences in expression. Seventy clones meeting this criterion were sequenced. The sequences were compared with database sequences by BLAST searching. Table 1 is a summary of these genes. Sixty seven percent of the clones (47 out of 70) sequenced were genes of mitochondrial origin. Of these several were featured more than once on the microarray, namely genes encoding 16S ribosomal RNA, 12S ribosomal RNA, and NADH dehydrogenase subunits



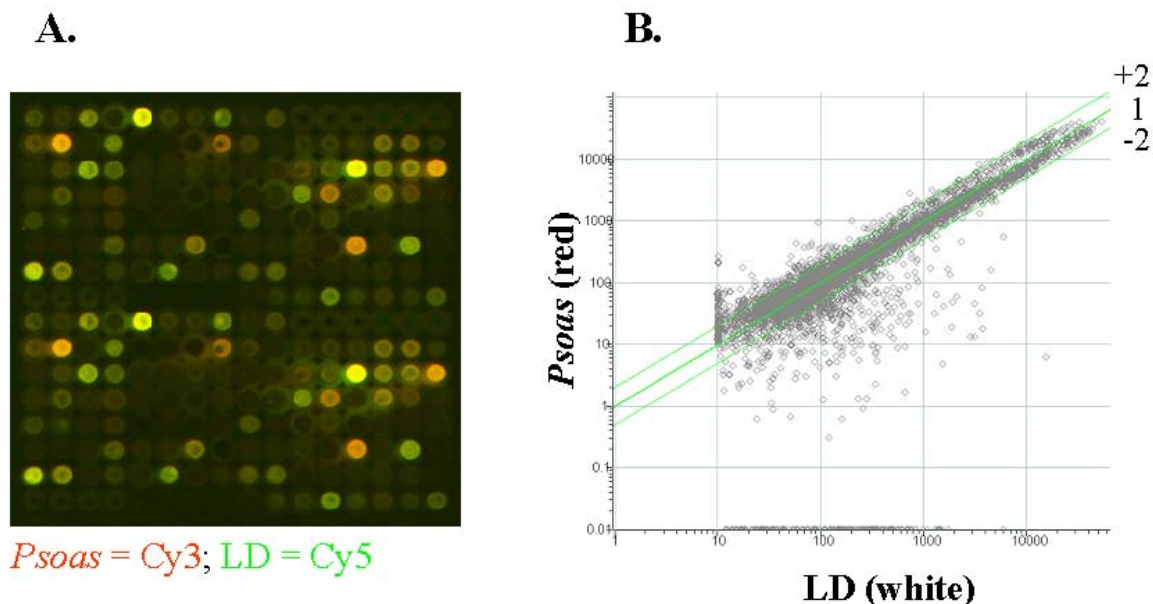
### Figure 1

Selection of clones for porcine skeletal muscle cDNA microarray assembly by colony hybridisation. **A.** A typical colony dot-blot hybridisation result of randomly selected plasmid clones, from a 50-day-old foetal back muscle cDNA library, probed with  $^{32}\text{P}$ -labelled total cDNA, derived from the same muscle of a 50-day-old foetus. **B.** A typical hybridisation result of selected plasmid clones, assessed by previous hybridisation (**A**) to be from weakly to moderately expressed genes. A reduction in the number of highly expressed clones could be seen in panel B.

3 and 6. Thirty percent of the clones (21 out of 70) did not show any homology with known mitochondrial or sarcomeric genes. The function of 9 of these clones was completely unknown. One gene encoded for fructose-1,6-biphosphatase, an enzyme that is necessary for muscle gluconeogenesis. The function of the remaining 11 clones were involved with aspects of transcription, translation or signal transduction, but their functions in skeletal muscle have not been characterised. Several genes were members of the casein kinase 2 signalling pathway. The  $\alpha 1$  subunit of casein kinase 2 (CK2) is one half of the holoenzyme [14,15]. The small muscle protein (smpx) is encoded by a recently discovered X-linked stretch response gene [16].

The tyrosine kinase A6-related protein binds ATP and actin, and interacts with protein kinase C zeta [17]. Although the function of the latter two proteins is not known, both were shown to be targets of CK2 phosphorylation.

