

# **Regulation of the Growth of Poultry Skeletal Muscle**

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## **Introduction**

Skeletal muscle myogenesis is a highly organized process regulated by complex interactions between muscle cells and their environment. Postnatal skeletal muscle development occurs due to the proliferation, differentiation, and fusion of myogenic satellite cells with existing muscle fibers. Muscle fibers are surrounded and supported by layers of connective tissue which is composed of cells and an extracellular matrix. The matrix contains fibrous and nonfibrous proteins including collagens and proteoglycans. Research has shown the extracellular matrix to be a dynamic action zone that functions to instruct cellular gene expression and phenotype, and is not just an inert ground substance in which the cells are merely embedded. Many of the activities of the extracellular matrix result from its interaction with other molecules in the extracellular environment resulting in the activation of signal transduction pathways. However, the mechanisms regulating muscle growth and the role of the extracellular matrix are not currently well defined.

It has long been known that steroids, both androgens and estrogens, influence skeletal muscle and whole body growth. In vivo studies using isolated muscle satellite cells have further expanded our knowledge of the involvement of steroids in skeletal muscle growth and physiology. Androgen receptors were identified in porcine skeletal muscle by Doumit et al. (1996). Testosterone administration increased receptor abundance in both satellite cells and myotubes, decreased differentiation, but had no effect on proliferation. Tissue histochemical analysis identified the presence of androgen receptors predominantly localized with the satellite cells (Sinha-Hikim et al., 2004). In addition, estrogen receptor alpha has been identified within the nuclei of porcine satellite cells and estrogen receptor beta in the cytoplasm only (Kalbe et al., 2007).

Satellite cell proliferation and differentiation are also regulated by changes in the extracellular matrix environment surrounding the satellite cells. Changes in the extracellular matrix will either inhibit or stimulate satellite cell proliferation and differentiation. These types of changes in satellite cell behavior can either positively or negatively affect muscle mass accretion. Although there are no reports in skeletal muscle, both estrogen and testosterone have been shown to alter the expression of extracellular matrix collagens and proteoglycans in cartilage (Ganesan et al., 2008). As shown by Johnson et al. (1998), satellite cell proliferation and differentiation is affected by steroids. This change in satellite cell characteristics is most likely due, in part, to changes in cell surface receptors that signal both proliferation and differentiation. In particular, the cell surface associated heparan sulfate proteoglycans are likely to play a significant role in these changes in satellite cell behavior. It is not known how steroids affect the expression of heparan sulfate proteoglycans. The research in this study addressed the effect of steroids on the heparan sulfate proteoglycans, syndecan-4 and glypican-1. Syndecan-4 and glypican-1 are differentially expressed during the proliferation and differentiation of satellite cells.

## **Materials and Methods**

*Turkey Pectoralis Major Muscle Proliferation Studies:* Satellite cells were isolated from 13-wk-old tom turkeys as previously described (McFarland et al., 1995). Cells were plated in 24-well culture plates in DMEM/F12 phenol red free + 10% chicken serum + 5% horse serum. Following a 24 h attachment period, cells were administered serum-free medium (McFarland et al., 2006) containing increasing levels of estradiol  $10^{-12}$  to  $10^{-5}$  M (treatment 1=control; treatment 2= $10^{-12}$  M; treatment 3= $10^{-9}$  M; treatment 4= $10^{-6}$  M; and treatment 5= $10^{-5}$  M. Plates were collected every 24 h for 72 h and stored at -70 °C until analysis.

*RNA Extraction and cDNA Synthesis:* RNA was be extracted from the satellite cells using Tri Reagent (Molecular Research Center) according to the manufacturer's protocol. The total RNA was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (M-MLV). The reverse transcription (RT) reaction was performed in a 25  $\mu$ L volume. An RNA-primer mix [1  $\mu$ L Oligo dT<sub>20</sub> (50  $\mu$ M), 2  $\mu$ g total RNA, and nuclease-free water up to 13.5  $\mu$ L] heated at 65 °C for 5 min. The mixture was incubated immediately on ice for 2 min and 11.5  $\mu$ L of the reaction mix [5  $\mu$ L of 5X First-Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>, 50 mM dithiothreitol), 5  $\mu$ L 10 mM deoxynucleoside triphosphate mix (dATP, dCTP, dGTP, dTTP), 0.5  $\mu$ L RNasin (40 U/ $\mu$ L), 1  $\mu$ L M-MLV (200 U/ $\mu$ L)] was added. The complete reaction was incubated at 55 °C for 60 min, and heated at 90 °C for 15 min to inactivate the reverse transcription (RT) reaction. The synthesized cDNA was diluted with 25  $\mu$ L of nuclease-free water prior to performing the real-time quantitative PCR.

