Temporal embryonic transcription of chicken fast skeletal myosin heavy chain isoforms in the single comb white leghorn

J. Griffin, N. St-Pierre, M. S. Lilburn, and M. Wick[1]

Department of Animal Sciences, The Ohio State University, Columbus, OH 43201

ABSTRACT There are numerous factors that can significantly influence embryonic development in poultry and thus make simple days of incubation (chronological age) a less than perfect metric for studying embryonic physiology. The developmental fast skeletal muscle myosin (MyHC), the predominant protein in the *Pectoralis major* (PM), is temporally expressed as a cadre of highly specific developmental isoforms. In the study described herein, a novel molecular technology (NanoString) was used to characterize the myosin isoform transcriptional patterns in the PM of Single Comb White Leghorn (SCWL) embryos. NanoString technology is based on quantitative analysis of the transcriptome through digital detection and quantification of target mRNA transcripts. Total RNA was isolated and gene transcription quantified using NanoString in embryonic muscle samples collected daily from 6 through 19 days of incubation. Data were analyzed using the LOESS smoothing function at a 95% confidence level. The temporal transcription of MyHC isoforms obtained in this study was consistent with the literature at higher specificity and resolution, thus validating NanoString for use in gene transcription analyses. The results support a hypothesis that the transcription patterns of the embryonic MyHC isoforms may be used as molecular clocks to further investigate the developmental relationships underlying embryonic fast skeletal muscle growth and development.

Key words: *Pectoralis major*, myosin heavy chain, isoform, myogenesis, transcriptome

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INTRODUCTION

The Pectoralis major (**PM**) is the predominant breast muscle in poultry and continued selection for proportional increases in breast muscle size in broilers and turkeys has increased the potential for correlated negative effects in both fresh and cooked muscle products. There are numerous papers that address various aspects of PM development post-hatch (Crow and Stockdale, 1986; Cerny and Bandman, 1987; Stockdale and Miller, 1987; Bandman and Bennett, 1988; Moore et al., 1992; Maruyama et al., 1993; Tidyman et al., 1997; Wick et al., 2003; Reddish et al., 2005; Huffman et al., 2012; Lee et al., 2012). However, the developmental relationships and mechanisms underlying embryonic myogenesis in the PM remain largely unresolved. Embryonic studies in poultry have traditionally used days of incubation as a basis for developmental comparisons although a more precise staging procedure based on embryonic morphology was initially described by Hamburger and Hamilton (1951).

The growth and development of the PM is associated with the temporal expression of fast skeletal muscle specific proteins, predominantly myosin. The tissue specific expression of myosin heavy chain (**MyHC**) isoforms during PM muscle development in poultry have been well documented at the protein level using immunochemical analyses (Bandman, 1985; Crow and Stockdale, 1986; Cerny and Bandman, 1987; Stockdale and Miller, 1987; Bandman and Bennett, 1988; Maruyama et al. 1993). In chickens the MyHC are temporally expressed as a series of six developmental isoforms covering the embryonic and post-hatch developmental stages of growth. Tidyman et al. (1997) characterized the temporal transcription of the fast skeletal muscle MyHC in the PM using semi-quantitative RNA dot blot analysis.

To better understand the biology underlying the temporal transcription of the MyHC isoforms, it is imperative to consider their expression in relation to overall muscle growth and development. To date, there have been reports on the relationship between MyHC isoform appearance and myogenic development of the PM

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[1]Corresponding author: wick.13@osu.edu

Table 1. Outline of targeted MyHC isoform genes, their corresponding NCBI reference and target mRNA sequence.

