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Decreased Satellite Cell Number and Function in Humans and Mice With Type 1 Diabetes Is the Result of Altered Notch Signaling

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Type 1 diabetes (T1D) negatively influences skeletal muscle health; however, its effect on muscle satellite cells (SCs) remains largely unknown. SCs from samples from rodents (Akita) and human subjects with T1D were examined to discern differences in SC density and functionality compared with samples from their respective control subjects. Examination of the Notch pathway was undertaken to investigate its role in changes to SC functionality. Compared with controls, Akita mice demonstrated increased muscle damage after eccentric exercise along with a decline in SC density and myogenic capacity. Quantification of components of the Notch signaling pathway revealed a persistent activation of Notch signaling in Akita SCs, which could be reversed with the Notch inhibitor DAPT. Similar to Akita samples, skeletal muscle from human subjects with T1D displayed a significant reduction in SC content, and the Notch ligand, DLL1, was significantly increased compared with control subjects, supporting the dysregulated Notch pathway observed in Akita muscles. These data indicate that persistent activation in Notch signaling impairs SC functionality in the T1D muscle, resulting in a decline in SC content. Given the vital role played by the SC in muscle growth and maintenance, these findings suggest that impairments in SC capacities play a primary role in the skeletal muscle myopathy that characterizes T1D.

The prevalence of type 1 diabetes (T1D) continues to rise globally in youth populations (1). This autoimmune disorder is characterized by the destruction of pancreatic β-cells, leading to hypoinsulinemia and the loss of glucose homeostasis. Although exogenous insulin therapy is currently available for these afflicted individuals, this treatment is not curative. Failure to properly maintain blood glucose through insulin therapy promotes periods of extreme glycemic levels and, over time, the development of diabetic complications.

Diabetic myopathy is an often-overlooked diabetic complication but is believed to adversely affect the health and well-being of individuals with T1D. Although skeletal muscle is a largely resilient tissue that is capable of adapting to changing conditions, the skeletal muscle of individuals with T1D exhibits a decline in physiological function and performance compared with healthy skeletal muscle, including significant impairments to its reparative capacities (2–7).

The skeletal muscle stem cell population, referred to as satellite cells (SCs), is a primary contributor to the maintenance and repair of skeletal muscle and has a central role in skeletal muscle plasticity (8). Although muscle SCs are fundamentally involved in maintaining the health of skeletal muscle, few studies have investigated the effect of T1D on the muscle SCs, and no study, to the best of our knowledge, has investigated SC populations in young human populations with T1D to ascertain whether the changes occurring in rodent studies are translatable to the human condition.

The purpose of the current study was to examine SC content and function in the *Ins2*^{Akita} (Akita) mouse model and in young adults with T1D. Single fiber isolation experiments were completed to examine markers of SC quiescence and activation in Akita and wild-type (WT) mice, allowing for the identification of intrinsic differences in SC function between experimental conditions. Disparities in SC function within biopsy specimens of young adults

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with T1D were also investigated to determine whether these changes in rodents were translatable. We hypothesized that SCs derived from muscle of subjects with T1D diabetes would display impairments in SC function that detrimentally affect overall muscle health and that this would be attributed to modifications to unique intracellular pathways that regulate SC quiescence and activation. Specifically, we assessed the influence of the Notch signaling pathway and its effect on SC function in T1D skeletal muscle due to its well-established role in postnatal myogenesis (9) and its regulation of SC self-renewal during muscle regeneration (10).

RESEARCH DESIGN AND METHODS

Animals

Male C57BL/6-Ins2$^{Akita/J}$ mice and their WT littermates were housed in a temperature- and humidity-controlled facility with a 12-/12-h light/dark cycle and given free access to food and water. Akita mice spontaneously develop T1D at ~4 weeks of age due to a heterozygous mutation in the *Ins-2* gene. Akita mice were monitored for diabetes onset (blood glucose >15 mmol/L) after weaning through the use of blood and urine analyses. All experimental protocols were carried out with approval of the McMaster University Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines.

Endurance Exercise Test

To compare the functional capacity of Akita and WT skeletal muscle, mice with 16 weeks of diabetes (i.e., 20 weeks of age) from each experimental condition underwent an endurance exercise test (n = 3 WT, n = 4 Akita). The acclimation period lasted for 2 days and consisted of placing mice on the treadmill (Columbus Instruments, Columbus, OH), with a gradual increase in treadmill speed and duration up to 10 m/min for 5 min. The exercise test was performed with mice starting at a speed of 8 m/min for 5 min, with a subsequent increase to 9 m/min for 3 min. After this, the speed was increased by 1 m/min every 10 min until the mice reached exhaustion (11).

