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Uterine leiomyomas exhibit fewer stem/progenitor cell characteristics when compared with corresponding normal myometrium

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Abstract

Uterine leiomyomas (also known as uterine fibroids) are the most common benign tumors of female reproductive tract and are the single most common indication for hysterectomies. Despite their high prevalence, the exact pathogenesis of these benign tumors is still unknown. One possible mechanism for leiomyoma formation is dysregulation of mesenchymal stem cell activity. Mesenchymal stem cells have been identified in both human and murine uteri and cancer stem cells have been identified in female reproductive malignancies. We compared stem/progenitor cell characteristics in both normal myometrium and the corresponding leiomyoma of patient's undergoing hysterectomies. We found that leiomyoma cells form fewer mesenchymal stem cell colonies and exhibit less Hoechst dye-excluding side population activity, which is a function associated with progenitor cells in other tissues, than cells isolated from normal myometrium. Whereas in normal myometrium we observed heterogeneous expression of CD90, a cell surface marker associated with differentiation potential of uterine fibroblasts, in leiomyomas, we observed homogenous expression of CD90, suggesting leiomyoma cells are more terminally differentiated. Furthermore, we found that while leiomyoma cells could only produce CD90 expressing cells, both CD90+ and CD90- myometrial cells could reestablish their original heterogeneous CD90 profile when expanded *in vitro*. These results suggest that normal myometrium contains cells with stem/progenitor cell activities that are absent in leiomyomas.

Keywords

uterine leiomyomas; fibroids; stem cells

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INTRODUCTION

Uterine leiomyomas (also known as uterine fibroids) are the most common benign reproductive tumor in women with a prevalence suggested to be as high as 77%. Additionally, uterine leiomyomas are the single most common indication for hysterectomy. While most are asymptomatic, clinical symptoms are present in 25% of cases, manifesting as menstrual bleeding, pelvic pain and possibly infertility. Despite its high prevalence, the exact pathophysiology underlying uterine leiomyomas is still unknown¹. Somatic stem cells are a subset of cells residing in normal adult tissues that, through asymmetric division, retain their ability to self-renew while producing daughter cells which go on to differentiate and play a role in tissue regeneration and repair. Likewise, cancer stem cells (or tumor initiating cells) are a subset of cells from a tumor population, which, also through asymmetric division, retain the ability to reconstitute tumors while producing non-tumorigenic daughter cells, which form the bulk of the tumor^{2, 3}. While there have been several studies reporting the prospective identification of these cells in a variety of malignant tumors including breast and ovary⁴⁻⁶, there are little data supporting the existence of these cells in benign tumors such as uterine leiomyomas.

In various organ systems, stem cells have been identified by specific cell surface markers that can be exploited for their isolation and characterization both *in vitro* and *in vivo*. CD90 (also known as Thy-1) is a small glycosylphosphatidylinositol (GPI)-linked glycoprotein which has been associated with neuronal development, cell-cell interactions, cell-matrix interactions and fibrosis though its exact biologic function is still unknown^{7, 8}. Originally identified on mouse thymocytes, CD90 is expressed by a variety of cells, including peripheral T-cells, hematopoietic stem cells, neurons, fibroblasts, and mesenchymal stem cells⁸⁻¹². In the human endometrium, CD90+ cells showed a trend towards higher colony formation than their CD90 – counterparts¹³. Additionally, CD90 has been validated as a phenotypic marker of human myometrium cells with different lineage preferences for differentiation¹⁴. It has not been characterized in uterine leiomyomas, however.

In this study, we sought to explore the possibility that the pathogenesis of uterine leiomyomas is a mesenchymal stem cell-driven process. We examine the differences between leiomyomas and normal myometrium with regards to inherent growth potential by evaluating colony-forming ability, mesenchymal stem cell markers by evaluating CD90 expression and side population (SP), and differentiation status by CD90 expression patterns after *in vitro* expansion. We find that normal myometrial cells have greater differentiation potential than those isolated from leiomyomas.