Gene	NCBI Accession #	Target Sequence
Cemb1	MYH1emb.1	TTCCCTGGAAGATCAACTCAGTGAAATTAAGACAAAGGAAGAGGAGCAACA GCGCACCATTAATGACATCAGTGCACAGAAAGCTCGTCTACAAACAGAG
Cemb2	NM_204228.1	GCTGAAGGCAGAAAGAAGCTACCACATATTTTATCAAATTATGTCCAACAA GAAACCAGAGCTAATTGGCATGCTTCTAATTACCACCAATCCATACGAC
Cemb3	NM_001113709.1	CAGAGGCTGGTGGTGGAGGCAAAAAGGGTGGCAAGAAGAAGGGTTCTTCTTTCC AAACAGTTTCTGCTCTTTTCCGGGAGAACTTAAACAAGCTGATGAC
Cneo	M74087.1	AGTGTGACCTGAGGCATGCATAAAATGTGAACTCTGTGTTGCTTTTTTATGTCA TTGTCATCTATGTCTAGGTAATAAAGAGAGTAGAGACCTTTGCATA
B-actin	NM_205518.1	CATTGTCCACCGCAAATGCTTCTAAACCGGACTGTTACCAACACCCACACCCC TGTGATGAAACAAAACCCATAAATGCGCATAAAACAAGACGAGATTG
GAPDH	AF047874.1	CAAGTGGTGGCCATCAATGATCCCTTCATCGATCTGAACTACATGGTTTACATGT TCAAATATGATTCTACACACGGACACTTCAAGGGCACTGTCAAGG
HPRT1	NM_204848.1	CTGTCTCTACTTAAGCAGTACAATCCAAAGATGGTGAAAGTGGCCAGTTTGTTGGTCA AAAGAACTCCTCGAAGTGTGGGATATCGGCCAGACTTTGTTG
HMBS	XM_417846.2	CGTTTGGAGGGTGGCTGTAGTGTCCCTGTTGCAGTTAACACCATGCTGAAAGATGGCC AGTTGTACTTGACAGGTGCAGTCTACAGTTTGGATGGATCCG
RPL4	NM_001007479.1	CCAAAGAAGAAGATTCACCGCAGAGTCCTGAAAAAGAACCCGCTGAAGAATCTGAGAGTC ATGATAAAGCTGAACCCATACGCCAAAACAATGCGACGCA

in both chickens (Bandman and Bennett, 1988; Cerny and Bandman, 1987) and turkeys (Maruyama et al., 1993; Huffman et al., 2012). Thus, in order to better understand the molecular and cellular events that drive skeletal muscle development, this study aimed to target specific stages of embryonic development in the Single Comb White Leghorn (**SCWL**) via the temporal transcription of the developmental MyHC embryonic isoforms.

The primary goal of this study was to validate the use of the novel NanoString molecular technology, which to date, has not been used to characterize temporal transcriptional events in commercial avian species. We compared the temporal transcription of the MyHC isoforms during myogenesis in embryos from a control SCWL genetic line with the data from Tidyman et al. (1997). This report served as the positive control for our preliminary research as these authors defined the temporal transcription of the MyHC isoforms using RNA from the PM of SCWL chicks, similar to the control experimental line used in the current study. It is important to emphasize the use of the SCWL as the control genetic line because a critical component of the work was to confirm, using NanoString technology, what has already been reported. Tidyman et al (1997) defined the temporal transcription of MyHC isoforms starting at embryonic day 10 through 240 days post-hatch. The experiment design herein was designed to emulate the study by Tidyman et al. (1997) as much as possible in order to make accurate comparisons with the NanoString molecular technology for gene transcription analysis. Our hypothesis is that if we can use this technology to confirm the embryonic MyHC isoform transcription pattern reported by Tidyman et al. (1997), the transcription of each MyHC embryonic isoform could serve as a potential biological marker in future studies which would be expanded to include the transcription of other myogenic growth factors.

MATERIALS AND METHODS

Pectoralis major (PM) Tissue Extraction and RNA Isolation

Control SCWL embryos used in this study were maintained by the Ohio Agricultural Research and Developmental Center at The Ohio State University. Embryos were incubated at the industry standard of 37.5°C. Embryos were removed from the incubator on chronological days 6 through 19. All embryos were sacrificed by decapitation, at which point, the PM breast muscle was exposed and removed from each side of the sternum. Total RNA was extracted according to the manufacturer's recommendation (Norgen Biotek Corp, Thorold Ontario, Canada). Total RNA concentration and integrity were evaluated using nanodrop and gel electrophoresis, respectively.