Eccentric Exercise Protocol

A fraction of WT and Akita animals (8 weeks of diabetes, n = 3 per group) were randomly assigned to a 4-day eccentric exercise training protocol to assess changes to muscle repair after subjection to a physiologically relevant stimulus to induce muscle damage. These mice were placed on a treadmill with a 15° downhill incline to promote eccentric exercise, as previously described (12), with modifications. Mice were tested at this specific age to compare and contrast data from a previous study completed by our laboratory that used a chemical means to induce muscle damage in this same mouse model (13).

Tissue Collection

Animals were euthanized by CO_2 inhalation, followed by cervical dislocation. The tibialis anterior muscles were excised from WT and Akita mice, with the left muscle coated in tissue-mounting medium and frozen in liquid nitrogen–cooled isopentane, and the right muscle was snap frozen in liquid nitrogen. Left and right extensor digitorum longus and peroneus muscles were harvested to isolate single muscle fibers, and the remaining hind limb muscles (gastrocnemius-plantaris-soleus complex and quadriceps) were snap frozen and used for protein analyses.

Patients and Ethics Statement

Skeletal muscle biopsy specimens were taken from the vastus lateralis using a 5-mm Bergstrom needle, as previously described (14). Samples were taken from healthy subjects without diabetes (control; n = 5) and men with T1D (n = 6) 18–24 years of age (Table 1). Differences exist in the number of samples used for each analyses based on the method of preparing the specimen, and the specific sample size used for each analysis is defined within the figure legends. Subjects gave written consent after being informed of the procedure and associated risks involved with the study. This portion of the study was approved by the Hamilton Health Sciences Research Ethics Board (REB #14-649), and conformed to the Declaration of Helsinki regarding the use of human subjects as research participants.

Single Muscle Fiber Isolation

Single muscle fibers were obtained from Akita and WT mice at 12 weeks of age (8 weeks of diabetes) from the left and right extensor digitorum longus and peroneus muscles, as previously described (15). Fibers were fixed immediately after isolation (referred to as Control fibers) or placed in culture dishes with plating media (10% normal horse serum, 0.5% chick embryo extract in DMEM) overnight (18 h; referred to as Activated fibers). For all single-fiber experiments, the minimum and maximum number of fibers analyzed is provided in a range (i.e., 25–40) and is derived from at least three mice per experimental group.

To investigate the role of Notch signaling, isolated myofibers were treated with 10 μm *N*-[2*S*-(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl-1,1-dimethyletyl ester glycine (DAPT; Sigma-Aldrich, St. Louis, MO) after myofiber isolation and subsequently left in culture, as

Table 1—Demographics of human subjects

Characteristic	Control subjects, n = 5	Subjects with T1D, n = 6
Age (years)	22 ± 0.55	20 ± 0.52
Weight (kg)	82.98 ± 3.85	72.20 ± 3.50
Height (m)	1.83 ± 0.01	1.78 ± 0.04
BMI (kg/m^2)	24.90 ± 1.19	22.80 ± 0.33
Diabetes duration (years)		7.80 ± 1.16
HbA_{1c} (%)		8.40 ± 0.27

previously described (16). Specifically, isolated single fibers from WT and Akita skeletal muscle were placed in the presence or absence of DAPT for 24 h to permit assessment of their capacity to become activated with DAPT treatment (DAPT Tx) or without Notch inhibition (Activated). Fibers were subsequently fixed and stained for $Pax7^+$ nuclei to determine changes in the quantity of SCs between experimental groups. A direct comparison of the number of $Pax7^+$ nuclei on DAPT Tx and Activated fibers from each experimental group was completed and presented as a fold-difference.

SC Activation

SC activation was assessed in floating cultures by adding 10 μmol/L BrdU to the plating media and incubating newly isolated single fibers for 24 h. Fibers were fixed and stained for BrdU (Abcam, Cambridge, MA), as previously described (15). SCs that became activated and entered the cell cycle incorporated BrdU. Because myonuclei are postmitotic, $BrdU^+$ nuclei represent SCs that became "activated" and have entered the cell cycle. SC activation was therefore analyzed by the number of $BrdU^+$ nuclei per muscle fiber.

Western Blot Analyses

Protein (~100 μg) from mouse or human whole muscle lysates was run out on a separate acrylamide gel, transferred to polyvinylidene fluoride membrane, blocked with 5% skim milk for 1 h at room temperature, and then incubated overnight at 4°C with primary delta-like 1 (DLL1) mouse (Abnova, Taipei, Taiwan) and human (Cell Signaling, Danvers, MA) antibody. The appropriate horseradish peroxidase-conjugated secondary antibodies were incubated for 1 h at room temperature, and the blot was visualized using SuperSignal Chemiluminescent reagent (Thermo Scientific, Waltham, MA). Images were acquired using a Gel Logic 6000 Pro Imager (Carestream, Rochester, NY), and the area density of each band was analyzed using Adobe Photoshop.