MATERIALS AND METHODS

Sample Acquisition and Preparation

Samples of human leiomyomas and corresponding normal myometrium from the same patient were obtained after hysterectomy according to an Institutional Review Board approved protocol. The samples were cut into 1–2 mm³ pieces, placed in a solution containing 7 ml DMEM, 2 mg/ml type II collagenase, 35mg/ml Amphotericin B and 1mg/ml DNase I, and 2% (PSG) penicillin/streptomycin/glutamine solution (Invitrogen, Carlsbad, CA, USA), and incubated overnight in a 37°C shaking water bath. The sample was then gently triturated to break up the remaining tissue and filtered through a 100 mm filter. The cells were collected, incubated in 2.5 ml of ACK lysis buffer (Cambrex, Walkersville, MD, USA) for approximately 1 min, washed twice with PBS before being counted and resuspended in an appropriate volume of DMEM. Portions of the original samples were saved for study by immunohistochemistry. Briefly, tissues were fixed overnight in 4% paraformaldehyde at 4°C, and then prepared by paraffin embedding and sectioning for immunohistochemistry or incubated overnight in 15%

sucrose solution for frozen sections. For immunofluorescence, tissues were next embedded in 7.5% gelatin/15% sucrose solution, frozen at -60°C in isopentane for sectioning. Paraffin sections were subsequently incubated in blocking solution then with 1/50 diluted anti-CD90 (Abcam, Cambridge, MA) followed by biotinylated F(ab)2 fragments (Jackson ImmunoResearch, West Grove, PA). Antibody detection was performed with a ready-to-use streptavidin-conjugated horseradish peroxidase (Lab Vision, Fremont, CA) and color development with a DAB kit (Vector Labs, Burlingame, CA). Sections were counterstained with hematoxylin prior to mounting and analysis. For immunofluorescence, frozen sections were incubated overnight at 4°C with the same anti-CD90 followed by an Alexfluor-488 conjugated second antibody (Invitrogen) and a second overnight incubation with Cy3-conjugated smooth muscle actin antibody (Sigma, St. Louis, MO) prior to visualization. Photomicrographs were taken with a Spot camera (Diagnostic Instruments, Sterling Heights, MI) on a Nikon TE-2000S microscope.

Flow Cytometry and Cell Sorting

All flow cytometry and cell sorting was performed in the Flow Cytometry Laboratory of the Department of Pathology, Massachusetts General Hospital, according to its published protocols¹⁵. For cell surface marker analysis, the cell suspensions obtained above were washed with and resuspended at 1×10^6 cells/ml in PBS with 2% FCS. Antibodies were then added and incubated for 15 min on ice and washed again in PBS with 2% FBS before analysis. The antibodies used were allophycocyanin (APC)-conjugated mouse anti-human CD45 (Caltag, Carlsbad, CA, USA) and FITC-conjugated mouse anti-human CD90 (Immunotech-Beckman Coulter, Marseille, FR). A non-stained sample was used as negative controls and to set all gates. For SP analysis, normal myometrial and leiomyoma cells were resuspended at a concentration of 1×10^6 cells/ml in DMEM with 2% FBS and 1mM HEPES and stained with 5 mg/ml of Hoechst 33342 for 90 min at 37°C . For selected samples, Verapamil was added at 25mg/ml prior to the addition of the Hoechst. The cells were then washed with and resuspended in cold PBS containing 2% FCS. SP cells were identified and electronically gated based on Hoechst 33342 red versus blue fluorescence linear emission patterns after excitation with 100 mW of a 350–360 nm ultraviolet laser on a FACSVantage Diva Cell Sorter (Becton Dickinson, San Jose, CA, USA). Side and forward scatter profiles were used to eliminate doublets and debris. Dead cells were eliminated using 4',6-diamidino-2-phenylindole (DAPI; 1 mg/ml final concentration). Hematopoietic cells were gated out by identification and omission of the CD45 + population.

Colony Formation and Analysis

Normal myometrium and leiomyomas were separated into single cell suspensions as described above. Cells were then plated in 6-well tissue culture plates and incubated for 14 days in MesenCult Human Mesenchymal Stem Cell Media according to manufacturer protocols (Stem Cell Technologies, Vancouver, BC, Canada). Cells were initially plated at increasing numbers from 1×10^3 , 2.5×10^3 , 5×10^3 , 1×10^4 , 2.5×10^4 and 5×10^4 to determine optimal plating efficiency. A cell number of 2.5×10^3 was chosen for all following colony growth experiments. After 14 days of incubation, cells were visualized using Giemsa staining. In brief, culture wells were washed twice with PBS, fixed with cold 100% methanol and stained with 1× Giemsa solution (Sigma). The plates were then dried and digitally scanned. All image analysis was performed using ImageJ software (NIH, Bethesda, MD, USA). Images were cropped to the appropriate well size and red/green/blue color split. Using the green channel image, a black-white image threshold was set at 0 to 150. Particle analysis was done using an area threshold of 0.001 to 2.0 inches² with a circularity threshold of 0 to 1. The resulting surface area % given by the software was then used for all comparisons. Quantification of colony growth was performed by measuring well surface area coverage percentage by cells rather than number of colonies because the growth differences between the myometrium and leiomyomas did not

