

# Twist2-expressing cells reside in human skeletal muscle and are responsive to aging and resistance exercise training

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## RESEARCH ARTICLE

# Twist2-expressing cells reside in human skeletal muscle and are responsive to aging and resistance exercise training

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## Abstract

Skeletal muscle is maintained and repaired by sub-laminar, Pax7-expressing satellite cells. However, recent mouse investigations have described a second myogenic progenitor population that resides within the myofiber interstitium and expresses the transcription factor Twist2. Twist2-expressing cells exclusively repair and maintain type IIX/b muscle fibers. Currently, it is unknown if Twist2-expressing cells are present in human skeletal muscle and if they function as myogenic progenitors. Here, we perform a combination of single-cell RNA sequencing analysis and immunofluorescence staining to demonstrate the identity and localization of Twist2-expressing cells in human skeletal muscle. Twist2-expressing cells were identified to be anatomically and transcriptionally comparable to fibro-adipogenic progenitors (FAPs) and lack expression of typical satellite cell markers such as Pax7. Comparative analysis revealed that human and mouse Twist2-expressing cells were highly transcriptionally analogous and resided within the same anatomical structures in vivo. Examination of young and aged skeletal muscle biopsy samples revealed that Twist2-positive cells are more prevalent in aged muscle and increase following 12-weeks of resistance exercise training (RET) in humans. However, the quantity of Twist2-positive cells was not correlated with indices of muscle mass or muscle fiber cross-sectional area (CSA) in young or older muscle, and their abundance was surprisingly, negatively correlated with CSA and myonuclear domain size following RET. Taken together, we have identified cells expressing Twist2 in human skeletal muscle which are responsive to aging and exercise. Further examination of their myogenic potential is warranted.

## KEYWORDS

aging, progenitor cells, sarcopenia, skeletal muscle, Twist2

**Abbreviations:** ALM, appendicular lean mass; BSA, bovine serum albumin; CSA, cross-sectional area; DMEM, Dulbecco's modified Eagle medium; DSHB, Developmental studies hybridoma bank; FAP, fibro-adipogenic progenitor; FBS, fetal bovine serum; IHC, immunohistochemistry; MHC, myosin heavy chain; PBS, phosphate buffered saline; PBST, phosphate buffered saline containing 0.02% Tween; PDGFR $\alpha$ , Platelet derived growth factor alpha; PFA, paraformaldehyde; qPCR, quantitative polymerase chain reaction; RET, resistance exercise training; SBB, Sudan Black B; SC, satellite cell; scRNAseq, single-cell RNA sequencing; SMC, smooth muscle cell; TA, tibialis anterior; TBST, tris-buffered saline containing Tween 20; UMAP, uniform manifold approximation and projection; WB, western blotting.

## 1 | INTRODUCTION

Aging results in a significant reduction of skeletal muscle mass and strength. This is associated with specific decrements in type II muscle fiber cross-sectional area (CSA) and quantity.<sup>1</sup> Current guidelines suggest that resistance exercise training (RET) is an effective countermeasure to reduce the impact of the age-related loss of muscle mass and to preserve strength and functionality.<sup>2</sup> The mechanisms that underlie muscle loss with age remain poorly defined and likely encompass multiple pathways including, anabolic resistance,<sup>3</sup> enhanced inflammatory signaling,<sup>4</sup> and altered mitochondrial dynamics,<sup>5</sup> in addition to disrupted muscle maintenance and repair activity. Indeed, aging decreases the quantity and function of muscle stem cells, known as satellite cells.<sup>6,7</sup> There are specific decreases in the number of Pax7-positive satellite cells associated with type II muscle fibers,<sup>8</sup> as well as cell-intrinsic changes such as enhanced senescence gene activity<sup>9</sup> and altered self-renewal mechanisms.<sup>10</sup> Importantly, RET has a positive impact on satellite cells, increasing their numbers in aged skeletal muscle.<sup>11,12</sup>

While satellite cells are thought to be the only muscle-resident cell population with innate myogenic potential, recent studies in rodents have challenged this notion.<sup>13,14</sup> Work from the Olson group has defined a population of progenitor cells residing within the muscle interstitium which express the transcription factor Twist2.<sup>13</sup> Twist2-expressing cells are anatomically and transcriptionally distinct from satellite cells but appear similar to fibro-adipogenic progenitors (FAPs). Twist2-positive cells can directly contribute to adult skeletal muscle, specifically giving rise to type IIb/x muscle fibers following cardiotoxin-induced muscle injury in mice. Further, when Twist2-positive cells are ablated, a reduction in muscle mass and type IIb/x muscle fiber CSA is observed. To date, it is unknown if myogenic progenitor cells expressing Twist2 reside within human skeletal muscle, if their quantity differs between age groups, and if they are responsive to RET. Here, using a combination of single-cell RNA sequencing analysis (scRNAseq) and immunohistochemistry (IHC), we define a Twist2-expressing cell population that is separate from satellite cells but highly similar to FAPs which reside in the muscle fiber interstitium of humans. By assessing skeletal muscle biopsies of young and older participants, we reveal that the quantity of Twist2-expressing cells is higher in aged muscle and increases following RET.

## 2 | METHODS

### 2.1 | Acquisition of human skeletal muscle

All procedures were approved by the Research Ethics Boards of the University of Prince Edward Island (REB

file #6007738) and Health Prince Edward Island. Samples utilized for quantitative polymerase chain reaction (qPCR) and western blotting (WB) were from the hamstrings muscle which was provided by orthopedic patients (2 males, 2 females, mean age =  $22 \pm 0.6$  y) at the Queen Elizabeth Hospital (Charlottetown, Prince Edward Island, Canada). Following surgical removal, the tissue was immediately placed into a cell culture media solution consisting of 20% fetal bovine serum (FBS) in Dulbecco's modified Eagle medium (DMEM) and transported to the laboratory where it was freed from blood and connective tissue, and snap frozen in liquid nitrogen (for qPCR, WB) or mounted in OCT and frozen in liquid nitrogen cooled 2-methylbutane (for IHC).

Samples utilized for the immunohistochemical (IHC) quantification of Twist2-positive cells in young participants, and older/older-exercised participants were isolated from the vastus lateralis using the Bergstrom biopsy technique with suction at McMaster University (young participants) and Maastricht University (older, older-exercised). Approximately, 80 mg of muscle tissue was obtained through one or two needle passes and samples were dissected from blood and connective tissue and mounted in OCT, and frozen in liquid nitrogen-cooled 2-methylbutane.

### 2.2 | Participants and exercise protocol

We examined a subset of 34, healthy, normoglycemic, older men (mean age =  $70 \pm 4$  y, participants' characteristics are found in Table 1) who were living independently and had not been participating in any structured exercise training programs within the last 3 years. Individuals were recruited for a 12-week whole-body progressive resistance exercise training (RET) regimen as described in detail previously.<sup>15</sup> Briefly, following a 5 min warm up on a cycle ergometer, participants performed 4 sets of 10 repetitions of leg press and leg extension and 2 sets of chest press, lateral pulldown, shoulder press, and horizontal row, at 70% of the one-repetition maximum with 2 min of rest between sets and 3 min rest between exercises. Training sessions were performed 3 times per week. The workloads were adjusted at the discretion of the investigators when >10 repetitions could be performed. At least 4 days before and 4 days after the last exercise session, anthropometric measures, DEXA, and muscle biopsies were taken and processed as described above. Participants were randomly assigned to ingest a 21 g leucine-enriched whey protein (3 g total leucine) beverage or an energy-matched placebo after exercise and each night before sleep, including rest days. Since the training was equally effective at increasing leg

TABLE 1 Participant characteristics

	Pre	Post 12 Weeks RET
Age	70 ± 3.5	
Body mass (kg)	78 ± 8.2	79.2 ± 8.17*
Lean tissue mass (kg)	60.8 ± 5.9	61.2 ± 5.8
Appendicular lean mass (kg)	26.2 ± 2.8	27.1 ± 2.9
Leg lean mass (kg)	13.0 ± 1.1	13.6 ± 1.5
Fat-free mass index (kg/m <sup>2</sup> )	19.4 ± 1.5	19.5 ± 1.6
BMI (kg/m <sup>2</sup> )	25.3 ± 2.5	25.7 ± 2.4
Bone density test ( <i>T</i> -score) • via DEXA scan	1.0 ± 0.0	1.0 ± 0.0
Type I muscle fiber cross sectional area (um <sup>2</sup> )	6007 ± 1391	6318 ± 1232
Type II muscle fiber cross sectional area (um <sup>2</sup> )	5709 ± 1554	6234 ± 1456*

\**p* < .05 from Pre.