Skeletal Muscle Histology

Hematoxylin and eosin stains were used for the determination of muscle morphology, with greater than 75 muscle fibers analyzed per section. Muscle injury induced by the eccentric exercise protocol was determined by the presence of centrally located nuclei, pale cytoplasm, and infiltrated muscle fibers, as has been previously established (12). Each incidence of muscle injury was annotated to obtain a value that was then corrected for by the total number of fibers analyzed. The amount of muscle injury in each experimental condition was then expressed relative to the degree of injury observed in the WT sedentary group.

Immunofluorescent Staining

Tibialis anterior muscle sections from WT and Akita mice were fixed with 4% paraformaldehyde, and human vastus lateralis muscle sections were fixed using the same protocol. Single muscle fibers isolated from WT and Akita mice were immediately fixed using 4% paraformaldehyde (Control fibers) or after an activation period (Activated fibers). Muscle sections from mice and human subjects were stained for Pax7 (Developmental Studies Hybridoma Bank, Iowa City, IA), using tyramide signal amplification, and Dystrophin (Abcam). Single fibers were stained for antibodies against Pax7 (Developmental Studies Hybridoma Bank), MyoD (Abcam), Myogenin (Novus Biologicals, Littleton, CO), Notch Intracellular Domain (NICD; Abcam), and Hes1 (Abcam). The appropriate secondary antibodies were applied: Alexa Fluor 594, biotinylated secondary antibody, and Alexa Fluor 488 (Thermo Scientific). Nuclei were counterstained with DAPI.

Image Analyses

All stained fibers were viewed using the Nikon 90 Eclipse microscope (Nikon, Inc., Melville, NY) and analyzed using Nikon Elements software. Analyses included examination of muscle morphology, quantification of protein expression on single fibers, and quantification of SC content ($Pax7^+/DAPI^+$) in muscle sections. All images were examined at original magnification ×20.

Statistics

Measures were assessed using a two-way ANOVA with Bonferroni post hoc test, or where appropriate, the Student *t* test. Significance was set at a P value of <0.05. All statistical analysis was performed using GraphPad Prism 5 software (La Jolla, CA). Data are presented as means ± standard error of the mean (SEM).

RESULTS

Diabetic Akita Mice Display Greater Evidence of Muscle Damage After Eccentric Exercise

After 8 weeks of overt diabetes (~12 weeks of age), there were significant reductions in skeletal muscle masses ($P < 0.05$, Fig. 1*A*) relative to nondiabetic (WT) controls, along with a 17% decrease in body weight and a 60% decrease in epididymal fat mass (data not shown), as has been previously observed in T1D rodent models (7,17,18). The reduction in muscle mass led to the evaluation of muscle function, determined by an endurance exercise test. Compared with their age-matched WT counterparts, Akita mice reached exhaustion faster ($P < 0.05$, Fig. 1*B*). We then investigated whether T1D rodent skeletal muscle was more susceptible to muscle damage after eccentric exercise. WT and Akita mice underwent a 4-day eccentric exercise training protocol. The increased presence of muscle injury in Akita muscle sections, as observed histologically (Fig. 1*C*) and in a graphic representation ($P < 0.05$, Fig. 1*D*,), confirms that T1D skeletal muscles are more susceptible (i.e., display a greater degree of damage) to a physiologically relevant muscle injury stimulus. This finding is consistent with previous work using Evans Blue Dye incorporation into the muscles of downhill-run diabetic and WT mice (7).

