Myogenic Progenitor Cells in Runt Pigs

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Abstract— **Runt pigs exhibit decreased postnatal growth compared to their normal littermates. Skeletal muscle satellite progenitor cells play a crucial role in muscle fiber development and postnatal muscle growth. The side population (SP) has recently been identified from skeletal muscle using fluorescence-activated cell sorting (FACS) and exhibits both hematopoietic and myogenic capabilities. To this end, we analyzed *semitendinosus* (ST) muscles of 2 d old littermate normal (1.5 ± 0.05 kg) or runt (0.8 ± 0.06 kg) piglets for satellite cell and SP numbers. Muscles of runt pigs possessed smaller circumferences ($P < 0.05$), total fiber numbers ($P < 0.05$), and average fiber sizes ($P < 0.05$) compared to those from normal pigs. Concentration of total protein ($P = 0.18$) or DNA ($P = 0.76$), however, was not different. Total RNA and DNA content were greater ($P < 0.05$) in ST from normal compared to runt pigs. Satellite cell yield per g ST muscle obtained by primary cell culture from both groups was not different ($P = 0.67$). Proliferative capacity was not different at 0, 24, and 48 h in culture, but tended to be higher for cells from normal pigs at 72 h compared to runt pigs ($P = 0.07$). Muscle from runts yielded lower SP frequencies (normal, 9.16% versus runts, 1.22%; $P = 0.034$). These results confirm runt pigs have decreased muscle size and fiber number and suggest aberrations in myogenic stem cell populations play a critical role in this growth retardation model.**

Keywords— **runt pigs, side population, satellite cell**

I. INTRODUCTION

A runt pig's birth weight is less than two thirds of the average birth weight of it littermates and is growth retarded due to prenatal under nutrition [1]. These pigs have a lower chance of surviving to weaning and have decreased postnatal growth performance, which is economically detrimental modern production systems.

Skeletal muscle fiber number and DNA content have been shown to be lower in runt pigs [2]. Because prenatal myogenesis determines the number of muscle fibers by the time of birth [2], satellite cells (SCs), as myogenic progenitor cells, serve as the source of muscle generation during postnatal growth [3]. Proliferation of SCs and their fusion with existing muscle fibers are associated with an increase in DNA and protein content. Although there is little doubt that SCs represent progenitors of muscle cells, another progenitor cell pool known as side population (SP) has recently been identified in skeletal muscle [4]. Skeletal muscle-derived SPs can be isolated by fluorescence-activated cell sorting (FACS) on the basis of Hoechst dye exclusion [5]. Previous studies showed that SPs require the paired box protein, Pax7 for the specification of skeletal muscle SCs [6]. Although the molecular regulation of SP in muscle growth remains unknown, these results suggest that SP might be precursors of muscle satellite cell population.

While quantitative differences of growth performance are well documented, the contribution made by these cell populations to skeletal muscle growth in runt pigs remains unclear. Therefore, the purpose of this study was to compare myogenic progenitor cell populations in muscles from normal and runt piglets.

II. MATERIALS AND METHODS

Animals- All procedures were conducted in accordance with the guidelines set by the Institutional Animal Care and Use Committee at Virginia Tech. The weights of individual pigs were recorded at birth (Table 1) and runt (< 0.9 kg) and normal birth weight (1.4 to 1.6 kg) littermates (3 pigs each) were euthanized at 2 d.

Satellite cell (SC) cultures - *Semitendinosus* (ST) muscles were dissected from 2-d-old piglets, minced, and digested with pronase (0.8 mg/mL in PBS). Cells were suspended in MEM supplemented with 10% FBS with antibiotics and stored in liquid nitrogen for future use. The proliferation assay for SCs was measured for 4 d by using the Promega Cell Proliferation Assay Kit (Promega, Madison, WI). At 80% confluence, cells were switched to 2% horse serum in MEM for differentiation. When fully differentiated, cells were fixed and stained with 0.03% Giemsa. Ten observations per well were made to determine the fusion index (% myotube nuclei/total number of nuclei/mm^2).

Immunochemistry - Cultured cells were fixed in paraformaldehyde and blocked in PBS containing 2% BSA, 5% goat serum, and 0.2% Triton X-100. Cells were then incubated with Pax7 primary antibody (R&D system, Minneapolis, MN) followed by Cy5-conjugated secondary antibodies. Nuclei were counter-stained with DAPI.

Muscle RNA, DNA, and protein - Muscle RNA, DNA and protein were quantified using a modified procedure by Clowes, et al.[7]

Muscle fiber number- ST muscles were mounted on corks and immediately frozen in liquid nitrogen-cooled isopentane. Frozen muscle cross-sections were cut, dried and washed in PBS, blocked in 5% goat serum, followed by incubation with wheat germ agglutinin conjugated with Oregon Green dye (Invitrogen, Carlsbad, CA) to visualize muscle fiber membranes. Ten random fields were chosen for analysis. The cross sectional area of ST was measured by drawing the outline of center cut of ST on paper and determining the area using image J software.