TABLE 2 Immunohistochemical reagents and procedures

Primary antibodies	Manufacturer	Blocking solution	Dilution	Secondary & tertiary antibodies (all thermo fisher)
Twist2	Abcam (ab66031)	10% Goat Serum 0.1% Triton	1:200	Goat anti-rabbit 555 (A32732) - 1:500
Myosin Heavy Chain I	DSHB (BA-F8)	10% Goat Serum	1:25	Goat anti-mouse 488 IgG2b (A21141) - 1:1000
Rat Laminin	Sigma Aldrich (L0663)	10% Goat Serum 0.1% Triton	1:500	Goat anti-rat 488 (A11006) - 1:1000
Rabbit Laminin	Thermo Fisher (MA532577)	10% Goat Serum	1:500	Goat anti-rabbit 488 (A32731) - 1:500
PDGFR $\alpha$	R&D (AF1062)	DAKO Serum Free Block (X0909)	1:500	Goat anti-mouse 555 (A21422) - 1:500
Pax7	DSHB	10% Goat Serum 0.1% Triton	1:1	Goat anti-mouse biotin (31800, 1:500) Streptavidin 555 (S21381, 1:500)

1-repetition maximum, quadriceps cross-sectional area (CSA), and type II muscle fiber hypertrophy with no significant differences between groups, all participants were combined for the analysis of Twist2-positive cell number pre/pre-post training. In order to characterize the effect of age on Twist2-positive cell number, muscle biopsies were analyzed from a subset of  $n = 7$  (mean age = 24 years) healthy, young untrained participants from a previous study.<sup>16</sup>

### 2.3 | Mouse skeletal muscle

All animal procedures and ethics were approved by the University of Prince Edward Island Animal Care Committee in accordance with CCAC guidelines (AUP #16-036). Five 8-week-old male C57BL/6 mice were purchased from Charles River Laboratories. Tibialis

anterior (TA) muscles were isolated and mounted in OCT compound and snap frozen in liquid nitrogen cooled 2-methylbutane and stored at  $-80^{\circ}\text{C}$ .

### 2.4 | Immunohistochemistry

Human and mouse snap-frozen muscles were cut at 8  $\mu\text{m}$  using a cryostat (Leica Biosystems, CM1860), mounted on slides, and stored at  $-80^{\circ}\text{C}$ . Full details of IHC procedures are provided in Table 2. Briefly, slides were thawed and air-dried at room temperature for 20 min followed by fixation with 4% paraformaldehyde (PFA) for 5 min. Following washing with phosphate-buffered saline (PBS) containing 0.02% Tween (PBST) (3  $\times$  5 min), sections were permeabilized with 0.3% triton x-100 in PBS for 10 min at room temperature before being covered in a blocking solution at room temperature for 1 h. Sections

were then incubated in primary antibodies overnight at 4°C. Following washes in PBST (3 × 5 min) sections were incubated in the appropriate fluorescently-conjugated or biotinylated secondary antibodies (then anti-biotin tertiary antibody where appropriate) in the dark at room temperature. Following washing with PBS for 3 × 5 min, sections were then treated with a 0.1% Sudan Black B (SBB) solution in 70% ethanol for 2 min to eliminate lipofuscin autofluorescence and quickly rinsed 5 times with PBST, before the final washes in PBST (3 × 5 min), nuclear labeling with DAPI and mounting. Images of skeletal muscle were captured using a Zeiss Axio-observer inverted fluorescent microscope.

## 2.5 | Quantification of muscle characteristics, satellite cells, and Twist2-positive cell number

Data analyses were performed using GraphPad Prism software (Version 6.01; GraphPad Software Inc., San Diego, CA). Fiber type specific, muscle fiber CSA, myonuclear domain, number of nuclei per fiber, fiber type specific satellite cells (sublaminar, Pax7-positive, DAPI-positive cells associated with a type I or II muscle fibers), and Twist2-positive cell number was quantified manually using an area comprising at least 75 total fibers. Twist2-positive cells were quantified per area and were not assigned to be associated with individual fibers due to their interstitial location. In all instances, the individual analyzing the muscle characteristics was blinded to the identity of the sample. The sarcopenia index was calculated by dividing the average CSA of type II muscle fibers by the CSA of type I fibers per subject. Fibers were considered type II if they were devoid of type I myosin heavy chain (MHC) staining as we performed previously.<sup>17</sup>

## 2.6 | PCR analysis

Analysis was performed on human skeletal muscle samples ( $n = 3$ , hamstrings muscle, isolated as described above) with primers purchased from Integrated DNA Technologies (IDT, Integrated DNA Technologies Inc., Coralville, Iowa) which underwent primer validation as per the MIQE guidelines.<sup>18</sup> Briefly, total RNA was isolated from ~20 mg of muscle by adding samples to pre-cooled (−80°C) 2 ml tubes with six 2.8 mm ceramic beads (OMNI inc, 19-646) in the presence of TRIzol (Thermo Fisher, 15 596 062) and underwent three cycles of 20 s of homogenization at 5 m/s using a beadmill (BeadRuptor Elite OMNI inc., Kennesaw Georgia) with 20 s of cooling on ice between rounds. RNA was purified using the

E.Z.N.A. total RNA kit (OMEGA, R6834-02) using the manufacturer's instructions. Samples were treated with DNase before reverse transcription into cDNA (Bio-Rad, iScript) and qPCR was performed using the Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, California). SsoAdvanced SYBR Green Supermix (Bio-Rad) was used for fluorescent detection. qPCR conditions were set as follows: 95°C for 15 min, 40× (95°C for 15s, 60°C for 30s, 72°C for 20s), and the melt curve at 65°C for 60 min to 95°C for 15 min. The resulting product was resolved on a 2.5% agarose gel and visualized using a Bio-Rad ChemiDoc imaging system (Bio-Rad, Hercules, California) using RedSafe for detection (FroggaBio Inc). The sequence for the Twist2 primers was—forward 5'- CAGAGCGACGAGATGGACAA-3', reverse 5'- CACACGGAGAAGGCGTAGC-3'.

## 2.7 | Western blotting

Human skeletal muscle ( $n = 3$ , hamstrings muscle) mouse ( $n = 3$ , TA muscle) and a mouse kidney ( $n = 1$ ) were homogenized in RIPA lysis buffer (Pierce) with protease inhibitors using the OMNI bead rupture elite (OMNI Inc, Kennesaw Georgia) for 4 cycles of 25s at a speed of 2.5 m/s, placing them on ice for 30s between cycles, and a BCA assay was used to determine protein concentration against a standard curve. 50 μg of each sample was added to laemelli loading buffer and boiled at 95°C for 5 min before being resolved by SDS-PAGE (12% polyacrylamide gel). Proteins were transferred to a PVDF membrane and washed with Tris-buffered saline containing tween-20 (TBST) and blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were incubated overnight at 4°C with an anti-Twist2 (Abcam, ab66031) primary antibody followed by 3 × 5 min of washing on a rocker prior to incubation with an anti-rabbit secondary antibody coupled to horseradish peroxidase. The membrane was then washed with TBST before applying enhanced chemiluminescence reagent (Bio-Rad) for 5 min. Images were captured using the Bio-Rad ChemiDoc imaging system.

## 2.8 | Single-cell RNA sequencing and bioinformatics

Publicly available human intercostal and mouse hindlimb muscle scRNAseq datasets were obtained from the Heart Cell Atlas web portal ([www.heartcellatlas.org](http://www.heartcellatlas.org)) and GEO database (GSM3520458/9), respectively. Raw count matrices were processed using a standard pipeline in scanpy 1.8.2 (<https://doi.org/10.1186/s13059-017-1382-0>). For the

human dataset, cells with less than 1500 UMIs or less than 500 unique genes, and nuclei were removed from downstream analysis. Batch integration was performed with scVI 0.6.7 (<https://doi.org/10.1038/s41592-018-0229-2>), using the sample as a covariate, keeping only the 2000 most variable genes across samples (selected by the 'cell\_ranger' method in scanpy), and default settings with the scVI model's recommended integration training parameters ( $n\_layers = 2$ ,  $n\_hidden = 30$ ,  $n\_latent = 128$ ). scVI's latent space was used to build a neighborhood graph, which was clustered and visualized using the Leiden and UMAP algorithms, respectively.