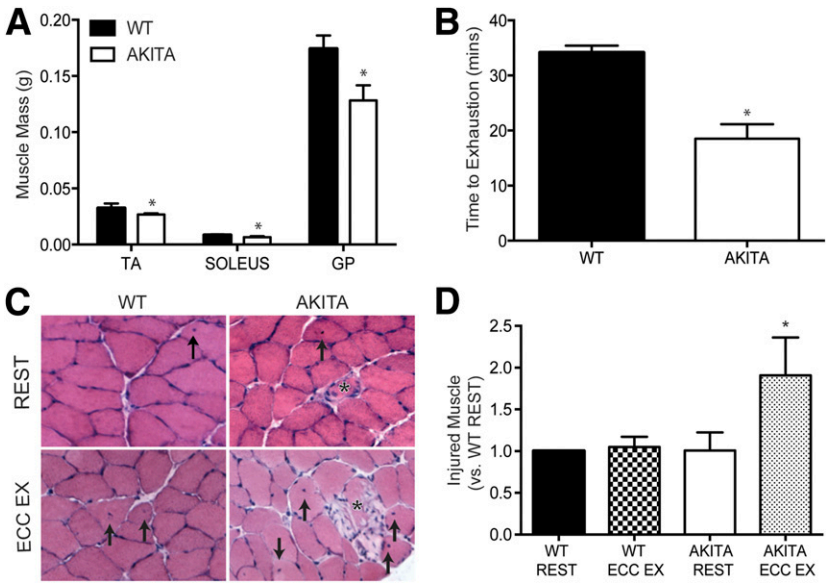

Figure 1—T1D skeletal muscle displays hallmark characteristics of myopathy. *A*: Muscle masses from tibialis anterior (TA), soleus, and gastrocnemius-plantaris (GP) muscles are decreased in 12 week Akita mice ($n = 3$). *B*: WT and Akita mice subjected to an endurance stress test demonstrate that Akita mice are quicker to exhaust than their WT counterparts ($n = 3$ WT, $n = 4$ Akita). *C*: WT and Akita mice were eccentrically exercised (ECC EX) to induce mild muscle damage, with exercised Akita mice displaying the greatest indices of muscle damage compared with rested mice. The black arrows identify centrally located nuclei, and the black asterisks identify necrotic tissue. *D*: Quantification of muscle injury (see RESEARCH DESIGN AND METHODS for criteria) indicates that Akita skeletal muscle is more damaged than WT muscle after eccentric exercise ($n = 3$). *$P < 0.05$ vs. WT.

T1D SCs Display Impairments in Activation and Content

The importance of SCs to skeletal muscle repair and regeneration has been well established (19,20) and was, therefore, a primary focus for the current study. Observations of a decline in skeletal muscle health in Akita mice, particularly after eccentric exercise, prompted our interest in evaluating the response of SCs. An important characteristic of SC function is the capacity to exit quiescence in response to a stimulus, a process termed "activation." We had hypothesized that SC activation would be enhanced given the myopathy that characterizes the skeletal muscle of subjects with T1D.

SC activation was examined using single fibers isolated from Akita and WT muscles that were fixed immediately after isolation or after an in vitro activation period. Fibers were stained for nuclei and Pax7, a transcription factor used to demarcate the SC (Fig. 2*A*). Compared with the quiescent period, SCs present on Akita myofibers did not increase in content after an in vitro activation period, as evidenced by a 40% difference in $Pax7^+$ nuclei on Akita-activated versus WT-activated myofibers ($P < 0.05$, Fig. 2*B*). Furthermore, BrdU incorporation at 24 h after isolation was lower in SCs on myofibers isolated from Akita muscle compared with WT ($P < 0.05$, Fig. 2*C* and *D*), further confirming that activation is lower in Akita diabetic SCs.

It is well established that a failure for SCs to properly activate and progress through myogenesis hinders their ability to replenish their own population, leading to an eventual decline in SC content (21). Given the impairments in SC activation we observed in T1D muscles, assessment of SC content was completed to determine whether a failure to properly activate SCs altered total SC density in T1D muscle. Quantification of SC density revealed a 31% reduction in Akita diabetic compared with WT skeletal muscle ($P < 0.05$, Fig. 2*E*).

After activation, most SCs will progress down the myogenic lineage (termed myoblasts), including expansive proliferation and fusion with one another or with existing, damaged myofibers (8). Additional markers of myogenesis, MyoD and Myogenin, were examined to assess the progression of Akita SCs down the myogenic lineage. In activated Akita myofibers, $MyoD^+$ nuclei were 2.7-fold lower in expression than the WT ($P < 0.05$, Fig. 2*F*), and 2-fold fewer $Myogenin^+$ nuclei were observed in Akita myofibers compared with the WT ($P < 0.05$, Fig. 2*G*).

Hyperactivation of Notch Signaling in T1D SCs

A tight regulation of Notch signaling is imperative for normal myogenesis (22), because it is typically found to increase with activation to promote Pax7 expression and SC self-renewal (23) but must return to a negligible level to facilitate the progression of the SC through the remainder of myogenesis. Given this, we hypothesized that Notch signaling would remain elevated in T1D muscle SCs, resulting in a reduced capacity for activation and progression down the myogenic lineage. Assessment of the active form of Notch-1, referred to as the NICD, and its downstream effector, Hes1, was achieved through immunofluorescent