Clones with a normalised *psaos*/LD ratio of 0.7 or less, which represented the most extreme 5% of clones were identified. Forty-five clones were sequenced and examined for homology by BLAST searching. Table 2 lists the genes that were more highly expressed in the LD than in the *psaos*. Only 4 out of 45 clones were of mitochondrial origin, and they were different from those expressed more



**Figure 2**

Differential gene expression between red and white porcine muscles. **A.** An illustration of a two-colour hybridisation. There were 48 such grids on each microarray slide. Each clone was printed twice. Clones on the top half were duplicated in the lower half of each grid. With some spots, the surrounding background was higher than the signal of the corresponding spots. **B.** Scatter plot displaying the median expression profile of all genes represented on the microarray, based on seven replicate slides. Genes equally expressed in red and whites would be positioned along the middle line (1). Points above the +2 or below -2 represent genes that were at least 2-fold more or less highly expressed in the *psoas* than the LD.

abundantly in the *psoas* (Table 1). Fast isoforms of sarcomeric proteins (myosin heavy chains [MyHCs] 2a, 2x, 2b, myosin regulatory light chain 2,  $\alpha$ -actinin 3, fast troponin C, and fast troponin T3) were well represented amongst the sequenced clones. Sarcomeric/structural genes made up nearly half of the total clones (22 out of 45). The other highly represented group of genes on the list (11 out of 45 clones) were involved in glycolysis, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The function of two non-sarcomeric genes was completely unknown. A few candidate regulatory genes (bin1, polyubiquitin, myomegalin-like, and HSPA8) were also found. Of particular interest is the tumour suppressor gene bin1 [18], which has recently been shown to play a role in C2C12 myoblast differentiation [19].

#### Quantitative expression of selected genes: validation by TaqMan real time RT-PCR

To assess the validity of the microarray approach to identify differentially expressed genes, quantitative real-time RT-PCR (TaqMan) was performed on four representative clones, GAPDH, MyHC 2b, bin1, and a novel gene (kc2725), each normalised to  $\beta$ -actin. In line with functional expectations, recent quantitative work performed has shown that the mRNA expression of GAPDH and MyHC 2b was higher in the *psoas* than in the LD within individual animals [20]. In this study, the relative levels of GAPDH and MyHC 2b of pooled total cDNA samples from three 22-week-old Berkshire pigs were measured (Fig. 3). Our results showed that the differential expression of GAPDH and MyHC 2b was sufficiently consistent between the two muscles to be detected in pooled cDNAs. A novel gene kc2725 of unknown function, found by

**Table 1: Genes more highly expressed in psoas than in LD**

Gene	Psoas/LD	Homology	# of clones	Accession #
<i>Mitochondrial origin</i>				
16S ribosomal RNA	2.07	97% (S)	12	AY011178
12S ribosomal RNA	2.18	100% (S)	7	AY012145
28S ribosomal RNA	2.67	99% (R)	1	V01270
Ribosomal protein S29	4.14	94% (B)	1	U66372
Ribosomal protein L23	30.05	92% (H)	1	NM_000978
Cytochrome oxidase subunit I	2.03	86% (B)	2	AF490529
Cytochrome oxidase subunit III	2.28	81% (T)	1	AF030272
Cytochrome oxidase subunit VIc	27.97	88% (H)	1	NM_004374
Cytochrome oxidase subunit VIb	2.51	96% (B)	1	X15112
NADH dehydrogenase subunit 2	2.51	77% (P)	1	AF414121
NADH dehydrogenase subunit 3	2.58	82% (B)	7	AY052631
NADH dehydrogenase subunit 6	2.15	81% (B)	4	AF416451
NADH dehydrogenase subunit B15	2.34	87% (B)	1	X64898
ATP synthase protein 6	2.1	96% (S)	2	AF190813
ATP synthase protein 8	2.17	100% (S)	1	AF039170
ATP synthase protein 9	20.55	96% (H)	1	NM_001689
tRNA	3.87	97% (S)	2	AF304202
Adenine nucleotide translocator SLC25A6	2.05	92% (H)	1	XM_114724
<b>Sarcomeric / structural</b>				
Ankyrin I	2.23	93% (H)	1	XM_016774
Tubulin alpha I	2.68	94% (H)	1	AF141347
<b>Non-sarcomeric</b>				
Pituitary tumor-transforming I interacting protein (tr)	2.98	84% (H)	1	BC031097
Par-6 partitioning defective 6 homolog gamma (tr)	30.36	92% (M)	1	XM_129044
TAFII140, a novel TAF component (TATA-binding protein associated factors) (tr)	2.16	94% (H)	1	AJ292190
STAF transcriptional activator (tr)	2.15	88% (H)	1	NM_003442
Prefoldin subunit 6 (KE2 protein) (tl)	2.65	85% (M)	1	M65255
EH domain-binding protein, Epsin (s)	2.23	93% (H)	2	NM_013333
Beta-catenin (s)	2.18	100% (S)	1	AB046171
Casein kinase 2, alpha I polypeptide (s)	6.47	87% (H)	1	NM_001895
Small muscle protein (smpx) (s)	7.34	88% (H)	1	BC005948
Tyrosine kinase 9-like (A6-related protein) (s)	2.04	90% (H)	1	BC016452
Fructose-1,6-biphosphatase (m)	25.07	92% (O)	1	AJ272520
Testis intracellular mediator protein (PEAS) (u)	2.12	88% (H)	1	BC021546
Hypothetical protein (FLJ12666) (u)	2.23	93% (H)	1	NM_024595
Unknown small protein (u)	3.93	94% (H)	1	BC005398
Unknown clone (kc2725), homologous to 3' untranslated region (UTR) of *RNA binding protein S1 (u)	2.57	*90% (H)	1	*XM_028847
Unknown clone, homologous to 3' UTR of *enhancer of rudimentary (u)	2.07	*81% (H)	1	*U66871
Unknown clone, homologous to Homo sapiens clone *K1AA0513 (u)	13.21	*81% (H)	1	*NM_014732
Unknown clones (kc2668, kc522, kc2469) (u)	3		3	