Cells with less than 200 genes or greater than 8% of reads mapping to mitochondrial genes were removed from the mouse dataset. The two mouse biological replicate datasets were integrated using BBKNN 1.5.1 (<https://doi.org/10.1093/bioinformatics/btz625>) with ridge regression; Leiden clustering and UMAP being performed on the corrected neighborhood graph. For both datasets, differentially expressed genes were identified using the Wilcoxon rank-sum test and Benjamini-Hochberg correction on the normalized, natural-log transformed counts data.

## 2.9 | Statistics

Two-tailed, paired student's *t*-tests were performed for pre- and post-resistance training measures with two-tailed, unpaired student's *t*-tests being utilized between young and older subjects. Pearson's *r* correlation analyses were performed pre- and post-resistance training for multiple factors including the number of Twist2-positive cells, type I and II CSA, number of type I and type II satellite cells (per 100 fibers), appendicular lean mass, lean tissue mass, myonuclear domain size, and sarcopenia index. Values for lean mass and appendicular lean mass and lean tissue mass were taken from a subset of participants as previously reported.<sup>15,16</sup> The significance for all analyses was set at  $p < .05$ .

## 3 | RESULTS

### 3.1 | Twist2 mRNA and protein are expressed in human skeletal muscle tissue

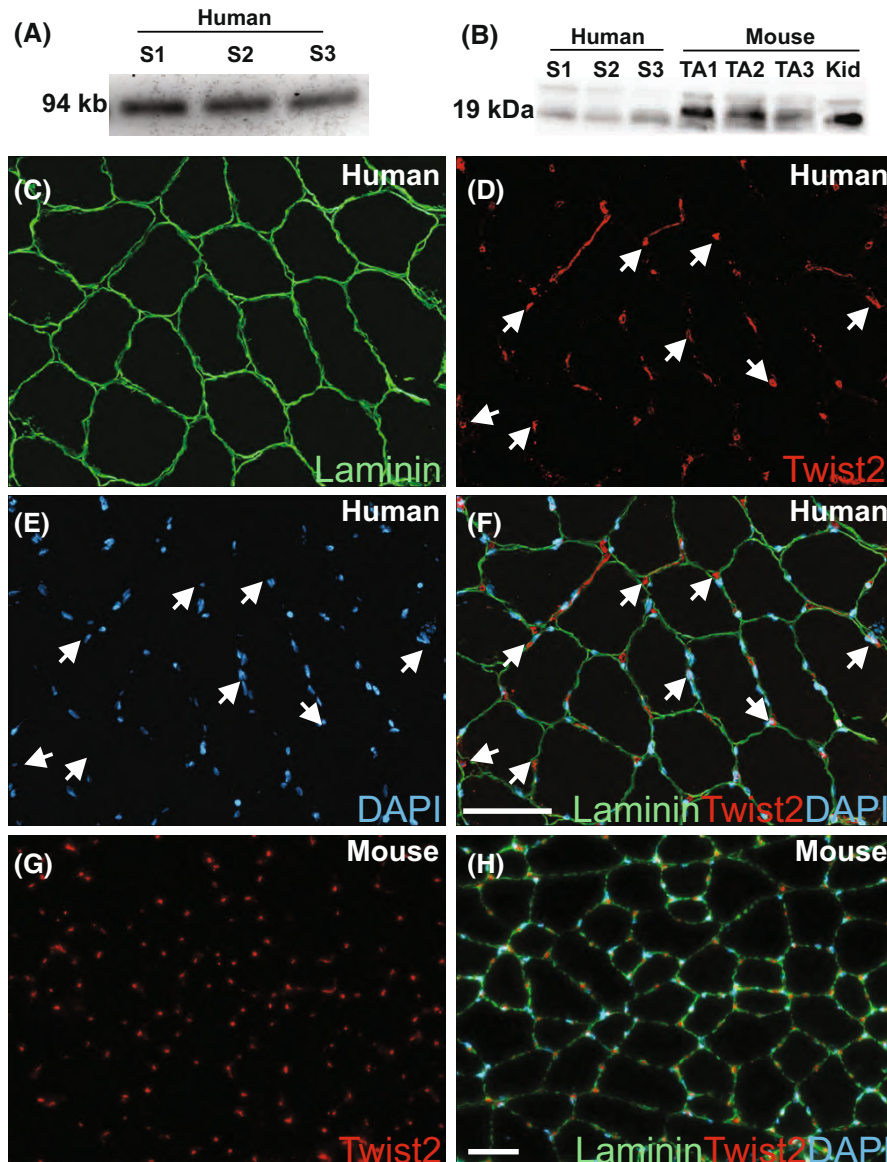
As a first step to describe Twist2 expression within the human skeletal muscle, PCR analysis was performed on whole muscle homogenates to detect *TWIST2* mRNA. Agarose gel electrophoresis demonstrated a single band at 94 kb (Figure 1A) which is consistent with the predicted product size. To assess Twist2 protein, we performed WB on whole muscle lysate from human

hamstrings and mouse TA muscles while mouse kidneys served as a positive control. We used an antibody specific to Twist2 that was previously validated to detect Twist2 protein within skeletal muscle.<sup>13,14</sup> WB analysis revealed bands in each sample at 19kDa, the predicted size for the Twist2 protein (Figure 1B). Next, we assessed where Twist2-expressing cells were located by examining muscle cross-sections and probed with anti-laminin antibodies to reveal myofibers (Figure 1C). This demonstrated that the Twist2 protein was generally cytoplasmic and exclusively localized between muscle fibers, outside the myofiber membrane (Figure 1D,F). Cells displayed elongated morphologies consistent with IHC staining of uninjured mouse T muscle (Figure 1G,H) and with previous reports in mice.<sup>13,14</sup>

### 3.2 | Single-cell RNA sequencing identifies *TWIST2*-expressing mesenchymal cells

To identify the cells within human skeletal muscle expressing *TWIST2* mRNA, we analyzed a published dataset comprised of sequencing of ~70 000 single-cells/single-nuclei derived from five adult human skeletal muscle donors.<sup>19</sup> This revealed the presence of the expected cell types including *PECAM1*-positive endothelial cells, *MHY11*-positive smooth muscle cells (SMCs), *KCNJ8*-positive pericytes, *CD68*-positive myeloid and *CD3D* and *NKG7*-positive lymphoid lineage cells, *NGFR*-positive perineural fibroblasts, *TTN*-positive myocytes, *PAX7*-positive satellite cells, and *PDGFRA*-positive mesenchymal/FAPs (Figure 2A,B). Feature plots demonstrated that the vast majority (89%) of *TWIST2*-positive cells were restricted to the greater mesenchymal cluster while a few *TWIST2*-positive cells were localized to satellite cell, SMC, pericyte, and myeloid clusters (Figure 2B,C). Mouse Twist2-positive cells do not endogenously express satellite cell genes such as *Pax7* and *MyoD*, but when cultured, they engage a myogenic program as they lose Twist2 expression and transition through a myogenic/satellite cell state.<sup>13</sup> Therefore, we closely examined the human satellite cell cluster, which largely expressed *MYF5* (Figure 2G) and was comprised of 3160 cells, 1410 (45%) of which expressed *PAX7* (Figure 2D). However, only 28 *TWIST2*-positive cells were restricted to this cluster (Figure 2E) and 11 co-expressed *PAX7* (Figure 2F).