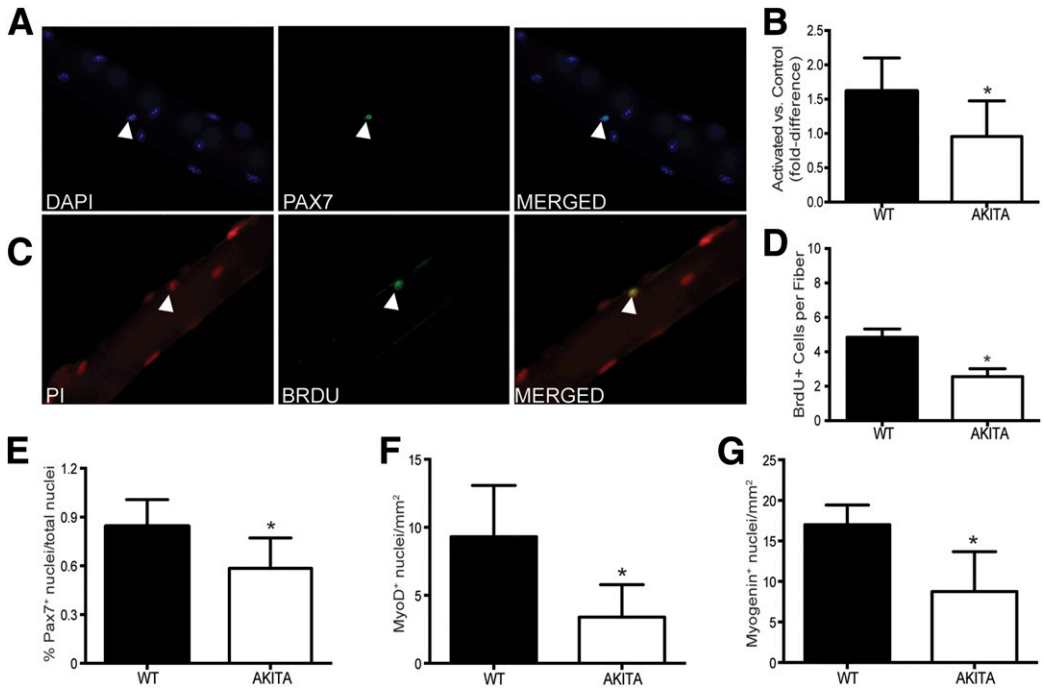

Figure 2—SC activation and content is decreased in T1D skeletal muscle. *A*: Single myofibers were isolated from WT and Akita muscle and stained for nuclei and Pax7. The white arrowheads note a positive signal for a SC. *B*: The difference in Pax7 content between Activated and Control myofibers was determined and indicates that Akita SCs demonstrate a failure to become activated compared with the WT SCs (n = 25–40 myofibers per experimental group). *C*: Representative images of BrdU incorporation, a measure of SC activation, are shown in a WT myofiber. Single myofibers were stained with propidium iodide (PI; a marker for nuclei) and BrdU. The white arrowheads indicate a positive signal for BrdU incorporation. *D*: SC activation was lower in Akita mice at 24 h after isolation compared with WT single myofibers (n = 7–21 myofibers per experimental group). *E*: SC content, determined by Pax7 expression in muscle sections, is lower in T1D skeletal muscle (n = 5). Activated WT and Akita single myofibers were stained for markers of myogenesis, MyoD (*F*) and Myogenin (*G*). Compared with WT, T1D SCs display reduced expression of MyoD (n = 14–16 myofibers) and Myogenin (n = 4–5 myofibers). *$P < 0.05$ vs. WT.

staining of single myofibers (costained with Pax7, Fig. 3*A*). No difference between groups was observed in the expression of $NICD^+/Pax7^+$ on quiescent fibers between WT and Akita myofibers, but a 1.9-fold increase in $NICD^+/Pax7^+$ nuclei was evident in activated Akita myofibers compared with WT ($P < 0.05$, Fig. 3*B*). Similar to the NICD data, we determined that the $Hes1^+$ SC number did not differ in expression between quiescent (control) Akita and WT SCs, but was downregulated in activated WT SCs while remaining significantly elevated in activated Akita SCs ($P < 0.05$, Fig. 3*C*). Pharmacological repression of the Notch signaling pathway was completed through use of the Notch inhibitor, DAPT, in vitro. Although no difference was observed in the expression of Pax7 on treated and untreated WT single fibers, a 1.6-fold increase in Pax7 expression was identified when Akita single fibers, with and without DAPT Tx, were compared ($P < 0.05$, Fig. 3*D*). Taken together, these data provide evidence that inhibiting Notch signaling facilitates an increase in Pax7 expression in Akita single fibers, thereby supporting a role for Notch in impairing SC activation in T1D.

To determine whether the increase in Notch signaling in Akita SCs was the result of increased Notch ligand presence on the myofiber, we quantified delta-like 1 (DLL1) by Western blot in WT and Akita skeletal muscle. No significant difference between groups was noted in DLL1 expression (Fig. 3*E*), suggesting that the Notch pathway is being activated by means other than a direct upregulation of DLL1.

