

# Impaired muscle stem cell function in cows with high concentrations of androstenedione in their follicular fluid<sup>1</sup>

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## INTRODUCTION

It is unclear whether androstenedione (A4) increases muscle mass and strength similar to testosterone or whether it produces primarily catabolic effects on muscle-like estrogen (Rasmussen et al., 2000). Summers et al. (2014) observed two populations of cows that exhibit either high (>40 ng/mL; High A4) or low (<20 ng/mL; Low A4) concentrations of A4 within the fluid of the dominant follicle just prior to ovulation. High A4 cows had decreased reproductive rates and shorter times before falling out of the herd, but those that did produce calves weaned them ~10-kg heavier than their low A4 counterparts (Summers et al., 2014). It appears that the difference in weights is due to faster growing and more efficient skeletal muscle. High A4 cows share many characteristics with women suffering from polycystic ovary

syndrome (PCOS), whose high levels of circulating androgens are associated with changes in body composition (Kirchengast and Huber, 2001).

Skeletal muscle growth is mostly impacted by the functional capacity of muscle stem cells known as myoblasts (Cadaret et al., 2017a). Myoblast function can be divided into two distinct processes of proliferation and differentiation. Both processes occur through a carefully orchestrated progression controlled by myogenic regulatory factors (MRF) that are each expressed at specific time points of cellular changes (Molkentin and Olson, 1996). Myogenic determining factor 1 is expressed primarily when myoblasts are actively proliferating in order to expand their numbers (Gillespie et al., 2009). Myogenin expression is the hallmark MRF biomarker of myoblasts that have exited the cell cycle and begun to differentiate (Hawke and Garry, 2001). As the differentiation process progresses, myoblasts begin to express desmin and continue to do so until fusing with existing muscle fibers to facilitate hypertrophic growth (Ludolph and Konieczny, 1995). The objective of this study was to determine whether differences in myoblast function, specifically proliferation and differentiation, exist between High A4 and Low A4 cows that might explain the greater growth rates of their offspring between birth and weaning. Moreover, we sought to determine whether any changes in functional capacity were related to altered responsiveness to tumor necrosis factor

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alpha (TNF $\alpha$ ) and/or testosterone, two known regulators of myoblast function and muscle growth.

## MATERIALS AND METHODS

### *Animals and Experimental Design*

The following experiments were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln, which is accredited by AAALAC International. A subset of mature commercial beef cows from the University of Nebraska Agricultural Research and Development Center were used in these studies. This herd consists of approximately 230 composite beef cows that are 75% Red Angus. Cows were classified as High A4 or Low A4 by human A4 ELISA kit (Alpha Diagnostics International). High A4 cows ( $n = 10$ ) exhibited  $>40$  ng/mL A4 within the fluid of their dominant follicle just prior to ovulation, and Low A4 cows ( $n = 10$ ) had  $<20$  ng/mL A4 within their follicular fluid. Myoblasts were isolated from external oblique muscle biopsies collected from randomly selected cows from each classification during ovariectomies for other studies. The biopsy ( $\sim 5$  g) was collected from the incision site, washed in cold PBS, and then finely minced for myoblasts isolation. Myoblasts were liberated via protease digestion and isolated through serial centrifugation as described previously (Yates et al., 2014). Isolates were purified by multiple preplate incubations until  $\geq 95\%$  pure (pax7-positive).

### *Myoblast Proliferation*

Myoblasts were plated on fibronectin-coated six-well plates at a density of 5,000 cells per well and grown in complete growth media (Dulbecco's Modified Eagle's Medium [DMEM] [Gibco Life Technologies] + 20% fetal bovine serum [FBS, Atlas Biologicals]) for 3 d (media was changed at day 2), and then incubated in treatment-spiked growth media for 24 h. Treatment-spiked growth media contained no additive (basal), TNF $\alpha$  (20 ng/mL), or testosterone (10 nM). After 24-h treatment, myoblasts were pulsed with EdU for 2 h, cooled on ice, and fixed in suspension with 4% PFA. Cells were then stained in suspension via Click-iT EdU Staining kit (ThermoFisher) according to manufacturer recommendations. Click-iT EdU cocktail was added at 50  $\mu$ M and incubated for 2 h at 37 °C. Myoblasts undergoing replication during

the pulse period were identified with AlexaFluor 555 and counted via flow cytometry (zEPI; Orflo Technologies).

### *Myoblast Differentiation*

Myoblasts were plated on fibronectin-coated plates at a density of 30,000 cells per well, grown in complete growth media overnight, and differentiated in treatment-spiked differentiation media (DMEM + 2% FBS) for 4 d (media was changed at day 2) that contained no additive (basal) or TNF $\alpha$  (20 ng/mL). After 4 d, myoblasts were cooled on ice, lifted from the plate with Accutase, washed, and fixed in 4% PFA. Fixed myoblasts were incubated for 1 h at room temperature with primary antibodies against myogenin (1:200; Abcam) or desmin (1:50; GeneTex). Myoblasts were then washed and incubated with PE Conjugate anti-Mouse secondary (1:250; Cell Signaling) for 1 h at room temperature. Percentages of myogenin-positive and desmin-positive myoblasts were determined by flow cytometry.