Four clusters of mesenchymal/FAPs cells were evident, which all expressed characteristic mesenchymal genes such as *PDGFRA* and *DCN* (Figure 2H). FAPs subclusters were defined by the enhanced expression of *PCOLCE2* (FAPs1), *SMOC2*, *ABCA8* (FAPs2), *GPX3*, *CXCL14*

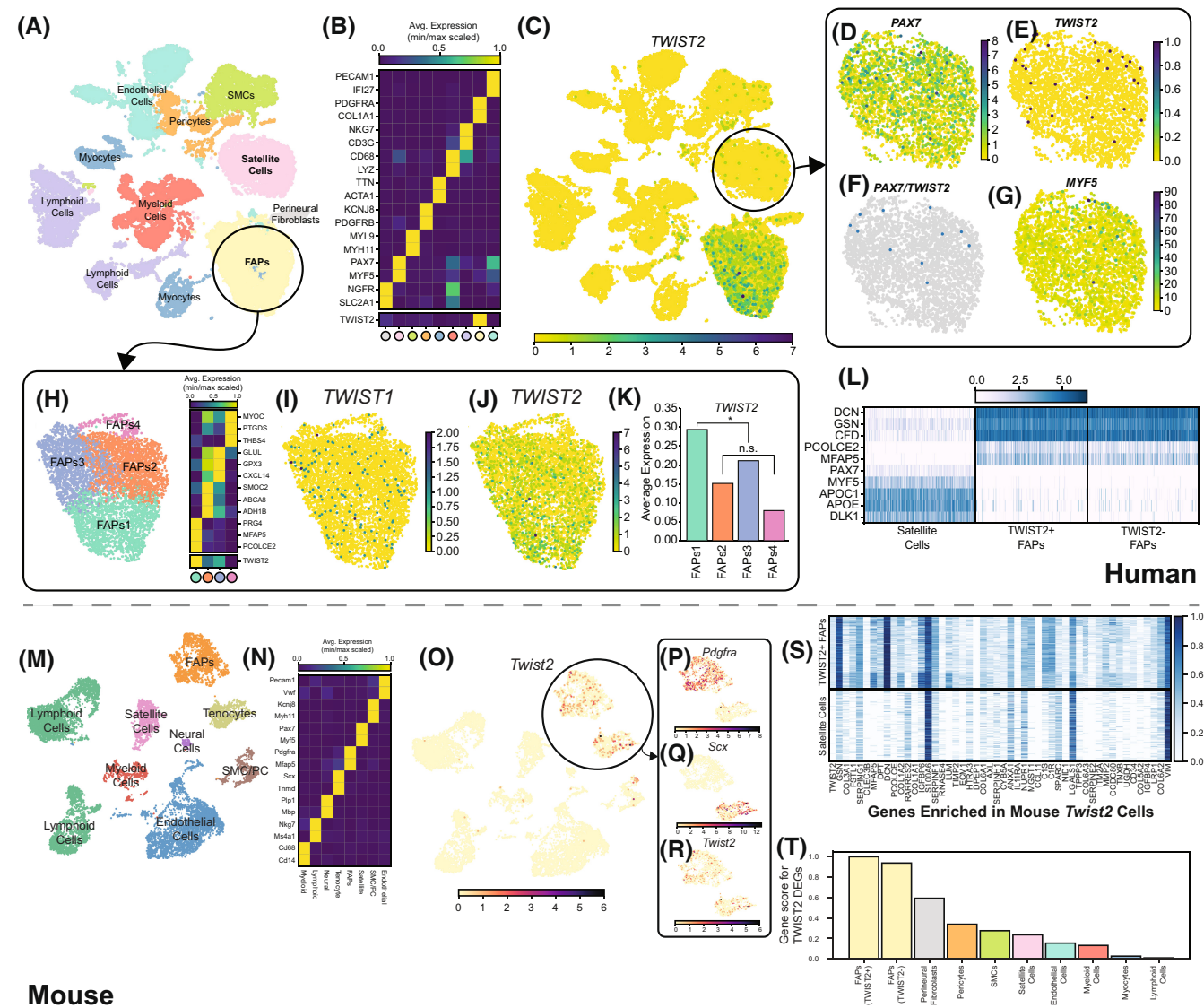


**FIGURE 1** Twist2 mRNA and protein are expressed in human skeletal muscle and are localized to the myofiber interstitium. (A) Agarose gel electrophoresis of *TWIST2* mRNA from three human skeletal muscle tissue samples (S1-3). A single band was evident at 94 kb, the predicted size of *TWIST2* mRNA. (B) WB for Twist2 protein in three human muscle tissue samples (S1-3), three mouse TA muscle samples (TA1-3), and one mouse kidney tissue sample (positive control). A band was evident at 19 kDa, the predicted size of Twist2 protein. Representative images of IHC staining of human skeletal muscle cross sections revealing laminin (C, green), Twist2 (D, red), DAPI (E, blue) and merge (F). Arrows indicate Twist2-expressing cells within the myofiber interstitium ( $n = 5$ ). Representative images of IHC staining of mouse TA muscle cross sections for Twist2 (G, red), Twist2 (red) with laminin (green), and DAPI (blue) to counterstain nuclei (H) ( $n = 5$ ). Scale bars = 50  $\mu\text{m}$ .

(FAPs3), and *MYOC* and *PTGDS* (FAPs4) (Figure 2H). The Twist subfamily of basic helix-loop-helix proteins has two family members, Twist1 and Twist2. Both *TWIST1* and *TWIST2* mRNA were expressed in mesenchymal/FAPs cells (Figure 2I,J) with *TWIST2* being more predominant as *TWIST1* was expressed in 7% of FAPs compared to 20% for *TWIST2*. *TWIST2* expression was enriched in the FAPs1 and FAPs3 clusters compared to FAPs2 and FAPs4 (Figure 2K). Indeed, of the 1799 cells in the FAPs1 cluster, 29% were *TWIST2*-positive compared to the 8% (16/202) which were *TWIST2*-positive in FAPs4. Next, we compared *TWIST2*-positive and negative FAPs, which did not reveal any striking transcriptional differences, as both groups expressed characteristic genes such as *GSN*, *DCN*, and *PDGFRA* (Figure 2L). 71% of *TWIST2*-expressing cells also expressed *TCF4* while 16% expressed the progenitor marker *HIC1*. We also compared these cells to satellite cells which demonstrated that characteristic satellite cell

genes such as *MYF5*, *PAX7*, and *APOE* were largely devoid in *TWIST2*-positive FAPs (Figure 2L).

Since mouse Twist2-positive cells have known myogenic potential, we wanted to assess how similar human *TWIST2*-expressing cells were to their rodent counterparts. To do this, we analyzed another published scRNA-seq dataset of ~12000 mouse skeletal muscle-derived single cells.<sup>20</sup> We first clustered the cells (Figure 2M) based on canonical marker expression (Figure 2N) which revealed the expected cell types including endothelial cells, SMCs/pericytes, myeloid cells, lymphoid cells, satellite cells, neural-related cells, tenocytes, and FAPs. In mice, *Twist2*-expressing cells were also restricted to *Pdgfra*-expressing FAPs in addition to *Scx*-positive tenocytes with 8.7% of FAPs and 9.1% tenocytes expressing *Twist2* mRNA (Figure 2O-R). While recent work has demonstrated that Twist2-expressing cells are highly related to FAPs, Twist2 expression in muscle resident tenocytes has not been



**FIGURE 2** Single-cell RNA sequencing of human and mouse skeletal muscle-derived cells identifies *TWIST2*-expressing FAPs. (A) UMAP plot demonstrating clusters identified by scRNAseq analysis of human skeletal muscle tissue. (B) Matrix plot demonstrating the average scaled expressed of canonical marker genes and *TWIST2* in each cell type cluster (circle color corresponds to clusters in A). (C) Feature plot demonstrating *TWIST2* expression in cell clusters. Feature plots of *PAX7* (D), *TWIST2* (E), *PAX7*, and *TWIST2* co-expressing cells (pseudocolored blue, F) and *MYF5* (G) in human satellite cells. (H) UMAP and matrix plot demonstrating 4 FAPs subclusters and differentially expressed marker genes in each subcluster. Feature plots of *TWIST1* (I) and *TWIST2* (J) in human FAPs. (K) Average *TWIST2* expression in human FAPs subclusters. (L) Single-cell heatmap demonstrating expression of canonical marker genes of satellite cells and FAPs in human *TWIST2*-positive and *TWIST2*-negative cells and satellite cells. (M) UMAP plot demonstrating clusters identified by scRNAseq analysis of mouse skeletal muscle. (N) Matrix plot demonstrating the average scaled expressed of canonical marker genes in each cell-type cluster. (O) Feature plot of *Twist2* expression in all mouse clusters. Feature plots of *Pdgfra* (P), *Scx* (Q), and *Twist2* (R), demonstrating expression of *Twist2* in mouse FAPs and tenocytes. (S) Single-cell heatmap of the 50 top differentially expressed genes in mouse *Twist2*-positive cells compared to all other clusters in human *TWIST2*-positive cells and human satellite cells. (T) *Twist2* score in each human cell type cluster based on the expression of all differentially expressed genes in mouse *Twist2*-positive cells. \* in K =  $p < .05$  compared with Faps1, n.s., non-significant compared to FAPs1.

previously defined. We examined the top differentially expressed genes in mouse and human *Twist2*-expressing cells in comparison to non-mesenchymal clusters which showed an overlap of 42% of the 50 top genes (Figure 2S). This relationship was also true when examining a large

number of genes, where 41% of the top 1000 differentially expressed genes were shared between mouse and human *Twist2*-expressing cells. Finally, we created a “*Twist2* score” for each human cell type based on differentially expressed genes identified in mouse *Twist2*-expressing cells.