SC Content Is Decreased in Young Adults With T1D

To determine if the observations made in T1D mouse SCs were comparable to SCs in human subjects with T1D, we assessed SC content and the expression of the Notch ligand, DLL1, in the skeletal muscle of young adults (18–24 years old) with and without T1D. A 39% reduction in Pax7 expression was observed in T1D skeletal muscle cross sections compared with healthy age- and sex-matched control subjects ($P < 0.05$, Fig. 4*A* and *B*). Because analyses of single muscle fibers from human skeletal muscle (including the aforementioned activation protocol) was not available using the Bergström biopsy procedure, we investigated changes to Notch signaling through quantification of DLL1 protein expression in whole-muscle lysates from skeletal muscle from healthy human subjects and those with T1D. In contrast to our findings in mouse skeletal muscle, DLL1 protein expression in skeletal muscle from human subjects with T1D was significantly elevated compared with muscle from human subjects without diabetes ($P < 0.05$, Fig. 4*C*)

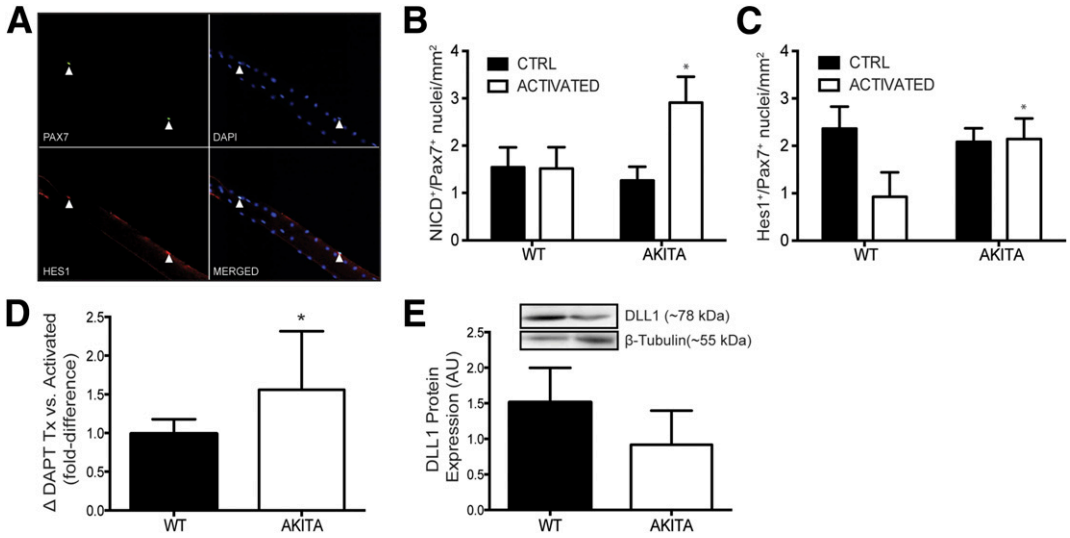

Figure 3—Hyperactivation of Notch signaling alters SC behavior in T1D muscle but is restored with a Notch inhibitor. *A*: Representative images of the evaluation of the Notch target, Hes1, in single myofibers. The white arrowheads identify a positive signal for $Hes1^+/Pax7^+$ SCs. *B*: Hyperactivation of Notch activity is evident in activated Akita SCs compared with WT SCs (n = 7–9 myofibers per experimental group). *C*: Hes1 is repressed in activated WT SCs but remains elevated in Akita SCs, confirming enhanced Notch activity in T1D SCs (n = 6–13 myofibers per experimental group). *D*: Activated WT and Akita single myofibers were treated with the Notch inhibitor DAPT (DAPT Tx) and compared with untreated activated single myofibers from each respective experimental condition (Activated). Notch inhibition with DAPT Tx led to a significant increase in Pax7 expression in activated Akita single myofibers, but no difference in Pax7 expression was determined in activated WT myofibers (n = 11 myofibers per experimental group). *E*: The Notch ligand DLL1 shows a trend (P = 0.09) toward a decrease in expression in whole muscle lysates from diabetic samples (n = 3). CTRL, control. *$P < 0.05$ vs. WT activated.

and may identify a species-specific difference in the availability of different Notch ligands.

DISCUSSION

Skeletal muscle represents the largest insulin-sensitive organ within the body and is the site for ~80% of whole-body glucose uptake (24). Given this level of contribution to glycemic control, one can appreciate that impairments to skeletal muscle health in T1D could be a primary factor in the progression of other diabetic complications. SCs play an important role in the maintenance of healthy skeletal muscle mass because of their function in maintenance and repair (25); however, little is known about this cell population after T1D development. In the current study, we demonstrate for the first time that exposure to the T1D environment adversely affected muscle SC content, a finding consistent in both rodent and human skeletal muscles. Akita diabetic mice exhibited a significant reduction in SC content that was mirrored in young adults with T1D. We also observed a significant impairment in SC activation in Akita mice that was consistent with our results. The mechanism for these defects appears to be impaired SC activation as a result of an overactivation of the Notch signaling pathway within this cell population. Indeed, inhibition of Notch activity in Akita myofibers through in vitro DAPT treatment led to an increase in the expression of the SC marker Pax7, and thus, an increase in SC activation, verifying the role of Notch in the regulation of T1D SC activation.