Possible involvement: (tr), transcriptional; (tl), translational; (s), signalling; (m) etabolic; (u) unknown. Accession # refers to porcine gene or close homologue: (S) = sus scrofa, (R) = rattus norvegicus, (B) = bos taurus, (H) = homo sapiens, (T) = tragelaphus buxtoni, (P) = physeter catodon, (M) = mus musculus, (O) = oryctolagus cuniculus, (C) = canis domesticus, (A) = ovis aries.

**Table 2: Genes more highly expressed in LD than in *psaos***

Gene	LD/Psoas	Homology	# of clones	Accession #
<b>Mitochondrial origin</b>				
Ribosomal protein L35A	2.2	86% (H)	1	AK055653
Ribosomal protein L7a	1.85	90% (H)	1	BC005128
Ribosomal protein S23	1.91	88% (R)	1	X77398
Ribosomal protein S12	1.61	100% (S)	1	X79417
<b>Sarcomeric / structural</b>				
Myosin heavy chain fast 2a	1.91	100% (S)	6	AB025260
Myosin heavy chain fast 2x	1.77	100% (S)	2	AB025262
Myosin heavy chain fast 2b	1.88	97% (S)	2	AB025261
Myosin regulatory light chain 2	2.95	91% (H)	2	AF363061
Alpha actinin 3	1.47	93% (H)	2	M86407
S-nexilin	1.77	70% (H)	1	AK057954
Fast skeletal troponin C	1.67	94% (O)	1	Y00760
Fast skeletal troponin T (TnT3)	1.7	93% (B)	3	AB085599
Alpha-1 skeletal actin (ACTA1)	1.53	93% (H)	2	BC012597
Alpha-2 smooth muscle actin (ACTA2)	1.62	96% (H)	1	BC017554
<b>Non-sarcomeric</b>				
Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (SERCA1) (g)	1.54	98% (S)	3	AY027797
Aldolase A (g)	1.47	87% (H)	1	BC010660
Phosphoglycerate kinase (g)	1.48	88% (H)	1	V00572
Glyceraldehyde-3-phosphate dehydrogenase (g)	1.53	100% (S)	1	X94251
Muscle pyruvate kinase (PKM2) (g)	1.63	91% (H)	1	BC007952
Phosphoglucomutase isoform 2 (PGM) (g)	1.81	94% (O)	1	M97664
Muscle glycogen phosphorylase (g)	1.65	92% (O)	2	X04265
Adenylate kinase isozyme 1 (myokinase) (g)	1.51	87% (S)	1	E03007
Calsequestrin (c)	1.7	83% (C)	2	J03766
Polyubiquitin (tl)	1.51	90% (A)	1	AF038129
Bridging-integrator protein-1 (bin1) (s)	2.17	85% (H)	1	AF001383
Myomegalin-like protein (u)	6.94	94% (H)	1	AB042557
Heat shock cognate protein (HSPA8) (u)	1.77	93% (B)	1	X53827
Unknown clone, homologous to Homo sapiens clone HSPCO40 (u)	2.08	86% (H)	1	BC000810
Unknown clone, homologous to 3' UTR of Homo sapiens *actin bundling protein (u)	1.99	*77% (H)	1	*U09873

Associated or possible role: (g), glycolysis; (c), contraction; (tl), translation; (s), signalling; (u) unknown. Accession # refers to porcine gene or close homologue. Genus abbreviations as in Table 1.

microarray analysis to be more highly expressed in the *psaos* than in the LD (Table 1), was individually quantified in 4 pigs (Fig. 4A). CK2 phosphorylation sites were also predicted in the deduced kc2725 protein (data not shown). All 4 pigs gave the same pattern of expression as that detected on the microarray. Interestingly, expression of kc2725 was ubiquitous and was much more abundant in other tissues than in skeletal muscle (Fig. 4B).