This demonstrated that *TWIST2*-expressing FAPs had the highest score followed by *TWIST2*-negative FAPs which were ~80% greater than human satellite cells. Taken together, these results demonstrate that human and rodent *Twist2*-expressing cells are highly similar while *TWIST2* is primarily expressed in subpopulations of FAPs in human skeletal muscle.

### 3.3 | *Twist2*-positive cells are consistent with FAPs and not satellite cells in vivo

Our scRNAseq analysis suggests that *Twist2*-expressing cells are highly-related to FAPs. Therefore, we co-stained *vastus lateralis* muscle cross-sections from healthy older males using antibodies to detect *Twist2*, PDGFR $\alpha$ , and the satellite cell marker Pax7.<sup>6</sup> *Twist2*-positive cells were again restricted to the interstitium while  $71 \pm 13\%$  expressed PDGFR $\alpha$  (Figure 3A–D). Conversely, IHC for *Twist2* and Pax7 (Figure 2E–H) revealed independent expression patterns with virtually all Pax7 positive cells localized within the satellite cell niche under the basal lamina and devoid of *Twist2* expression. This data is in agreement with other reports,<sup>13</sup> indicating that *Twist2*-positive cells represent a separate lineage from Pax7-positive satellite cells and are more closely related to the PDGFR $\alpha$ -expressing FAPs. Taken together, these results suggest that cells expressing *TWIST2* mRNA and protein are found within skeletal muscle and are restricted to the myofiber interstitium and express genes and proteins consistent with FAPs.

### 3.4 | *Twist2*-positive cell abundance is greater in muscles from older compared with younger adults but does not correlate with measures of muscle mass

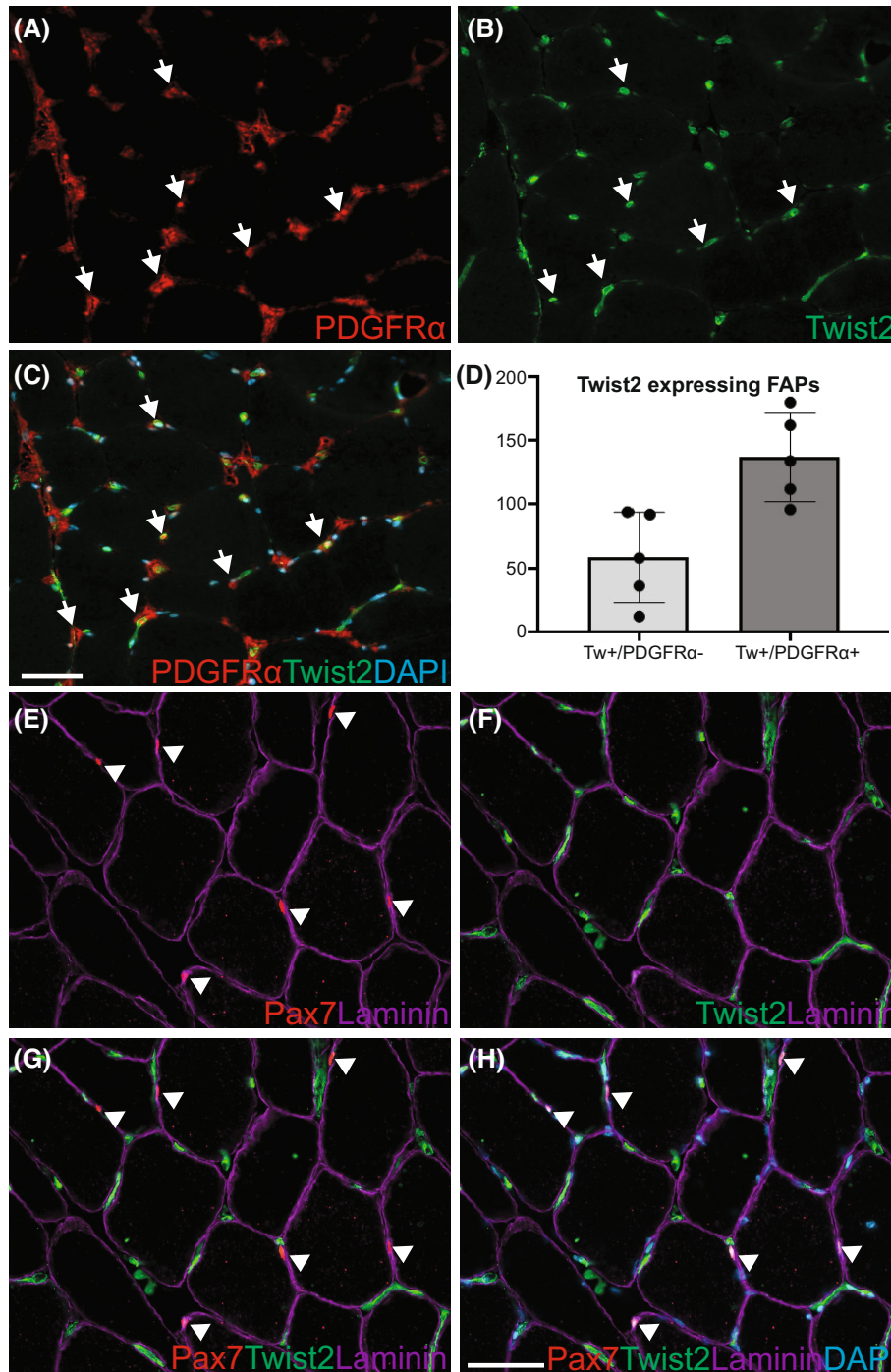
In mice, *Twist2*-positive cells serve to maintain and regenerate type IIb/x muscle fibers.<sup>13</sup> In this regard, skeletal muscle mass declines appreciably with age with specific decrements observed in type II, high force-generating, muscle fibers.<sup>7</sup> Based on this, we hypothesized that the quantity of *Twist2*-positive cells would be lower in the muscle tissue of older compared with young adults, in line with the age-related decline in type II muscle fiber CSA. Therefore, we examined muscle biopsies isolated from the *vastus lateralis* of healthy young and older males. Analysis of IHC for MHC demonstrated the expected significantly smaller type II muscle fiber CSA in older compared with younger individuals (Young:  $6410 \pm 1575 \mu\text{m}^2$ , Older:  $5710 \pm 1555 \mu\text{m}^2$ ). Next, we quantified the number of *Twist2*-positive cells which surprisingly, were significantly higher in muscle tissue collected in the older

compared with young participants (Young:  $59 \pm 16$  per  $\text{mm}^2$ ; Older:  $213 \pm 61$  per  $\text{mm}^2$ ) (Figure 4A–C).