Side population Cells (5×10^6/mL) were pre-warmed to 37°C, Hoechst 33342 was added to a final concentration of 5μg/mL, incubated for 90 min, centrifuged, and resuspended in Hanks's balanced saline solution containing 2% FBS and 2mM HEPES buffer. Cells were then kept on ice and analyzed with Coulter ALTRA Cell Sorter (Beckman Counlter Inc. Miami, FL).

Statistics - Data were analyzed with JMP using Student's t-test. Differences were determined significant at $P < 0.05$. Data are presented as means ± standard error.

III. RESULTS AND DISCUSSION

The difference in birth weight between normal and runt littermates was significant (table 1; $P < 0.01$). The weight and area of ST muscles from normal piglets were greater ($P < 0.01$) than those from runts. The DNA and protein concentration was not different between normal and runt animals, but total DNA content from the runt ST was lower ($P < 0.05$) than those from normal (Table 1). The STs from the runts tended to have lower RNA to DNA ratio ($P = 0.08$) than normal suggesting that runts have a reduced potential for postnatal protein synthesis.

Table 1. Birth weight and ST muscle characteristics

	Normal	Runt	Pooled SE
birth weight, kg	1.52	0.79**	0.03
ST weight, g	4.0	1.78*	0.57
ST area, mm^2	87.4	36.6*	8.6
ST protein, mg/g	68.5	65.1	1.4
ST DNA, mg/g	0.84	0.77	0.15
ST total DNA, mg	3.16	1.28*	0.26

**$P < 0.01$ and *$P < 0.05$ vs. normal within row

In agreement with studies from Wigmore [8] and our DNA data, runt piglets formed a lower $P < 0.05$) total fiber number as well as average fiber size in ST muscle compared to normal animals ($P < 0.05$; Fig. 1). Birth weight and ST fiber number showed a high linear correlation ($r = 0.61$; data not shown).

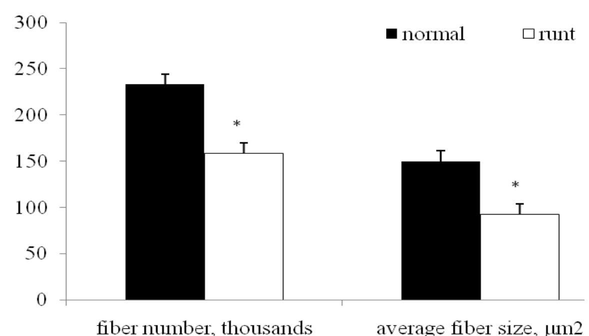

Figure 1. Total fiber number and average fiber size. *$P < 0.05$ vs. normal.

SCs are identified as the major source of nuclei additions to the muscle fiber postnatally, and more

than 50% of the cells are produced by SC division during the early stages of postnatal growth [3]. Therefore, we hypothesized that skeletal muscle mass differences between normal and runt piglets are associated with differences in the population of SCs and their proliferation and differentiation capacity. To this end, we isolated SCs from normal and runt littermates and analyzed their growth characteristics in culture. There was no difference in total cell yield between SCs of normal and runt piglets (data not shown). Because muscle primary cultures contain a mixed population of different cell types, we analyzed fusion index to better estimate myogenic cell.

Furthermore, since Pax7 plays a critical role in the satellite cell lineage development and is a specific marker for satellite cells [6], we stained myoblasts for Pax7. As expected, satellite cells from normal piglets possessed higher Pax7+ cells compared to those from runts (59% and 43%, respectively, $P < 0.05$, n=12; Fig. 2).

There was no significant difference in proliferation rates of SCs from normal and runt pigs at proliferation 0, 1, 2, and 3 d, but SCs from normal piglets tended to have a higher rate at 72 h ($P < 0.07$; Fig. 3). Despite this, SCs from runts reached confluence approximately 12 to 24 h later than SCs of normal pigs.

The fusion index including myotubes with more than two nuclei of SC culture from normal piglets was not significantly higher than that from runts (66.1% and 55.2%, respectively; data not shown). It is somewhat surprising that the fusion index is not significantly different between normal and runt cell culture when Pax7+ cells are significantly higher in ST from normal weight piglets.

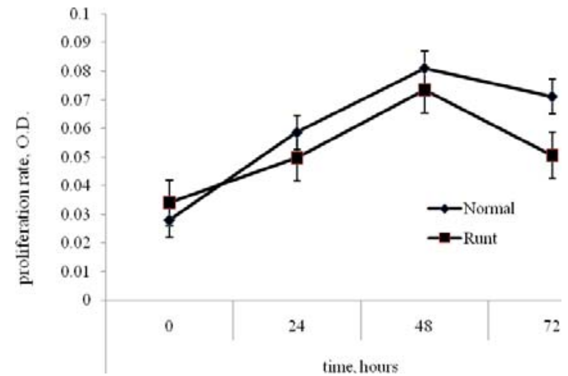

Figure 3. Proliferation rate of SCs from normal and runt littermates at 0, 24, 48, and 72 h.