### *Statistical Analysis*

Values are expressed as mean percentages  $\pm$  SEM. Cow is the experimental unit. Two replicates per incubation condition were performed for each fetus and averaged. Data were analyzed for effects due to A4 classification, incubation condition, and their interaction using the Mixed procedure in SAS (SAS Institute, Cary, NC) with culture condition as a repeated variable.

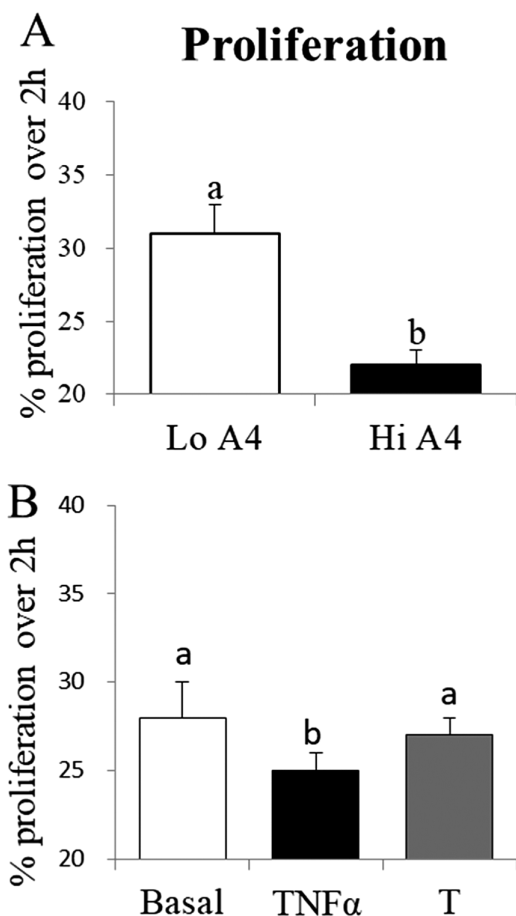
## RESULTS

### *Proliferation*

No interactions were observed between A4 classification and incubation conditions for proliferation rates (Figure 1). Proliferation rates were  $\sim 9\%$  less ( $P < 0.05$ ) in myoblasts isolated from High A4 cows compared with their Low A4 counterparts regardless of incubation condition. Incubation of myoblasts from all cows in growth media containing TNF $\alpha$  decreased ( $P < 0.05$ ) proliferation rates by  $\sim 3.5\%$  compared with basal media. Conversely, incubation of myoblasts from all cows in media containing testosterone had no significant effect on proliferation rates.

### *Differentiation*

No interactions were observed between A4 classification and incubation conditions for percentages

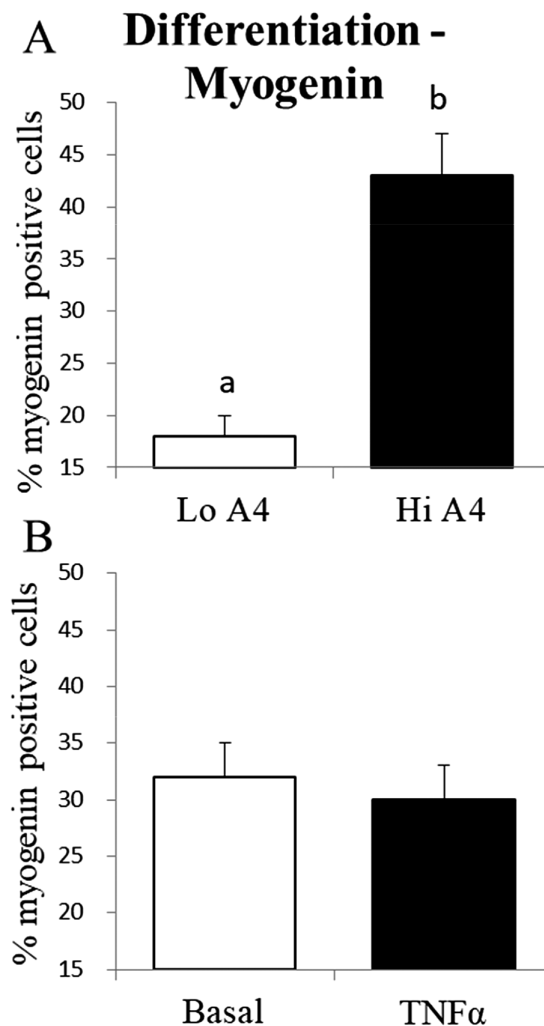


**Figure 1.** Proliferation rates of myoblasts from High A4 and Low A4 cows during a 2-h EdU pulse. Differences were observed between High A4 and Low A4 cows (A) and among basal, tumor necrosis factor alpha-spiked, and testosterone-spiked growth media (B). a,b,c represent means with different superscripts differ ( $P < 0.05$ ).

of myogenin-positive or desmin-positive myoblasts after 4-d differentiation. The percentage of myogenin-positive cells was ~25% greater ( $P < 0.05$ ) in myoblasts isolated from High A4 cows compared with Low A4 cows (Figure 2) regardless of incubation conditions. Likewise, the percentage of desmin-positive cells was ~15% greater ( $P < 0.05$ ) in myoblasts isolated from High A4 cows compared with Low A4 cows, regardless of incubation conditions (Figure 3). Unlike proliferation rates, the percentages of myoblasts that were positive for myogenin or desmin did not differ between basal media and TNF $\alpha$ -spiked media.