To explore this further, we assessed whether the number of *Twist2*-positive cells was related to indices of muscle mass using Pearson's correlations. First, we examined young participants which revealed no significant correlations between the quantity of *Twist2*-positive cells and the CSA of type I or type II muscle fibers, fiber type distribution, number of satellite cells associated with type I or II muscle fibers, or quantity of lean mass or fat mass as quantified by DEXA (Figures 4D–F and S1A–D). Similar results were observed in older participants where no significant correlations were observed with the number of *Twist2*-positive cells and CSA of type I or type II muscle fibers, fiber type distribution, the number of satellite cells associated with type I or II muscle fibers, or quantity of appendicular lean mass (quantified by DEXA) in addition to the calculated sarcopenia index (ratio of type I CSA: type II CSA) (Figures 4G–J and S1E–G). Additionally, IHC analysis of *Twist2* and Pax7 in young muscle confirmed the same anatomical location and mutual exclusivity in expression (*data not shown*). Collectively, these data suggest that the quantity of *Twist2*-expressing cells in muscle increases with age, but this is not correlated with muscle size or satellite cell number.

### 3.5 | Resistance exercise training increases the number of *Twist2*-positive cells

Resistance exercise training in older adults increases muscle mass, strength,<sup>21</sup> and satellite cell numbers.<sup>6,22</sup> Here, we assessed the impact of 12-weeks of RET on *Twist2*-positive cell content in the same cohort of older males used in the young versus old comparison. As reported previously,<sup>15</sup> resistance exercise training in these older adults resulted in significant increases in measures of muscle strength in addition to increases in body mass, and type II muscle fiber CSA. Twelve-weeks of resistance exercise training increased the number of *Twist2*-positive cells by 21% (from  $213 \pm 61$  to  $260 \pm 73$  cells per  $\text{mm}^2$ ;  $p < .05$ ; Figure 5C). Resistance exercise training did not change the localization as all *Twist2*-positive cells were found within the muscle fiber interstitium prior to and following prolonged exercise training (Figure 5A,B). To better understand the dynamics of the increased number of *Twist2*-positive cells following exercise training, we performed Pearson's correlations between the number of *Twist2*-positive cells prior to and the percent change in *Twist2*-positive cell number following 12-weeks of exercise training. This demonstrated a significant negative correlation ( $p < .001$ ,  $r = -.62$ ), whereby the participants

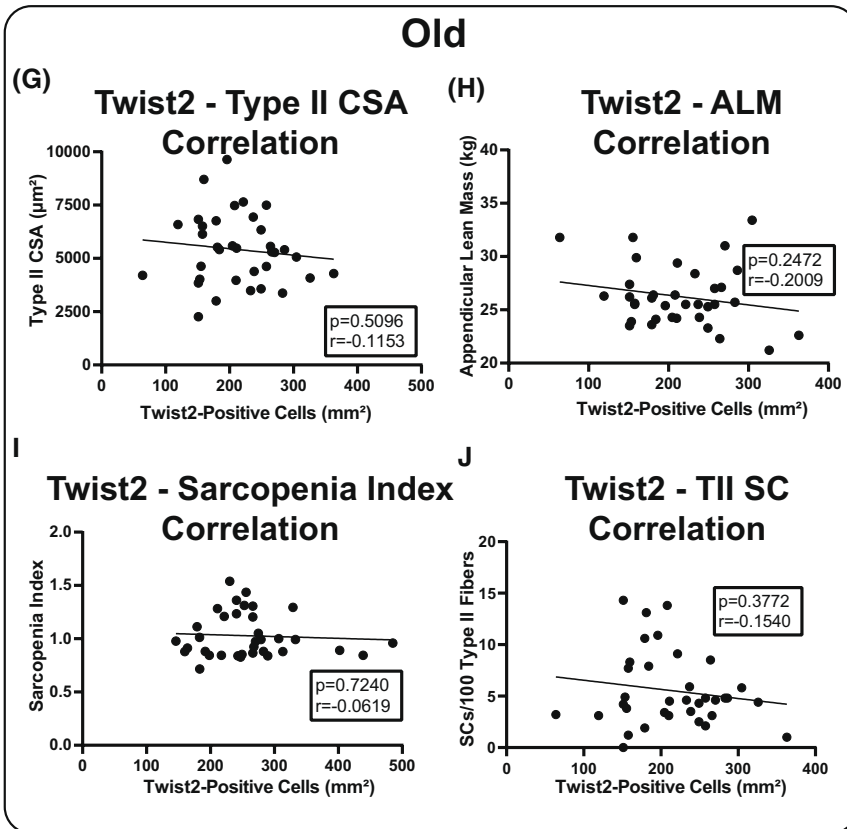
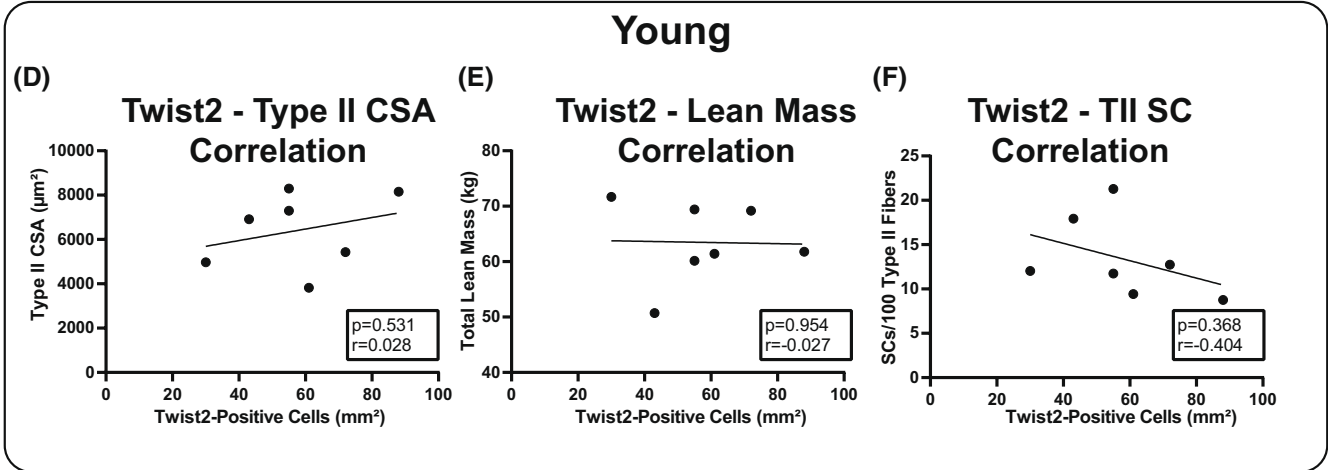
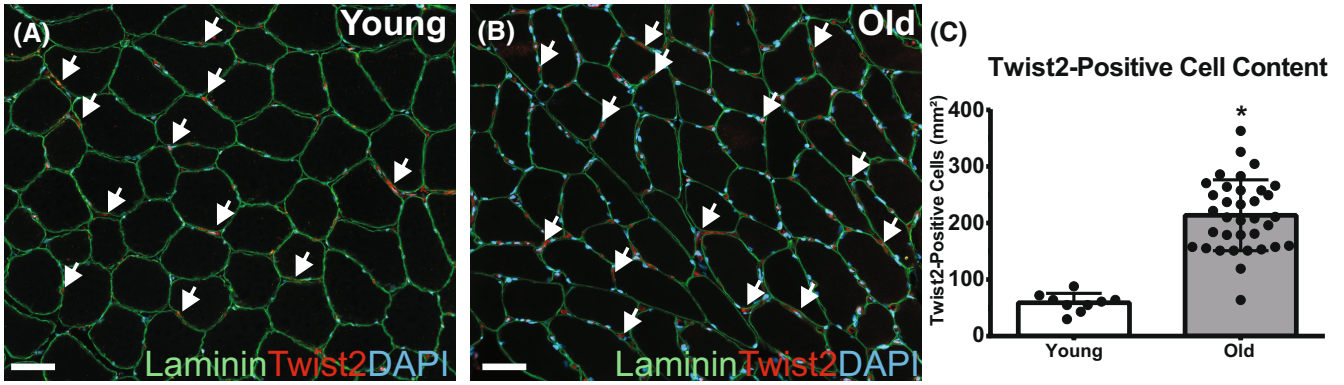


**FIGURE 3** Twist2-positive cells do not express satellite cell markers and are consistent with FAPs in vivo. IHC staining for PDGFR $\alpha$  (A, red), Twist2 (B, green) and merge (C) with DAPI (blue) in human muscle cross-sections. (D) Quantification of the average number of Twist2-positive and negative FAPs within human skeletal muscle cross sections ( $n = 5$ ). Arrows indicate Twist2-positive, PDGFR $\alpha$ -positive cells. IHC staining for Pax7 (E, red), Twist2 (F, green), merge (G) with DAPI (H, blue) to highlight nuclei. Sections were stained with laminin (pseudocolored purple) to reveal myofibers. Arrowheads indicate Pax7-positive cells. Scale bars = 50  $\mu$ m.

with the fewest Twist2-positive cells at baseline showed the greatest increase and those with the most Twist2-positive cells at baseline had the smallest gains, suggesting a ceiling limit in cell content (Figure 5D).