#### ***Real-time quantitative PCR:***

The real-time quantitative PCR was performed using DyNAmo Hot Start SYBR Green qPCR kit (New England Biolabs). Reaction components were assembled in low-profile multiple plates and sealed with Thermal Seal RT. Primers used for the amplification of syndecan-4, glypican-1, MyoD, myogenin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed from published sequences. Following the reaction assembly, plates were put into a DNA Engine Opticon 2 real-time system (MJ Research). The cycling program consisted of denaturation (95 °C for 15 min), followed by amplification and quantification (35 cycles of 94 °C for 30 s, 60 °C or 55 °C for 30 s, and 72 °C for 30 s with a single fluorescence measurement at the end of 72 °C for 5 min. The melting curve program was analyzed on a 1.5% agarose gel to check amplification specificity. Standard curves were constructed for syndecan-4, glypican-1, MyoD, myogenin, and GAPDH with serial dilutions of purified PCR products amplified from each gene. The PCR products were purified by agarose gel electrophoresis using the QIAquick gel extraction kit. All the sample concentrations were checked to ensure they fall within the values of the standard curves. The amount of sample cDNA for each gene was interpolated from the corresponding standard curve. The expression of syndecan-4, glypican-1, MyoD, and myogenin was normalized to GAPDH expression and calculated as an arbitrary unit.

## **Results**

Estradiol effects were measured on turkey pectoralis major satellite cell proliferation (Table I). Insulin levels were maintained at a basal level (5 ng/mL) and

insulin growth factor (IGF-I) concentrations ranged from 0 ng/mL to 100 ng/mL. Estradiol was included in the treated cultures at  $10^{-12}$  M. These results suggest that the addition of estradiol possibly stimulates endogenous production of IGF by satellite cells. A positive effect on proliferation was observed with lower levels of IGF-I. At higher levels of IGF-I, proliferation was inhibited by the presence of estradiol suggesting that estradiol is preventing further increases in proliferation by an unknown mechanism.

**Table I: Estradiol Effect on Turkey Pectoralis Major Muscle Satellite Cell Proliferation**

| IGF concentration | Control Fold increase in DNA from zero time | Treatment Fold increase in DNA from zero time | P value    | Estradiol effect |
|-------------------|---------------------------------------------|-----------------------------------------------|------------|------------------|
| 0 ng/ml           | 3.66 ± .11                                  | 3.14 ± .13                                    | $P = .017$ | inhibit          |
| 5 ng/ml           | 6.19 ± .25                                  | 6.75 ± .11                                    | $P = .092$ | not significant  |
| 10 ng/ml          | 6.76 ± .09                                  | 7.45 ± .12                                    | $P = .002$ | stimulate        |
| 25 ng/ml          | 7.46 ± .12                                  | 8.37 ± .27                                    | $P = .023$ | stimulate        |
| 50 ng/ml          | 8.15 ± .21                                  | 7.35 ± .25                                    | $P = .043$ | inhibit          |
| 75 ng/ml          | 9.33 ± .49                                  | 8.52 ± .09                                    | $P = .099$ | not significant  |
| 100 ng/ml         | 9.69 ± .27                                  | 8.45 ± .18                                    | $P = .011$ | inhibit          |

The effect of increasing concentrations had differential effects on the expression of myogenin, glypican-1, and syndecan-4 (Figure 1) during the proliferation of the turkey pectoralis major satellite cells. Myogenin, a muscle specific transcription factor, expressed during differentiation is decreased by increasing estradiol. In contrast, both syndecan-4 and glypican-1 expression are increased in response to estradiol. Syndecan-4 is thought to function during muscle cell proliferation and glypican-1 during differentiation.