NanoString nCounter Transcript Abundance Analysis

To gain insight into transcriptional events underlying PM embryonic development, we quantified mRNA transcripts of MyHC isoforms utilizing Nanostring nCounter technologies (Geiss et al., 2008; Malkov et al., 2009). Digital counts for 4 MyHC isoforms and 5 housekeeping genes (Table 1) were adjusted for binding efficiency with background subtraction using positive and negative controls from the manufacturer (Nanostring Technologies, Seattle, WA), as per Nanostring nCounter data analysis guidelines.

Probe Architecture. The nCounter analysis system uses unique color-coded molecular barcodes that hybridize directly to nucleic acids through the use of gene specific color-coded probe pairs. In solution, the probes capture and count specific nucleic acid molecules in a complex mixture, through hybridizing directly to the target mRNA for digital detection. The digital

color-coded barcodes consist of unique combinations of 4 spectrally non-overlapping dyes arranged at 7 contiguous regions, making it possible to generate hundreds of unique transcripts, each corresponding to 1 target gene, in a single reaction. Each target gene of interest is detected using a pair of reporter and capture probes carrying 35- to 50-base target-specific sequences. Each reporter probe is the "barcode", carrying a unique color code assigned to each target mRNA sequence at the 5′ end that enables the molecular barcoding for detection of the target mRNA in solution. This technology has been shown to achieve superior gene expression quantification results when compared to RT-Polymerase Chain Reaction (Reis et al., 2011).

Statistical Analyses

Using nSolver software provided by NanoString, housekeeping gene normalization was performed in order to adjust the digital counts of all probes that were not expected to vary between samples. A normalization factor was calculated based on the average of the geometric mean of all 5 housekeeping genes for each data point. The average of the geometric means across all data points was used as a reference against which each lane was normalized.

Following normalization, gene transcription measurements were regressed on days of incubation using the LOESS (i.e., Local regression) procedure of SAS V9.2, a non-parametric regression method that combines multiple regression models in a k-nearest neighborhood model (Cleveland and Devlin, 1988). LOESS regressions allow great flexibility in estimating response curves because they make no assumptions about the parametric form of the regression. The optimal smoothing parameter was determined using cross-validation. As with conventional regression methods (e.g., least-squares), LOESS produces prediction estimates as well as standard errors of these estimates. Comparisons were done using t tests based on the predictions and standard errors of MyHC isoforms on each embryonic day. Rates of change in gene transcription were calculated using finite forward and backward differences (half-days). Significance was declared at $P < 0.05$.

RESULTS

The relative percentage of total MyHC isoform transcription from embryonic day (**ED**) 6 through ED19