Next, as we did at baseline, we examined correlates of muscle mass with Twist2-positive cell number

post-training. Surprisingly, there were negative correlations between the number of Twist2-positive cells and type I and II muscle fiber CSA, and the myonuclear domain of type I and II muscle fibers (Figure 5E–H). There were no significant correlations between the number of Twist2-positive cells and appendicular lean mass or the



**FIGURE 4** Twist2-positive cell content increases with age and is not associated with indices of muscle mass and myofiber size. Representative images and quantification (C) of IHC staining for laminin (green), Twist2 (red), and DAPI (blue) in young (A) and old (B) muscle cross-sections. The \* in C indicates a significant difference ( $p < .05$ ) compared to young. Pearson's correlations of the quantity of Twist2-positive cells and type II fiber CSA (D), lean tissue mass (E), and satellite cells associated with type II muscle fibers (F) in skeletal muscle tissue collected from young adults ( $n = 7$ ). Pearson's correlations of the quantity of Twist2-positive cells and type II fiber CSA (G), appendicular lean mass (H) sarcopenia index (I), and satellite cells associated with type II muscle fibers (F) in skeletal muscle tissue collected from older adults ( $n = 34$ ). No significant correlations were observed, and  $p$ - and  $r$ -values are indicated on each graph. Scale bars in A, B = 50  $\mu\text{m}$ .

quantity of satellite cells associated with type I or II muscle fibers (Figures 5I,J and S2A–C).

## 4 | DISCUSSION

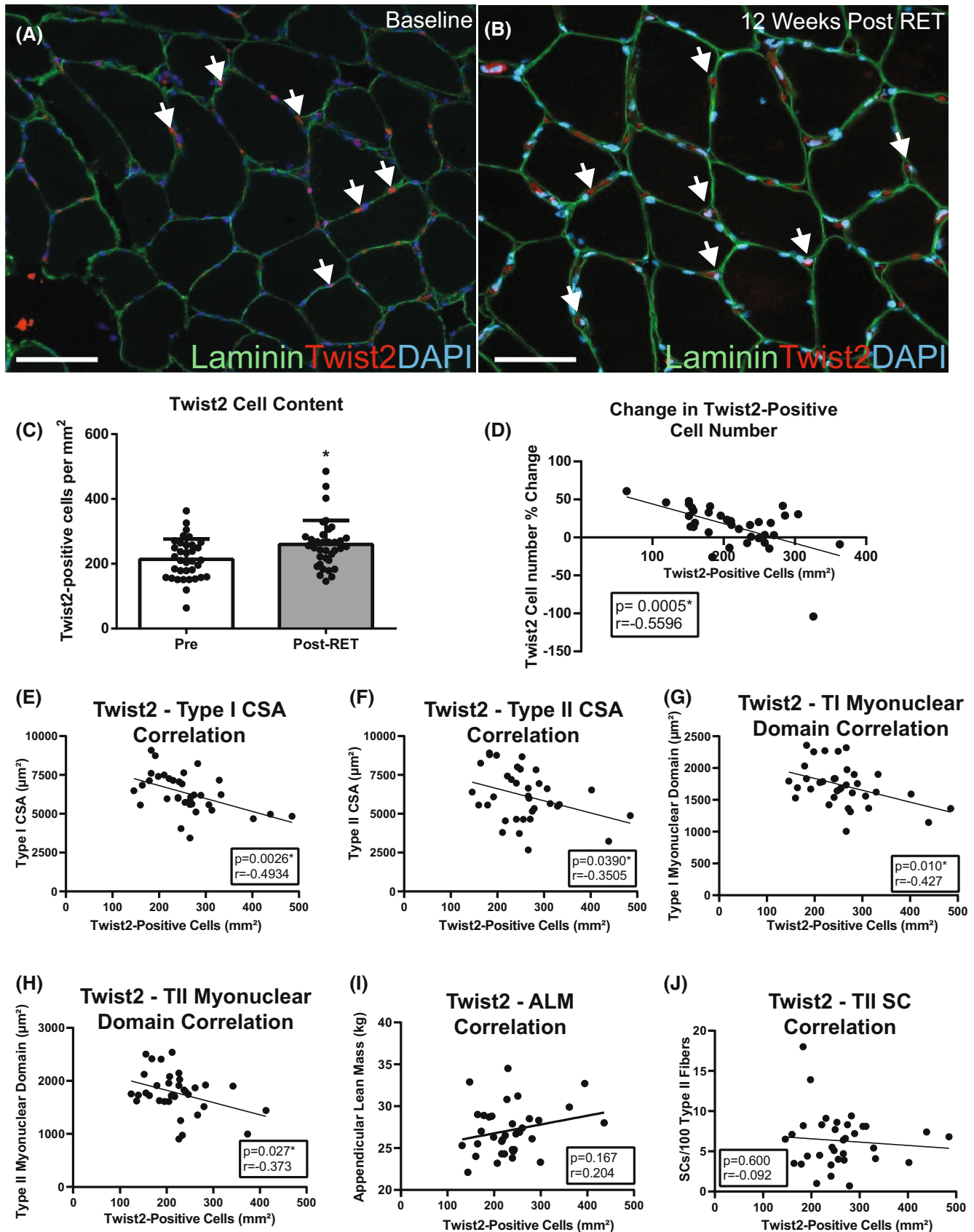
In the current investigation, we provide data that, (1) identifies mesenchymal/FAPs cells within human skeletal muscle that express the progenitor marker Twist2, (2) demonstrates that Twist2-positive cell quantity is higher in muscle tissue collected in older compared with young adults, (3) reveals a resistance-type exercise training-induced increase in muscle tissue Twist2-positive cell content in older adults and, (4) shows that Twist2-positive cell quantity does not seem to be related to muscle fiber size or muscle mass.

Satellite cells are the primary muscle intrinsic stem cell population that serve to repair and maintain skeletal muscle throughout the lifespan.<sup>22</sup> However, with advanced age, satellite cells are inhibited in their capacity to activate, and self-renew<sup>10</sup> and are ultimately reduced in association with type II muscle fibers.<sup>11,23</sup> It was not until recently that interstitial cells expressing Twist2 within rodent skeletal muscle were identified as definitive myogenic progenitors.<sup>13,14</sup> Depletion of Twist2-expressing cells reduced muscle mass of type IIb/x muscle fibers, identifying for the first time, a muscle fiber type-specific progenitor. Here, we identify cells within human skeletal muscle that express *TWIST2* mRNA and protein which reside within the muscle fiber interstitium. scRNAseq demonstrated that *TWIST2*-expressing cells are highly related to mesenchymal/FAPs with very few expressing characteristic satellite cell genes, in agreement with rodent Twist2 cells.

We are only now beginning to understand the complexity of mesenchymal cell populations which reside in human skeletal muscle. Here, we identified four clusters of mesenchymal/FAPs cells which is in general agreement with other scRNAseq investigations of human skeletal muscle, including De Micheli et al.<sup>24</sup> who describe three clusters of fibroblasts and a cluster they identified as adipocytes. Indeed, they identified a *PCOLCE2*-, *MFAP5*-expressing cluster (similar to our FAPs1), a *SMOC2*-, *ADH1B*-, and *ABCA8*-expressing cluster (similar to our FAPs2) a cluster of *GPX3*-, *CXCL14*-expressing adipocytes (similar to our FAPs3) and a *MYOC*-, *PTGDS*-positive cluster (similar to

our FAPs4). We demonstrate that *TWIST2*-expressing cells are enriched in the *PCOLCE2*-, *MFAP5*-, *CXCL14*-, *GPX3*-expressing clusters of FAPs. This is in agreement with our recent investigation where we identified a small number of *TWIST2*-expressing FAPs through scRNAseq of the hamstrings muscle<sup>25</sup>. The functional divergence of FAPs subclusters is poorly understood and a topic of intense scrutiny, however, it is attractive to hypothesize that a fraction of FAPs in these subclusters may possess direct myogenic potential. Further investigations profiling greater numbers of cells will be necessary to determine differentially expressed genes between *TWIST2*-positive and negative fractions of FAPs. In this regard, it should be noted that scRNAseq technologies are only capable of capturing and sequencing a proportion of mRNA transcripts per cell and, therefore, it is conceivable that *TWIST2* is expressed in additional cells/clusters below the detectable limit.