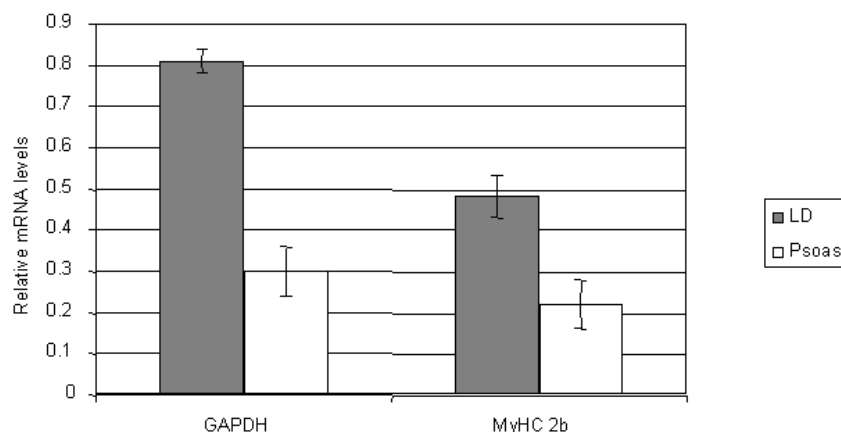
However, the expression of a gene from the same muscle can vary between similar individuals (Fig. 4A and Fig. 5A). Bin1 was identified by microarray analysis as being more highly expressed in the LD than in the *psaos* (Table 3). However, this pattern of expression was found in only 3 out of 4 pigs (Fig. 5A). In pig 2, there was no significant difference in bin1 expression between LD and *psaos*. Bin1

was previously found to be ubiquitously expressed by Northern analysis [18,21]. Quantitative PCR showed that bin1 expression was between 1 to 2 orders of magnitude greater in the heart and brain than in skeletal muscles (Fig. 5B). Therefore, TaqMan real-time quantitative PCR on selected representative genes had (1) demonstrated the functional integrity of our newly constructed porcine cDNA microarray in the appropriate identification of differentially expressed genes between red and white muscles, and (2) highlighted the existence of variation in muscle gene expression among similar individuals.

## Discussion

### Development of porcine skeletal muscle cDNA microarray

We have constructed a composite porcine skeletal muscle cDNA microarray consisting of 5,500 clones from two de-



### Figure 3

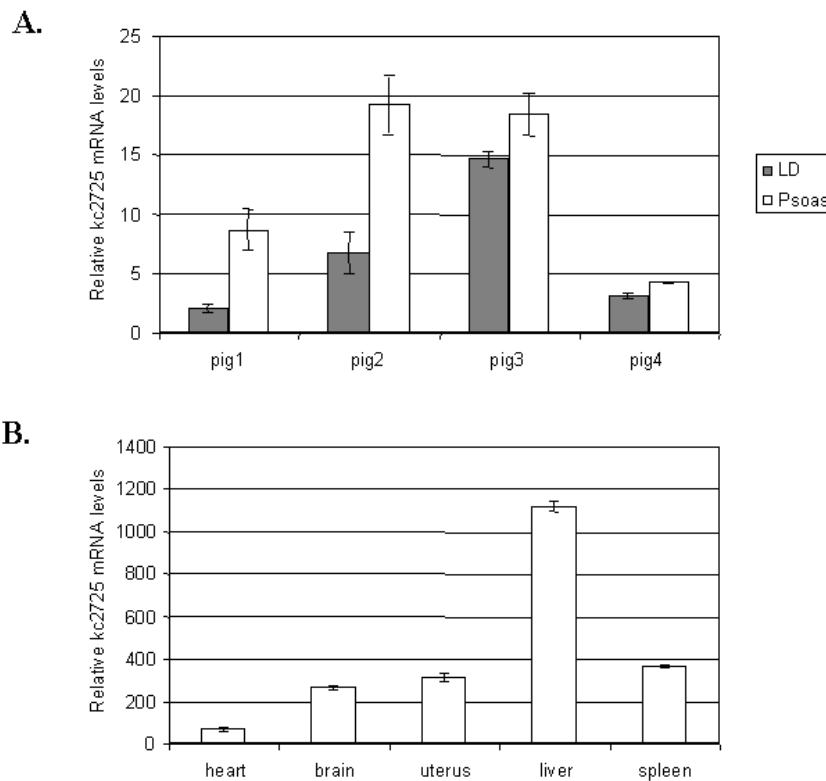
TaqMan quantitative real-time RT-PCR analysis of GAPDH, and MyHC 2b mRNAs in porcine *psosas* (red) and LD (white) muscles. Results of pooled total cDNA samples from three 22-week-old Berkshire pigs. GAPDH and MyHC 2b were about 2.7 and 2.5 times more highly expressed in the LD than *psosas*, respectively. Error bar = standard deviation.

developmentally distinct libraries, one derived from a 50-day foetal longissimus dorsi muscle, and the other derived from a 3-day-old gastrocnemius muscle. The choice of two developmentally distinct libraries was to increase the range of temporally-regulated genes represented in the microarray, to extend its suitability for use in different microarray-based muscle experiments.

