Author's response to reviews

Title: Differential gene expression associated with postnatal articular cartilage maturation

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Author’s response to reviews:

Dear Editor-In-Chief and Referees,

We thank the Referees for their constructive criticism of our manuscript entitled, “Differential gene expression associated with postnatal equine articular cartilage maturation.” We believe the suggested revisions improve the manuscript. Each revision and suggestion has been addressed in the pages which follow. We hope that the manuscript will now be judged worthy of publication in BMC Musculoskeletal Disorders. We look forward to hearing from you.

Sincerely,

Michael Mienaltowski
**Referee 1**

**Revisions**

“Title: It would be sensible to include the fact that the tissue is equine in the title”
The authors agree with the suggestion and have included “equine” in the title.

“Abstract – methods – may be worth putting in age range of animals in methods. It would be nice to indicate that qPCR was performed on only 4 or 5 genes (and possibly include the names of these genes).”
The authors have included the age ranges in the Abstract and Methods. In addition, RT-qPCR experiments were expanded to include total RNA from articular cartilage of sixteen horses (seven 0-10 day-old neonates and nine 4-5 year old adults). In addition to assaying endogenous control genes, six target genes (COL2A1, COMP, P4HA1, TGFβ1, TGFβR3, and TNC) were validated. Gene symbols have been included in the abstract.

“Background: Second paragraph – maybe worth stating which joint Sasano examined...”
Sasano et al. examined the collagen content in the articular cartilage of the proximal tibia. The authors have revised the manuscript to make this point clear.

“Materials and methods: No mention of where the cartilage was harvested from (i.e. which joint and wherein the joint). Not clear whether they discarded tissue with cartilage canals. I assume this was done but it isn’t clear.”
Revisions have been made to specify the sites of cartilage collection and to confirm the exclusion of cartilage with vascular canals.

“Primer design: It is not clear whether these primers were human sequences (bought from ABS) and cross reacted with the equine sequence – if this is the case, it should be mentioned. The efficiencies are quite a broad range (0.9 - 1.2) and are greater than some commentators have deemed to be acceptable – This should be discussed. It would be interesting to know whether any of the housekeeping genes featured in the microarray and whether they showed differences between different aged tissues.”
The primers used in this study were based on equine genomic sequence data. This has been made clear in the revised manuscript. The 0.9-1.2 efficiency range represent thresholds for manual serial dilution efficiency testing that are defined by Applied Biosystems in their manufacturing of the primer-probe sets. All primer-probe sets performed within this range. Actual efficiencies used in the expression assays were determined by the LinRegPCR program. Management of efficiencies was performed by a strategy defined by Ramakers et al. (2003) and Schef et al. (2006) as described in the manuscript. Endogenous control genes (GUSB, PGK1, RPLP0, and B2M) evaluated for stable expression by RT-qPCR were also represented on the cDNA microarray and did not achieve a p < 0.05 significance for differential expression. Endogenous control genes GUSB and RPLP0 were selected for normalization of the six target genes as is described in the Materials & Methods.

“What was the selection criteria for the 5 genes selected for qPCR analysis – it would be nice to see some hypothesis on why these were chosen (from 544 candidates) – why is data for fibronectin not mentioned in results or fig 4, whilst it is mentioned in methods”
Candidates for RT-qPCR validation were selected because of their significant differential gene expression according to the microarray data and because of functional annotation of their encoded proteins as
discussed in the manuscript. Validation of TGFβ1 and TGFβR3 by RT-qPCR were added in the revised manuscript in response to recommendations of Referee 3. Fibronectin was not validated by qPCR and should not have been listed in the Materials & Methods.

“Quantification of housekeeping genes stability - Would this stability been better proven using one of the housekeeping gene algorithms, such as Genenorm or Normfinder??”
As recommended, housekeeping gene stability has been quantified with geNorm. The two genes determined to be the most stable were GUSB and RPLP0 (M=0.552). These were used for normalization of target gene RT-qPCR data. Data for these two endogenous control genes are included in the revised manuscript.

“I have some concerns that the classification/categorization of neonatally upregulated genes is anecdotal and no association with these groups has been performed. An example of this is P9 4th line from the bottom – where the data is described as “seems to have greater up-regulation” - I am surprised that this cannot be proven using some mathematical/statistical method to progress the data from being qualitative to quantitative.”
In the revised manuscript, the microarray data have been applied to the Expression Analysis Systematic Explorer (EASE) available from the National Institutes of Health. Figure 2 from the original manuscript has been removed and replaced by Table 2 in the revised version of the manuscript, which displays those ontological categories demonstrating significant contribution to differential expression.

“Discussion – some genes discussed which are not mentioned in results, nor proven by qPCR – such as NID2 – why is this discussed in such detail – it seems to be a bit out of context?”
The authors have modified the Results section to include those genes mentioned in the discussion. Genes are now cross-referenced within the results, discussion, tables, and figures. For genes not validated by RT-qPCR, agreement amongst all comparisons is reflected by concordance in M value (foreground minus background given as log2 Neonate/Adult ) and statistical significance across redundant probe sets.
Referee 2

Discretionary Revisions

“From this manuscript, I did not fully understand the utility and/or necessity of microarray slides using for this study. I want the authors to describe additional information about the microarray, specifically why to have used that for this research.”

Microarray hybridizations were used to assess differential expression between neonatal and mature articular cartilage across a large subset of genes in the equine genome. By expanding the focus of the analysis to the 9367 probe sets, the authors intended to investigate ontological categories represented by the expression differences, instead of being limited to individual genes representing cartilage biomarkers. The Background section has been revised to state these objectives.
Referee 3

“The differential gene expression patterns of developing tissues is informative about gene signatures. The authors of this MS have compared in the equine differences between two ages: foals and adults. This is a major weakness. It would be important to assess differences between foals, adolescents and at least three age groups during and upto adulthood. The methods are straightforward. The current version of the MS must be deferred until additional data is forthcoming.”

The authors agree that an assessment of additional age groups will be a valuable extension to the findings reported in the current manuscript. In the near term, samples from additional age groups are not available to the authors with sufficient biological replicates for microarray-based transcriptional profiling experiments. The opportunity to build on the current study by assessing additional age groups and a consideration of anatomical site-dependent variations by age have both been added to the Discussion.

“1. The authors must add additional age groups.”

See above.

“2. Figure 1 is unnecessary photographs of foals and adults. The diagrammatic representation of the cartilage must be replaced by actual photomicrographs of the articular cartilage histology.”

Figure 1 has been revised to now include both schematic drawings and photomicrographs of H&E stained articular cartilage sections from neonatal and mature equine femoral articular cartilage.

“3. The list of analyses of the microarrays is limited. The authors must analyze the expression patterns of key regulators such as BMPs, IGF I and II and TGF Beta.”

The specific assessment of all microarray probe sets representing genes encoding growth factors and growth factor receptors has been added in the revised manuscript (Table 5 and Discussion). Transcripts encoding TGFβ1 and TGFβR3 have been added to the list of target genes evaluated by RT-qPCR.

“4. The INTRODUCTION must include a rationale for the micro array experiments.”

The authors have revised the Background section to include the rationale for utilization of transcriptional profiling experiments.