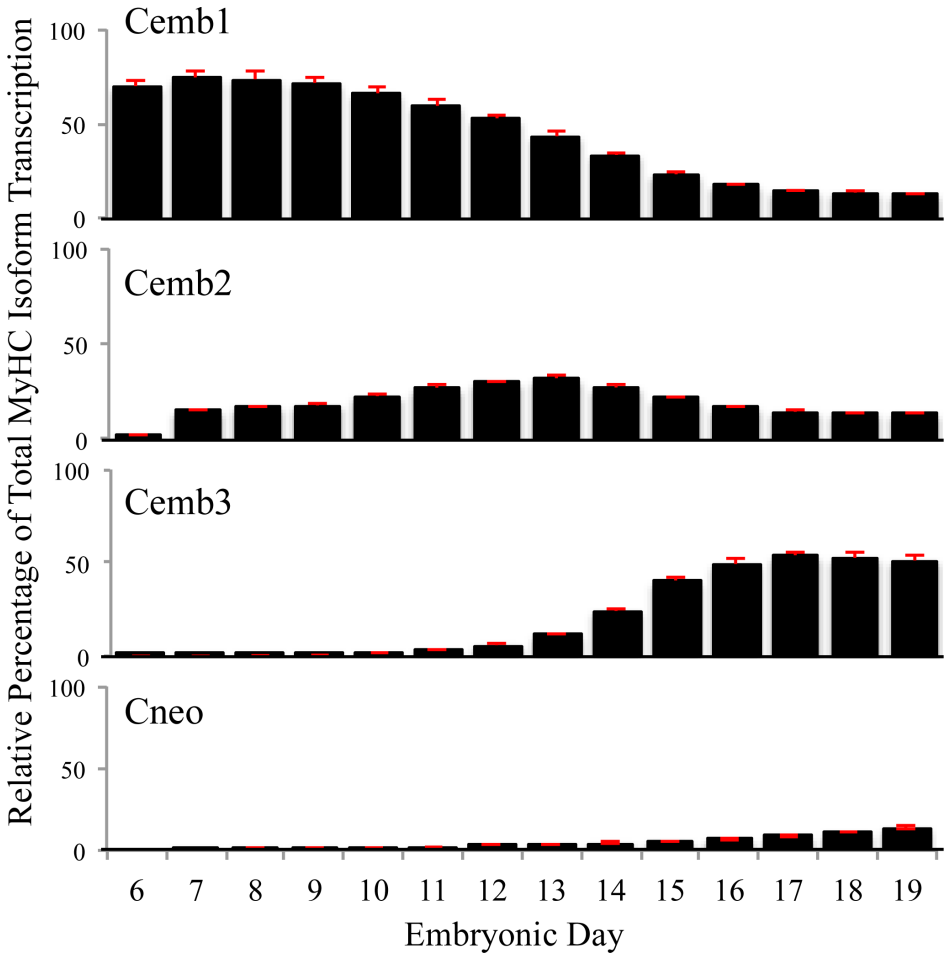

Figure 1. Relative transcription of the developmental MyHC isoforms in the SCWL using NanoString Technologies for gene transcript quantification. Significance determined at $P < 0.05$ using LOESS Smoothing function in SAS.