The selection emphasis for inclusion on the microarray was on lowly or moderately expressed clones. This clone selection reduced the representation of highly expressed genes, such as those encoding for sarcomeric proteins, and increased the likelihood of including rare transcripts, including those of regulatory importance. An alternative method of normalising clone selection, which has been used by others, is by reassociation of single-stranded library plasmids at relatively low  $C_{ot}$  to remove highly expressed clones [22,23]. Another commonly used and commercially available method of selection normalisation is subtractive suppression hybridisation [24]. We chose not to use this method for microarray clone selection because of the possibility of excluding lowly expressed non-muscle specific clones.

A possible disadvantage of our porcine microarray, at least at the beginning, is the lack of knowledge of the identity

of each clone. However, there is, at present, insufficient sequence information on farm animals to design a comprehensive oligonucleotide-based microarray. To date, we have sequenced about 10% of our microarray clones (data not shown). A major advantage of our microarray is that we are likely to be in possession of the corresponding full length cDNA clones, whose inserts were unidirectionally cloned into a CMV-promoter driven expression plasmid (pBK-CMV vector). These clones could be readily used for downstream expression studies. As the identity of each clone is unknown, we do not know for certain how representative is the porcine skeletal muscle cDNA microarray. On the one hand, considering that there might be fewer than 30,000 human genes [25], and assuming that 50% of all genes are transcriptionally active at one time in a given tissue [26], it is possible that around 20% of the genes that are expressed in skeletal muscle are found on our microarray [25,27]. On the other hand, a group of 4080 human skeletal muscle genes, which included both skeletal muscle-specific genes and genes expressed in skeletal muscle as well as in other tissues, was found to correspond to 80% of the total number of genes expressed in skeletal muscle as reported so far in Unigene (including foetal muscle and rhabdomyosarcoma) [26]. Hence our microarray may represent substantially more than 20% of all genes expressed in porcine muscle.



**Figure 4**

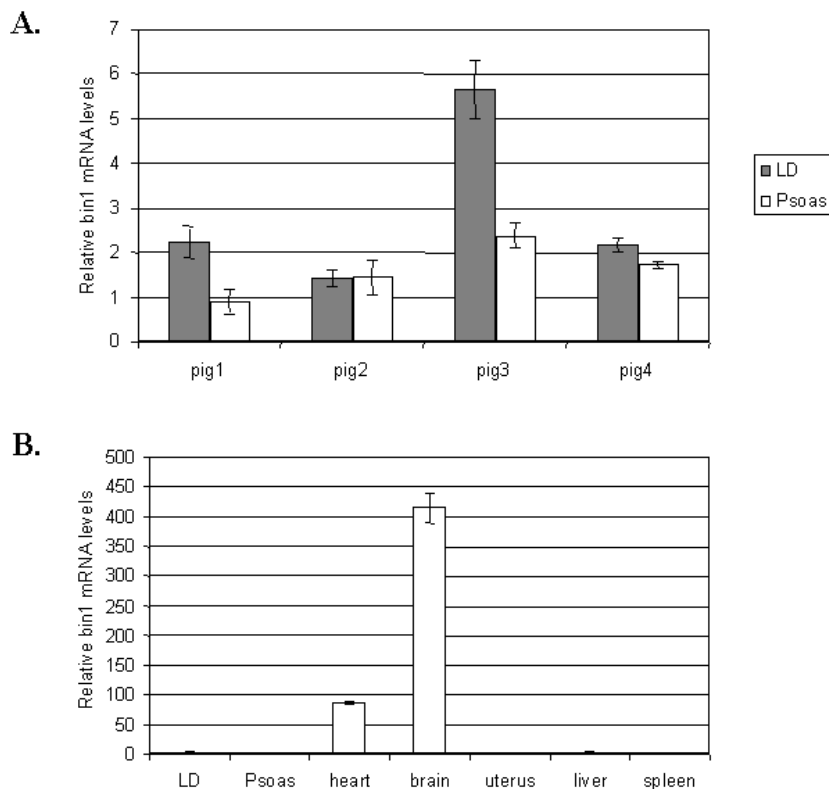
Tissue distribution of a novel gene *kc2725*. **A.** Expression of *kc2725* was higher in the *psoas* than in the LD in all 4 pigs. Pigs 1 to 3 were 22-week-old Berkshires, and pig 4 was a 7-week-old cross breed. **B.** The expression of *kc2725* was ubiquitous and much more abundant in other tissues. Error bar = standard deviation.