The decreased exercise capacity of Akita mice observed in this study is supported by previous work in rodent models of T1D (26,27) as well as in human subjects with T1D (28–31). Although the precise cause for this diminished capacity remains controversial, a number of factors are thought to contribute to this decline (30,31). A paucity of information is available regarding the response of T1D skeletal muscle to a more physiologically relevant stimulus, such as exercise-induced damage (32,33). Literature from our laboratory has demonstrated that Akita skeletal muscle displays functional deficits (17) and supports work done by others in regenerating and uninjured Akita skeletal muscle (6). We and others have established that rodents with T1D demonstrate a failure to repair after extreme damage, such as transplantation or toxin-induced injury (5,6,13,34), but the data presented here are the first to show a decline in skeletal muscle function after exposure to a mild muscle-damaging stimulus, such as eccentric exercise, and corroborates work from Howard et al. (7), who found that myocytes from diabetic mice failed to repair from laser- and contraction-induced plasma membrane injuries in vitro. We predict that the decline in Akita skeletal muscle function, as demonstrated by the rapid time to exhaustion, is a result of a slow rate of muscle repair after damage, as has been identified previously (34). Given our data, diabetic skeletal muscle is clearly more susceptible to muscle injury and likely endures a downward spiral of repeated damage and delayed repair that ultimately hinders normal functionality.

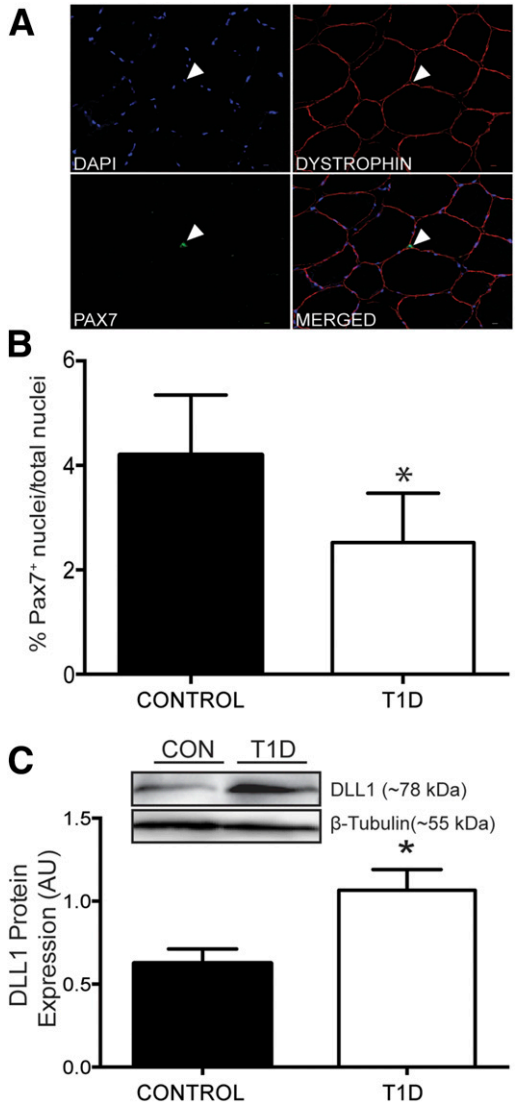

Figure 4—SC content is decreased in the skeletal muscle of people with T1D. *A*: Representative image of SC content in the muscle section from a young adult with T1D. Sections were costained with DAPI, Dystrophin, and Pax7. The white arrowheads indicate a positive signal for a SC. *B*: The corresponding quantification of SC density is shown in subjects with T1D (n = 5) compared with control subjects (n = 5). *C*: To ascertain whether activation of the Notch pathway was evident, protein expression for the Notch ligand DLL1 was quantified, showing enhanced expression in muscle from subjects with T1D (n = 4) compared with control subjects (n = 3). CON, control. *P < 0.05 vs. control subjects.

The more pronounced damage in Akita mice compared with WT mice exposed to the same stimulus led us to investigate the effect of T1D on the SC population, a pivotal player in muscle growth and repair. We hypothesized that SCs from the diabetic group would be more activated or would be more readily activated (a state referred to as G_{alert} [35]) because SCs are known to respond to stimuli such as muscle injury (36). Unexpectedly, we found a reduction in Pax7$^+$ cells in Akita muscles after an activation stimulus compared with WT. We verified this observation by investigating BrdU incorporation into activated/proliferating satellite cells on isolated single fibers as well as the number of MyoD$^+$ and Myogenin$^+$ SCs on isolated fibers. In all of these analyses, a significant impairment in SC activation was noted, in agreement with past work (37). A previously published report in rats given STZ had also noted a decreased expression of myogenic factors by Western blotting (38). Although consistent with our present findings, that study was investigating the effect of oxidative stress induced by chronic hyperglycemia on genes involved in protein muscle synthesis; thus, a specific analysis of the muscle SC was not undertaken.