Because Pax7 is expressed in quiescent as well as newly activated SCs [6], it is possible that not all satellite cells are activated in our cell culture system. This warrants us to find better way to recruit SCs to the cell cycle.

Recently, a stem-like cell population other than SCs, known as side population (SP), has been reported to be a multipotent progenitor cell population capable of myogenic differentiation in

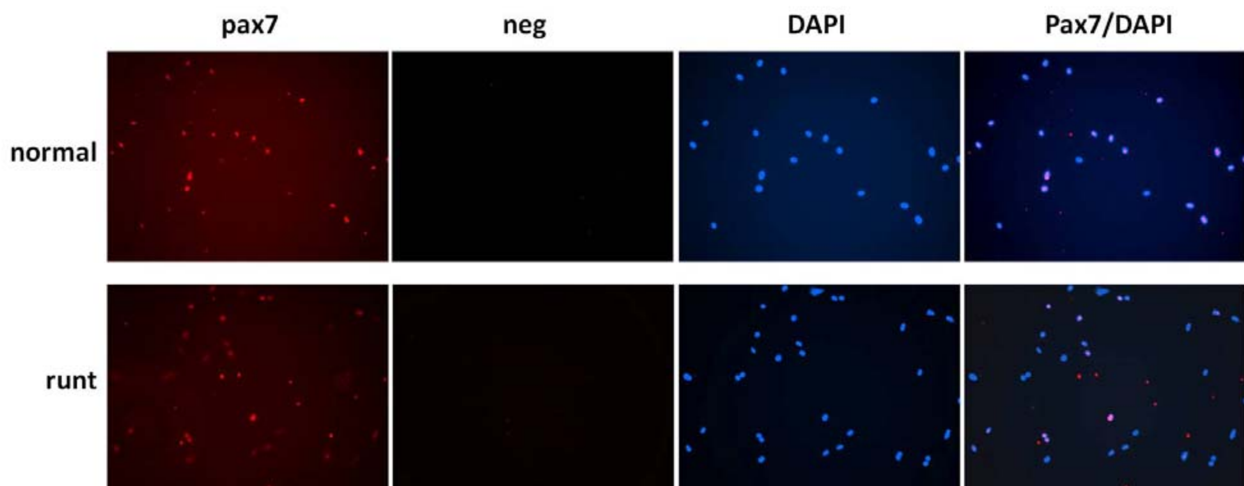

Figure 2. Pax7+ SC population from normal and runt ST. Immunostaining of freshly isolated SCs labelled with Pax7 (in red). Nuclei revealed with DAPI.

vitro and in vivo and to express Pax7 [4]. To check the contribution of SP in ST muscle cell culture, we next analyzed the SP population by FACS, on the basis of Hoechst dye exclusion (Fig. 4). We found approximately 7 fold higher SP in cells from normal ST (Fig. 4a) than those from runt piglets (Fig. 4b). Mean SP content of normal and runt cells is 9.16% and 1.22 %, respectively ($P < 0.05$; Fig. 4).

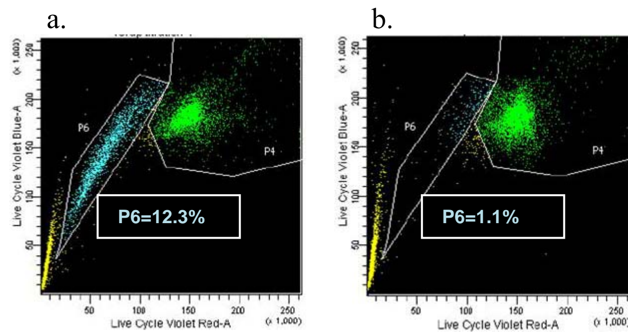

Figure 4. Flow cytometry analyses of cells isolated from ST. Cells were stained with Hoechst 33342. Representative FACS image of cells from normal (a) and runt (b) piglets. SP was gated in P6.

Although further investigation is needed to address the relationship between prenatal under nutrition and skeletal muscle SP content, these data suggest that SP could be another factor for postnatal muscle growth. Furthermore, runts posses less SPs and SCs compared to their normal weight littermates implying that SPs and SCs may not compensate for each other. Therefore, identifying factors regulating the population of SCs, the interrelationship of SCs and SP, and the subpopulations of SP are of particular interest.

IV. CONCLUSION

The results presented in this study show that runt piglets have decreased ST muscle size, fiber number, and DNA content. Muscle cell culture data showed that there is a tendency towards a lower proliferation and differentiation rate, less number of Pax7+ cells and SP in ST muscles from runts compared to those from normal weight littermates. Altogether, it is suggested that prenatal under nutrition may alter the rate of cell division of muscle progenitor cells including SCs and SP and these cell populations play a critical role in muscle growth and development.

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