#### Differential gene expression in red and white muscles

One objective of the red-white muscle experiment was simply to test the function of the newly assembled porcine microarray, from which two gene lists were generated (Tables 1 and 2). Genes that were expected to be differentially expressed and genes that were novel were found on each list. The microarray results validated our prior hypothesis of differential gene expression in red and white muscles, thus demonstrating the functional integrity of our newly constructed microarray. One of the well established distinguishing features of red muscle is its relatively high oxidative phosphorylation capacity, reflected by an abundance of mitochondria in red muscles. It is reassuring that genes from the mitochondrial genome were well represented in the red muscle pool of differentially expressed

genes (Table 1). White muscles comprise predominantly more fast-glycolytic fibres than red muscles. Our findings were consistent with expectations, in that most of the 45 clones selected as more highly expressed in white muscle (Table 2) were either fast isoforms of structural genes, or enzymes connected with anaerobic glycolysis. With highly expressed genes, such as mitochondrial genes and structural genes, there were detectable levels of redundancies (repeats) on the microarray. However, in the presence of redundancies, no gene was found to be present on both lists. Differentially expressed clones originating from both the foetal and neonatal libraries were found in comparable proportion (data not shown). A recent report comparing red and white murine skeletal muscles on generic commercial oligonucleotide chips (Affymetrix





### Figure 5

Bin1 mRNA expression detected by TaqMan quantitative real-time RT-PCR. **A.** In pigs 1, 3 and 4, bin1 mRNA was more abundant in the LD than in the *psaos*. In pig 2, bin1 expression in both muscles was similar. Pigs 1 to 3 were 22-week-old Berkshires, and pig 4 was a 7-week-old cross breed. **B.** Although ubiquitous, bin1 expression was by far most abundant in the brain and heart. Error bar = standard deviation.

GeneChips), which comprised the equivalent of 3,000 different genes, yielded a differential list of 49 known genes [28]. Our results of known differentially expressed genes were comparable in both number and in the different types of genes found.

One problem underlying the analysis of microarray data is the large number of comparisons required, which can produce false positive results [29]. We compared the expression of clones in *psaos* and LD muscle as the median of 14 comparisons (each microarray clone was printed twice on each slide and 7 slides were used), and restricted further analysis to the clones demonstrating the most consistent and extreme differences. Therefore, the majority of

our identified clones are likely to represent real differences between muscles. However, any specific gene assignment should be regarded as provisional until it can be confirmed in an independent study, such as another microarray or in another assay such as quantitative PCR.

The interpretation of microarray results can be complicated. Firstly, members of the same gene family could cross-hybridise to the same spots on the microarray. Interpretation of differential expression of individual isoforms should therefore be made with caution. In the case of MyHC genes (Table 2), a plausible interpretation is that fast MyHC mRNA isoforms (2a, 2x and 2b) were more abundant in the LD than in the *psaos*. However, we have

**Table 3: Oligonucleotides and TaqMan fluorogenic probes**

Gene	Primer	Sequence 5'→3'
β-actin	S	CCA GCA CCA TGA AGA TCA AGA TC
	A	ACA TCT GCT GGA AGG TGG ACA
	Ps	CCC CTC CCG AGC GCA AGT ACT CC
GAPDH	S	AGG CTC GGG CTC ACT TGA A
	A	TGC CCA TCA CAA ACA TGG G
	Ps	AGC CAA AAG GGT CAT CAT CTC TGC CC
MyHC 2b	S	CAC TTT AAG TAG TTG TCT GCC TTG AG
	A	GGC AGC AGG GCA CTA GAT GT
	Ps	TGC CAC CGT CTT CAT CTG GTA ACA TAA GAG G
Bin1	S	GCC AGC AAT GTG CAG AAG AA
	A	CAT CTG CCT TCC CCA GTT TC
	Pa	CCT TCT CCT GCG CGC GAG TGA G
Novel gene (kc2725)	S	TTT TTT CCA TTC CCT GGT TGA
	A	AGG GAC CCT GTA AGC CAA CA
	Ps	CCA GTC TGG TGG CCT AGT CAT GCC C

S, sense; A, antisense, Ps, TaqMan sense probe; Pa, TaqMan antisense probe

previously found by quantitative real time RT-PCR that in at least 4 out of 6 pigs, of the same sex, age and breed as the one used in the microarray hybridisation, MyHC 2a and 2x were, in fact, more highly expressed in the *psaos* than in the LD [20]. The 3 fast MyHC isoforms found in Table 2 might have been the result of cross hybridisation by the relatively large amounts of MyHC 2b specific probe generated from the LD muscle. Secondly, in comparing the profiles of two normal physiological states, such as red and white muscles, large variation in normal gene expression between individual pigs could present a major problem [30]. This variation is mainly attributed to genetic differences that exist between individual pigs; even pigs of the same breed are not genetically the same. The use of pooled porcine mRNA samples could inadvertently increase the genetic variation within each experimental group of animals. Therefore, in the context of porcine red-white muscle microarray analysis, there may be no advantage in pooling mRNAs, derived from the same muscle of several pigs. On the other hand, the use of different muscles from the same pig could minimise the effects of environmental variation, which could exist between individuals. The use of inbred lines in laboratory rodents largely eliminates the problem of genetic variation between individuals of the same line. Hence, the use of labelled cDNA from pooled inbred individuals would enhance experimental reliability without increasing genetic variation. However, inbred pig strains are not widely available and limited to a few lines of mini-pigs. Whether the microarray probes were derived from an individual or pooled from a group of individuals, extensive validation, such as by quantitative PCR or Northern analysis, is necessary to demonstrate that the differential expression of a

gene identified on a microarray is consistent in a wider context.