## Discussion

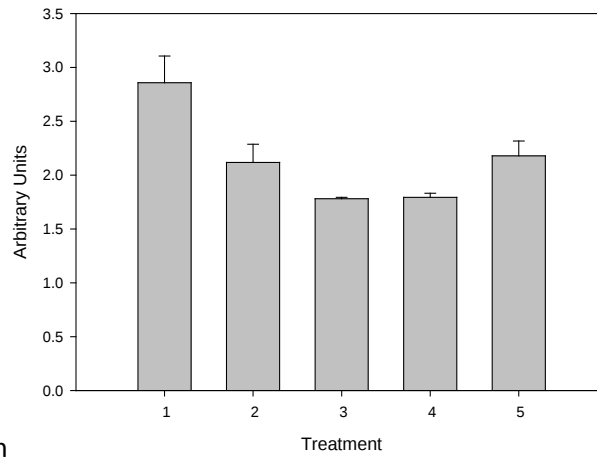
In domestic animals, Johnson et al. (1998) examined the effect of the cattle implant Revalor®-S, which consists of a combination of trenbolone and estradiol, on bovine satellite cell physiology. Implantation of steers elevated serum IGF-I levels, whereas IGF-I levels in non-implanted animals either decreased or remained the same. Cultures of satellite cells derived from implanted animals exhibited increased proliferation and differentiation from control cattle. These data suggest that steroids may influence muscle growth by the activation of the satellite cells. In poultry, little has been reported about the effects on muscle growth mechanisms (Fennell and Scanes, 1992) and to date, no information has been generated on their possible effect on satellite cell populations. The results from the current study suggest that at higher concentrations of IGF-I, satellite cell proliferation is inhibited by the presence of estradiol. Estradiol also differentially affects the expression of syndecan-4, glypican-1, MyoD, and myogenin. These data further support the hypothesis that estradiol is affecting the proliferation and differentiation of satellite cells in the turkey pectoralis major muscle, as syndecan-4 and MyoD are involved with satellite cell proliferation,

whereas glypican-1 and myogenin are regulators of the differentiation process. Taken together, these changes in gene expression will affect breast muscle weight. In support of these findings, Fennell and Scanes (1992) found in turkeys that body weight, average daily gain, and breast muscle weight were increased and abdominal fat was decreased in response to anabolic androgens.

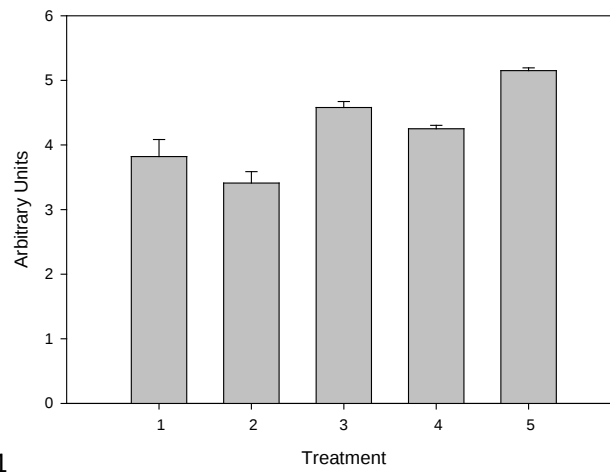
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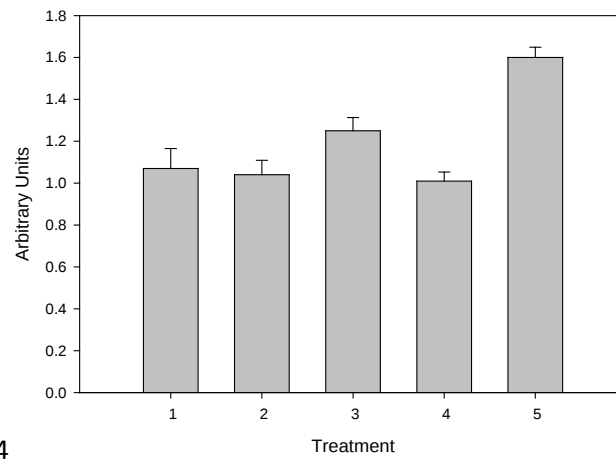
**Figure 1**



**A) Myogenin**



**B) Glypican-1**



C) Syndecan-4