## DISCUSSION

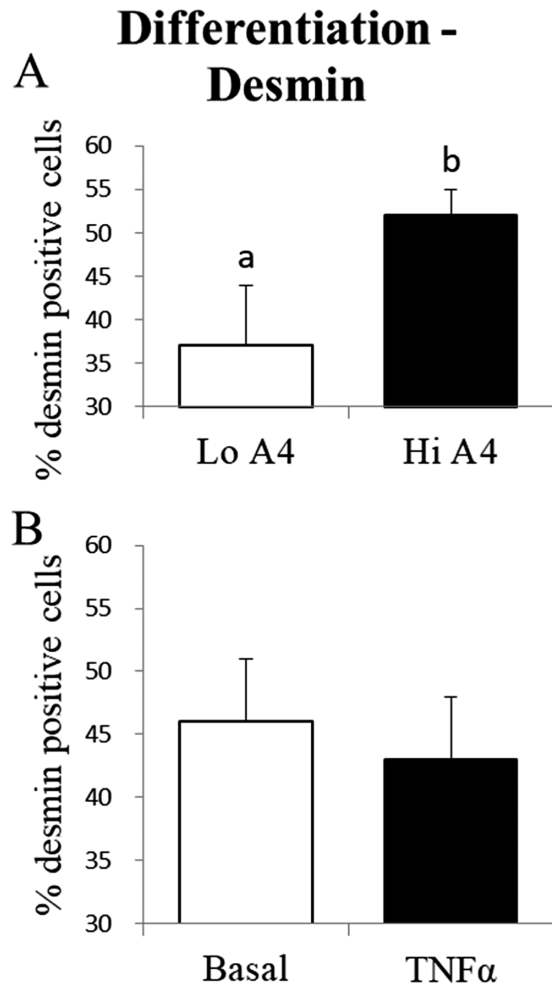
In this study, we show that cows with high A4 concentrations in their follicular fluid also exhibit intrinsic reductions in myoblast proliferative capacity but greater expression of major myoblast differentiation markers. This indicates that conditions responsible for the increase in follicular fluid A4 are also causing precocious differentiation in



**Figure 2.** Percent of myoblasts expressing myogenin after 4 d. Differences were observed between High A4 and Low A4 cows (A) and among basal and tumor necrosis factor alpha-spiked growth media (B). a,b,c represent means with different superscripts differ ( $P < 0.05$ ).

myoblasts. This may be due simply to impaired proliferation rates that push cells to differentiate because they have exited the cell cycle or due to an unidentified stimulator of differentiation. Regardless, this intrinsic deficit in myoblast function ultimately slows the rate-limiting step and presumably restricts muscle growth potential. Because calves born to High A4 cows are of normal size at birth but heavier by weaning, it is reasonable to postulate that exposure to the unidentified conditions of this “High A4” environment in utero reduces their responsiveness to impediments of myoblast function, allowing greater muscle growth from birth to weaning.

Our somewhat surprising observation that testosterone had no effect on myoblast proliferation rates in any cows leads us to believe that A4 (a presumed analog) does not elicit anabolic effects by enhancing myoblast proliferation. Of course, we cannot rule out the potential for different outcomes



**Figure 3.** Percent of myoblasts expressing desmin after 4 d. Differences were observed between High A4 and Low A4 cows (A) and among basal and TNF $\alpha$ -spiked growth media (B). a, b, c represent means with different superscripts differ ( $P < 0.05$ ).

with different media concentrations than the one used in this study. Many other studies have shown that testosterone works to increase muscle proliferation and muscle protein synthesis (Sinha-Hikim et al., 2003; Kadi, 2008). The small effect of TNF $\alpha$  on proliferation and lack of effect on differentiation were also unexpected compared with findings by (Cadaret et al., 2017b), although the fact that these myoblasts were from mature rather than growing animals could have contributed to these results.

### IMPLICATIONS

The decrease in proliferation and increase in differentiation rates indicates that High A4 cows have intrinsic myoblast dysfunction. As myoblast function serves as the rate-limiting step for muscle growth, this does not explain greater birth-to-weaning growth rates in calves from these High A4 cows. Rather, their calves likely develop a reduced

responsiveness to myoblast-restricting conditions of the High A4 cows in utero, which would explain their increased growth between birth and weaning.

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