We compared human and rodent Twist2-expressing cells which revealed that they are transcriptionally very similar. This is evidenced by the fact that both human and mouse Twist2-expressing cells do not cluster independently but are rather encompassed in their respective mesenchymal cell clusters and express similar characteristic genes such as *PDGFRA*, *DCN*, and *GSN*. Further, ~40% of differentially expressed genes in mouse and human Twist2-expressing cells were shared. Interestingly in mice, Twist2 was also expressed in a proportion of *Scx*-positive tenocytes which was not reported previously and not found in human muscle tissue as cells corresponding to tenocytes were not evident. These collective observations support the notion that Twist2-expressing cells within humans and rodents may be equivalent, or at least highly related. Therefore, it is a priority to identify if human Twist2-positive cells possess direct myogenic potential like their rodent counterparts and function to support and repair type IIx muscle fibers. In mice, the type IIx/b muscle fiber specificity of Twist2 cells is regulated by a mechanism involving Sema3A secretion by type I and IIa muscle fibers which signal through Nrp1 on Twist2 cells to direct their migration away from these fiber types and toward type IIx/b muscle. Here, we observe that *NRP1* is expressed in *TWIST2*-expressing cells (*data not shown*) suggesting a similar mechanism may be relevant in humans.



The greater quantity of Twist2-positive cells in muscle tissue collected in the older adults compared with the young was unexpected based on the notion that Twist2

cells may contribute to the maintenance of type IIX fiber CSA and the number which typically decrease with aging.<sup>1</sup> Our correlational analysis did not reveal evidence linking

**FIGURE 5** Resistance exercise training increases Twist2-positive cell number in old skeletal muscle. Representative images and quantification (C) of IHC staining for laminin (green), Twist2 (red), and DAPI (blue), in muscle cross-sections pre (A) and post (B) 12-weeks of resistance exercise training in older males ( $n = 34$ ). The \* in C indicates a significant difference ( $p < .05$ ) compared to pre. (D) Pearson's correlation of the number of Twist2-positive cells before training and the change (%) of Twist2-positive cells with 12-weeks of RET. Pearson's correlations of the quantity of Twist2-positive cells post RET and type I fiber CSA (E), type II fiber CSA (F), myonuclear domain size of type I fibers (G), myonuclear domain size of type II fibers (H), appendicular lean mass (I) and satellite cells associated with type II muscle fibers (J) in skeletal muscle tissue collected from older adults after 12-weeks of RET.  $p$  and  $r$  values are indicated on each graph with significance set at  $p < .05$ . Scale bars in A, B = 50  $\mu\text{m}$ .

the quantity of Twist2-positive cells with indices of muscle fiber size (of type I or II fibers), fiber type, or muscle mass in younger or older individuals. This is in general contrast to satellite cells which are maintained in higher numbers in larger, resistance-trained skeletal muscle.<sup>26</sup> A limitation of the current study was that we did not determine the CSA of individual type II muscle fiber subtypes (i.e., type IIa, ax, x). However, it should be noted that recent work has highlighted variability in the results from IHC-based approaches to quantify type II muscle fiber subtypes.<sup>27</sup> Therefore, we cannot conclusively rule out an association between Twist2-positive cell content and type IIx fiber CSA or content. The collective interpretation of these results suggests that the abundance of Twist2-positive cells is not an obvious indicator of muscle mass or the size of type II muscle fibers and highlight that our understanding of human Twist2 cell biology is lacking. Human skeletal muscle lacks the expression of the type IIb isoform of MHC and the percentage of fibers expressing type IIx is low (<10%) even considering hybrid fibers coexpressing type IIa and type IIx.<sup>28</sup> This is in contrast to mouse skeletal muscle where between 15% and 40% of muscle fibers express MHC type IIx and IIb isoforms depending on anatomical location.<sup>28</sup> Therefore, it is conceivable that the relative “importance” of Twist2-positive cells in human skeletal muscle maintenance may be less than in rodent skeletal muscle. Further, aging is typified by conversion of type II muscle fibers to that of type I through denervation and collateral reinnervation.<sup>29</sup> The function of Twist2-positive cells in this process is currently unknown.

Another possible explanation for the increase in Twist2-positive cell number with aging comes from work in cultured mouse cells and human rhabdomyosarcoma cells (tumors of skeletal muscle) where Twist2, acting as a transcription factor functions to repress myogenesis.<sup>30</sup> It would be provocative to interrogate if Twist2 performs a similar role in human muscle mesenchymal cells and if Twist2 expression increases with age, to potentially repress myogenic potential. Alternatively, there is accumulating evidence that Twist proteins participate in fibrotic diseases.<sup>31</sup> In this regard, the aging of skeletal muscle has been associated with increased muscle fibrosis through the deposition of collagens and extracellular matrix proteins.

However, the older adults who participated in this investigation were healthy and active. Therefore, future studies should focus on muscle Twist2-positive cell function and abundance under conditions of clinical sarcopenia, long-term disuse, and fibrosis to gain additional insight into Twist2-positive cell function.

RET increases skeletal muscle mass and strength and forms an effective countermeasure to attenuate or prevent muscle loss with age.<sup>32</sup> RET also increases satellite cell quantity<sup>26</sup> and increases type II muscle fiber-associated satellite cells in senescent skeletal muscle tissue.<sup>11</sup> Here, we demonstrate that Twist2-positive cell number is also increased in skeletal muscle tissue following RET in older adults. Interestingly however, following RET Twist2-positive cell quantity was negatively correlated with muscle fiber CSA and the myonuclear domain size of type I and type II fibers. These results show that while Twist2-positive cells are responsive to RET, their potential role in the skeletal muscle adaptive response to exercise is unclear.

There were no direct relationships between the quantity of Twist2-positive cells and satellite cells in the young or older adults or following RET. scRNAseq analysis of both human and mouse skeletal muscle demonstrated that very few satellite cells simultaneously expressed *TWIST2* mRNA or protein in conjunction with Pax7. Therefore, the endogenous role of *Twist*-positive cells in regulating, contributing to, or replenishing the satellite cell pool remains to be defined but is not obvious within unperturbed human skeletal muscle. Further studies using acute damaging exercise protocols (i.e., eccentric contractions) coupled to scRNAseq technologies should be implemented to interrogate Twist2-positive cell activity under reparative conditions. Collectively, the current results identify the presence of Twist2-expressing cells in the interstitium of human skeletal muscle which are responsive to aging and exercise, the myogenic potential of which merits further investigation.

## AUTHOR CONTRIBUTIONS

Nick Gaulton performed analysis, created figures, wrote and edited the manuscript. Griffen Wakelin performed computational analysis, created figures, wrote and edited the manuscript. Laura V. Young assisted with experiments, performed data analysis, edited the manuscript.

Scott Wotherspoon provided the human skeletal muscle and edited the manuscript. Michael Kamal and Gianni Parise recruited and isolated human skeletal muscle from young participants and edited the manuscript. Joshua P. Nederveen, Andy Holwerda, Lex B. Verdijk, Luc J. C. van Loon, and Tim Snijders recruited, trained, and isolated skeletal muscle from older participants and edited the manuscript. Adam P. Johnston conceived the experiments, aided in analysis assisted in creating figures, wrote and editing the manuscript.

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### DISCLOSURE

The authors declare no conflicts of interest.

### DATA AVAILABILITY STATEMENT

Data on human intercostal muscle are available at the Heart Cell Atlas web portal ([www.heartcellatlas.org](http://www.heartcellatlas.org)), while data from mouse skeletal muscle are available from the GEO database (accession GSM3520458/9).

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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