#### **Candidate genes for phenotype determination**

From the red-white muscle microarray results, a list of novel candidate regulatory genes that could influence muscle phenotype, such as hypertrophy, differentiation and isoform-specific expression, was identified. Most of the genes listed as unknown (Table 1 and 2) were found with major open-reading frames, suggesting that they code for protein products. Candidate regulatory genes with putative identities were based on homology comparison. Even for these genes, with the possible exception of bin1, their functional roles in skeletal muscle are largely unclear. CK2  $\alpha 1$  subunit, *smpx*, and tyrosine kinase A6-related gene are particularly interesting. They were found to be more highly expressed in red muscle and are connected to the casein kinase 2 signalling pathway. CK2 is a serine/threonine kinase that has been implicated in cell growth and proliferation [31,32]. CK2-mediated phosphorylation of Myf-5, a member of a family of myogenic transcription factors, was reported to be required for Myf-5 activity [33]. Presently, the contribution of the CK2 signalling pathway to skeletal muscle function is not known. Its role in muscle phenotype determination requires further evaluation. The gene for heat shock 70 kD protein 8 (HSPA8) seemed to be upregulated in white muscle. Heat shock proteins are considered to be molecular chaperones and indicators of cellular stress [34]. The same gene was found to be upregulated in human hypertrophic cardiomyopathy [35]. It is not clear if this finding was related to cellular stress of myopathy or muscle hypertrophy.

Our results demonstrate the power of microarray analysis in identifying candidate genes that influence muscle phenotype. The challenge now is to confirm these associations and demonstrate how these genes are involved in the relevant muscle phenotypes.

## Methods

### Construction of porcine skeletal muscle cDNA microarray

Mass phagemid excision was performed on two porcine skeletal muscle  $\lambda$ ZAP-Express cDNA libraries (50-day foetal *longissimus dorsi* muscle and 3-day-old *gastrocnemius* muscle), developed in-house, according to manufacturer's protocol (Stratagene). Both libraries, each with a million primary plaques, had been extensively characterised by sequencing and screening, and were used to isolate several full-length cDNA clones, including MyHCs (6.0 kb in size) and transcription factors, such as NFAT2 and GATA2. One study, performed on adult human skeletal muscle, found that highly expressed genes represented only 9.1% of the transcript variation, whereas moderately and weakly expressed genes made up 27.5% and 63.5% of the variation, respectively [26]. In order to ensure that the clones chosen for the porcine microarray assembly represented as many different genes as possible, clones were screened by colony hybridisation to assess their relative abundance of expression. About 400 bacterial clones were picked onto each square agar plate (Bio-Assay dish, Nunc) for colony hybridisation with  $^{32}$ P-labelled cDNA probe, derived from mRNA extracted by oligo(dT)<sub>25</sub> Dynabeads (Dynal) from the same muscle type and stage of development as that used for the library construction. Based on signal intensity on the autoradiograms, each clone was classified as a weakly, moderately or highly expressed gene [26]. Clones picked for microarray construction were judged to be lowly or moderately expressed. Miniprep plasmid DNA was prepared from selected clones using the QIAprep 96 Turbo kit (Qiagen).

Insert amplification of each pBK-CMV-based plasmid clone (Stratagene) was performed with T7 primer (5'-GTA ATA CGA CTC ACT ATA GGG C-3') and T3 primer (5'-CGA AAT TAA CCC TCA CTA AAG GG-3'), using a Hot-StarTaq Master Mix kit (Qiagen). PCR was conducted in a 100  $\mu$ l volume, using the equivalent of 0.1  $\mu$ l of miniprep plasmid (about 15 ng), for 35 cycles at 55°C for 45 s, followed by 72°C for 3.5 min, and 94°C for 1 min, with an initial activation step of 95°C for 15 min. PCR products were purified with QIAquick 96 PCR Purification kit (Qiagen) and printed onto CMT-GAPS coated slides (Corning) using a Microgrid II arraying robot (BioRobotics, Cambridge, UK). Spotted DNA was immobilised by baking at 80°C for two hours. Each clone was printed in duplicate on each slide. Appropriate positive controls to help with orientation and vector controls were incorporated on the microarray.