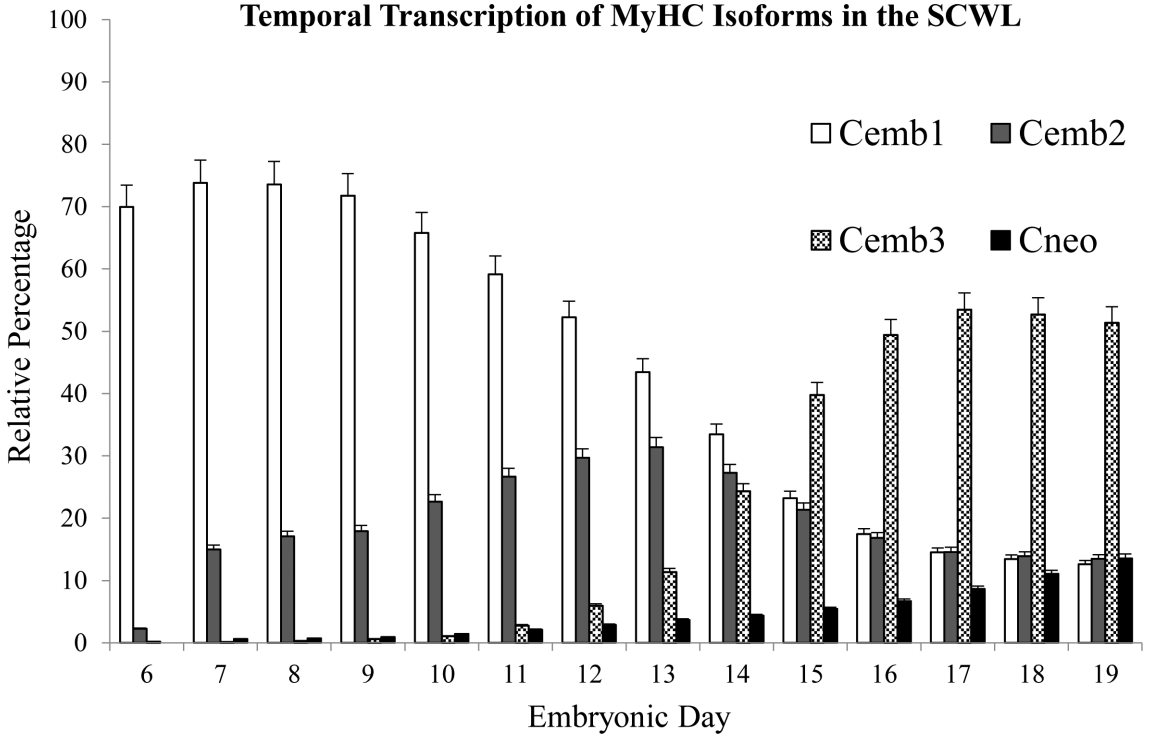

Figure 2. Relative transcription of the developmental MyHC isoforms in the SCWL at embryonic days 6 through 19. Significance determined at $P < 0.05$ using LOESS Smoothing function in SAS.

in the SCWL is shown in Figure 1. The 5% error bars are shown in red for each data point that was determined significant at a value $P < 0.05$. These data show chicken embryonic myosin heavy chain 1 was present in the greatest relative amount at 7 ED with 74% of total MyHC mRNA, chicken embryonic myosin heavy chain (Cemb 2) was greatest at 13 ED with 31% of total MyHC mRNA, chicken embryonic myosin heavy chain 3 (Cemb 3) was greatest at 17ED with 54% total MyHC mRNA, and chicken neonatal myosin heavy chain (Cneo) was greatest at 19 ED with 14% total MyHC mRNA (Figure 2). Although all MyHC isoforms were detected to some degree during embryogenesis, their temporal transcription reveals key transition points that may prove useful in characterizing developmental stages in the embryonic PM in the SCWL.

DISCUSSION

The results from this study provide a high-resolution transcriptional analysis of the developmental fast skeletal muscle MyHC isoforms for 14 timeframes, 6 ED through 19 ED. These analyses are consistent with those reported by Tidyman et al. (1997) who only reported 6 time frames, 10 ED through 20 ED. These results validated the use of NanoString Technologies for transcriptional analysis in avian species. The results obtained were at a much higher resolution due to the fact that this technology does not require the conversion of mRNA to cDNA by reverse transcription or the amplification of the resulting cDNA by Polymerase Chain Reaction (**PCR**) and has been shown to achieve superior gene expression quantification results when compared to RT-PCR (Reis et al., 2011). In addition to obtaining consistent results at a higher resolution, the MyHC isoform transcription analysis reported herein encompassed a larger embryonic timeframe, starting at 6 ED through 19 ED, compared to what was reported by Tidyman et al (1997), which started at 10 ED. The increased range of ages during embryonic development thus allowed for a more comprehensive characterization of the temporal MyHC isoform transitions during embryonic PM muscle development, in which we observed a potentially critical transition from embryonic to neonatal MyHC isoform transcription in the beginning of the third trimester of embryogenesis (Figure 2). It is worth noting that this same timeframe has been shown to be a critical transition period during avian skeletal myogenesis, in which there is a shift from primary to secondary myotube formation (Miller and Stockdale, 1986). Thus, it is reasonable to hypothesize that this transition period is crucial for the successful growth and development of the PM breast muscle in poultry. Therefore this timeframe warrants further transcriptional analyses. In conclusion, the study described herein could provide a method for elucidating the association between the transcription of myogenic regulatory factors and the developmental fast skeletal muscle MyHC isoforms.

In conclusion, the current study established the tools and initial framework necessary to develop a staging system based on the temporal transcription of developmental fast skeletal muscle myosin (MyHC) isoforms during the course of embryogenesis. Using the

same molecular technique, developmental comparisons between MyHC isoforms and several myogenic regulatory genes are made in a subsequent study.

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