Given the observed decrements to SC activation, we next wanted to ascertain whether SC content would be negatively influenced, because this relationship has previously been described (21). Here we examined SC density in both rodent and human T1D muscle samples. Despite our T1D mouse model being provided no exogenous insulin and our young adult cohort with T1D receiving exogenous insulin, a similar decrement in SC density was observed. To our knowledge, this is the first quantification of SC density in young adult patients with T1D, and although these patients receive exogenous insulin therapy, it is interesting to note that the decline in SC density is comparable to data derived from rodents with acute (8 weeks) uncontrolled T1D. As such, it appears that aberrant changes to the T1D SC population may be largely independent of insulin availability. Clearly, future studies using insulin pellets in rodents will shed further light on the temporal changes in SC density with exposure to T1D.

The impaired SC activation observed on isolated single fibers suggested that the declines in SC function were intrinsic to the SC or were mediated through the myofiber-SC microenvironment, a niche that is maintained in the isolated fiber protocol. Because the Notch pathway fit this theory and has been implicated in the maintenance of the SC population and SC quiescence (39,40), it seemed the most appropriate pathway to interrogate. In the adult, Notch signaling plays an important role in SC expansion (41), while constitutive Notch activity results in inhibition of MyoD and Myogenin expression (42) and impaired muscle regeneration (23). Therefore, the elevated Notch signaling observed in Akita skeletal muscle would repress MyoD and Myogenin expression in response to an activation stimulus and, ultimately, delay the exit of SCs from quiescence. Interestingly, a reduction in Notch activity has also been reported to delay regeneration in aged skeletal muscle (43). Thus, the influence of Notch activity on SC function appears to be situation-specific and suggests that changes to the SC niche may alter the availability of those factors (such as Notch ligands) that modulate Notch signaling.

Although we expected to identify an increase in the Notch ligand, DLL1, as a primary mechanism through which Notch activity was enhanced in skeletal muscle from rodents and human subjects with T1D, this was not

observed in both species. Instead, a discrepancy exists in the expression of DLL1 between human and rodent T1D skeletal muscle. The lack of increase in DLL1 in T1D rodent muscle could be attributed to alternative Notch ligands that regulate Notch signaling in rodent skeletal muscle. For instance, Jagged-1 is expressed in activated murine SCs and has been used to determine SC activation status (44). In another study, Jagged-2 was highly expressed in regenerating/damaged myofibers in both experimental cohorts examined and was higher in abundance than DLL1 after the injury stimulus (16), suggesting that the availability of Notch ligands may only be quantified when the muscle has been subjected to a stimulus (such as injury-induced exercise) that disrupts its environment. Future studies will aim to evaluate various Notch ligands in exercised and/or damaged Akita skeletal muscle to determine whether differences in their quantity are observed when compared with the WT.

The underlying cause for an increased DLL1 in human skeletal muscle was not elucidated in this study, but exposure of cells to high glucose has been found to alter Notch signaling pathway members (45,46). Hyperglycemia observed in diabetic mice (and consistent with poorly controlled young adults with T1D [47]) coincides with enhanced Notch signaling in T1D skeletal muscle. In addition, extracellular matrix remodelling is important for SC function (13,48), and it is clear that the capacity for extracellular matrix remodelling, through reduction in matrix metalloprotease activity, is negatively affected in T1D skeletal muscle (13,30). Because these proteases (matrix metalloprotease, a disintegrin and metalloproteinase, etc.) are known to cleave Notch ligands (DLL1), a reduced capacity or abundance of these extracellular proteases, as seen in T1D, could account for the persistent Notch signaling. The influence of a high glucose environment and aberrant protease activity on Notch signaling in T1D SCs represents an interesting area for future investigation.

The data collected from our human subjects are the first to identify that such impairments in skeletal muscle health, via the SC, occur in young adults with T1D despite the availability of insulin therapy. The comparable changes to SC density observed in samples from rodents and human subjects with T1D is promising as an avenue for future investigation in translational research because it suggests that, like what has been observed in rodent T1D SCs, SC function in human subjects with T1D may be hindered in skeletal muscle as a result of dysregulated Notch activity.

In summary, our present data highlight losses to the primary muscle stem cell population in human subjects and rodents with T1D, a novel finding that we would propose is the result of hyperactivated Notch signaling impairing SC function. Given the vital role of the SC in the maintenance of skeletal muscle health, identification of intrinsic changes to the SC in T1D is integral to the development of therapeutic strategies to attenuate diabetic myopathy.

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