### Red-white muscle microarray hybridisation

Messenger RNA was extracted by oligo(dT)<sub>25</sub> Dynabeads (Dynal) from the *longissimus dorsi* (LD), a white muscle, and the *psosas*, a red muscle, of a 22-week-old pig (Berkshire breed). Dual Cy-dye labellings were performed with a CyScribe First-Strand cDNA labelling kit (Amersham), which incorporates the use of oligo(dT) primers and random nanomers. Labelled cDNAs were purified in AutoSeq G-50 columns (Amersham). In a typical Cy3 or Cy5 labelling reaction, using 0.5 to 1.0  $\mu$ g mRNA, a final volume of 32  $\mu$ l was obtained, which was used on two microarray slides. Prior to pre-hybridisation, microarray slides were denatured in distilled water by heating to 95°C for 2 min. Pre-hybridisation was then performed in a plastic 2-slide holder containing 15 ml of 3  $\times$  SSC (sodium chloride and sodium citrate), 2% bovine serum albumin (B4287, Sigma) and 0.1% SDS at 65°C for 20 min. After a brief rinse in distilled water at room temperature, the slides were dehydrated in absolute ethanol and centrifuged at 100 g for 2 min.

Labelled probe was not quantified. Equal volumes of Cy3 and Cy5 labelled probes (16  $\mu$ l each) were mixed with 1.0  $\mu$ l (8.0  $\mu$ g/ $\mu$ l) of poly(dA) oligonucleotide (27-7836, Amersham), heated to 95°C for 2 min, and mixed with 33  $\mu$ l of 2  $\times$  hybridisation buffer (GHB-200, Genpak). A microarray slide was layered with the hybridisation mixture, then covered with a 22 mm  $\times$  64 mm cover glass (BDH), to the exclusion of air bubbles, and placed in a hybridisation chamber (ArrayIt hybridisation cassette AHC-1) in a 45°C dry incubator for 24 h. Slides were washed once in 300 ml of 1  $\times$  SSC and 0.2% SDS for 10 min at 45°C, twice in 300 ml of 0.2  $\times$  SSC and 0.2% SDS for 10 min at 45°C, and twice in 300 ml of 0.1  $\times$  SSC for 10 min at 37°C. After drying in a centrifuge at 100 g for 2 min, scanning was performed with an Affymetrix 428 scanner. A total of seven microarray slides (replicates) were subjected to two-colour Cy-dye hybridisation and scanned for the red-white muscle experiment.

### Microarray expression analysis and clone identification

Image analysis (grid generation and dye quantification) of scanned slides was performed with ImaGene v4.2 (Bio-Discovery). Data mining was conducted with GeneSpring v4.2 (Silicon Genetics), in which 3 normalisation steps were performed: per spot, intensity-dependent, and per chip (slide). In per spot normalisation, after background subtraction, the fluorescent intensity (e.g. Cy3) of each clone was divided by its control or reference channel intensity (e.g. Cy5). Values of the control channel that fell below 10 were adjusted to 10 prior to taking the ratio between signal and control value. Intensity-dependent (non-linear or LOWESS) normalisation was applied to correct for artefacts caused by differential Cy3 and Cy5 dye incorporation and non-linear rates of fluorescence be-

tween the two dyes. Per chip normalisation was used to take into account intensity variation across the entire slide, by dividing the signal strength of a clone on a slide by the 50<sup>th</sup> percentile signal of all of the measurements taken from the same slide. Additionally, the GeneSpring Global Error Model, based on the replicate measurement samples of all genes on the microarray was used to estimate differences between medians, the standard errors among the different clones, and the extreme tails of the distribution of differences. Differences among muscles in expression were standardised for different clones by dividing the difference between muscle medians by the square root of the common variance. Sequencing reactions with T7 and T3 primers were conducted with an ABI BigDye Terminator v3.0 PCR-based sequencing kit (Applied Biosystems). Performa DTR gel filtration cartridges (EDGE Biosystems) were used for purification of PCR products prior to loading onto the ABI PRISM 3100 Genetic Analyzer. All sequence data were subjected to BLAST (Basic Local Alignment Search Tool) searches for gene identification by sequence similarity.

#### Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed using TaqMan (Applied Biosystems) on five selected porcine genes:  $\beta$ -actin, GAPDH, MyHC 2b, bin1, and a novel gene (kc2725) (Table 3). The protocol, based on the use of the relative standard curve, was as previously described [20]. In the relative standard curve method of quantification, comparisons of relative expression should only be made between samples of the same gene. It is not appropriate to compare expression levels between two different genes. A reference cDNA panel, comprising a number of different tissue templates (LD muscle, *psaos*, heart, uterus, brain, liver and spleen) of a 7-week-old pig, along with LD and *psaos* cDNAs, from three additional 22-week-old Berkshire pigs, were used to evaluate the quantitative distribution of selected genes.

#### Authors' contributions

QB generated the microarray clones. CM, NdC, MJM and KCC helped with the validation and analysis of the microarray. DS and GE were responsible for the production of the microarray and advised on its experimental use. KCC conceived the study and was responsible for writing the manuscript. All authors read and approved the final manuscript.

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