allow us to choose a cell plating number which would allow both countable growth from the leiomyoma wells and distinct colonies from the myometrial wells.

Tissue Culture

Cells sorted into CD90+ and CD90- populations were plated at 1×10^5 cells in tissue culture-treated T75 flasks. Cells were incubated at 37°C and 5% CO₂ in DMEM/F-12 with 10% FBS and 1% Penicillin/Streptomycin/Fungizone (GIBCO, Carlsbad, CA, USA) for three weeks (~60% confluent) before trypsinization and reanalysis by flow cytometry.

Data Analyses

Flow cytometry data were analyzed using FlowJo 8.7.3 software. Statistical analysis was performed using Prism Graph Pad 5 software. Data between the two cohorts were analyzed using an unpaired two-tailed t-test with 95% confidence intervals resulting in significance with p-values less than 0.05.

RESULTS

Colony Formation of Normal Myometrium and Leiomyoma Cells

To analyze the inherent growth potential of the cells of myometrium and leiomyoma, we performed a colony forming unit (CFU) assay by plating unsorted single-cell suspensions prepared from both tissues in mesenchymal stem cell media for 14 days. Normal myometrium showed more robust colony growth plated at 2.5×10^3 cells per well with an average of 41.1% well surface area coverage compared to 6.1% by leiomyoma tissue plated at the same cell density ($p < 0.009$, $n = 5$; Fig. 1). In fact, the difference in growth between the myometrium and leiomyoma necessitated the use of cell surface area coverage rather than individual colony counts for quantification. At 2.5×10^3 cells plated, the myometrium was still nearly confluent while the leiomyoma colonies were sparse (Fig 1, insets). These results suggest that the cells of normal myometrial tissue show increased inherent growth potential as compared to the cells of leiomyoma tissue.

Side Population Analysis of Normal Myometrium and Leiomyomas

Given the significant difference in colony forming potential between cells isolated from the myometrium and leiomyomas we explored whether this finding could be accounted for by differences in mesenchymal stem cell activity. Since the SP phenomena has recently been shown to identify putative myometrial somatic stem cells in mice¹⁶ and humans¹⁷, we evaluated differences in the SP between the myometrium and leiomyomas. Single cell suspensions of normal myometrium and leiomyoma tissue prepared from several patients were each stained with Hoechst 33342 dye and analyzed by flow cytometry for SP. DAPI staining was used to evaluate cell viability and the CD45- population was analyzed to exclude hematopoietic cells. In the example shown in Fig. 2A, there were nearly three-fold more SP cells in the normal myometrium of this patient compared to that found in her leiomyoma tissue. When Verapamil, a reversible inhibitor of ATP binding cassette (ABC) transporter activity was included, none of the SP cells were apparent, suggesting that the Hoechst dye efflux was due to ABC transporter activity. Comparison of the two tissues from 7 patients showed that the normal myometrium had a consistently and significantly higher CD45- SP than leiomyomas ($1.69\% \pm 0.75$ vs. $0.40\% \pm 0.11$, $p < 0.05$, $n = 7$; Fig. 2B).

CD90 Differences Between Normal Myometrium and Leiomyomas

We identified CD90 as a marker that showed consistent and significant differences between the two tissues and, given its association with mesenchymal stem cells, was chosen for further study. Flow cytometry analysis of normal myometrium and leiomyoma cells from 5 patients

shows that less than half of the CD45- non-hematopoietic normal myometrial cells are CD90+ while over two-thirds of the leiomyoma cells are CD90+ ($41.5\% \pm 5.9$ vs. $70.5\% \pm 2.3$, $p < 0.005$, $n=5$; Fig. 3A). Closer examination of a representative flow cytometry histogram, however, shows that while leiomyomas consist of a homogeneous CD90+ population, normal myometrium exhibits a robust CD90- population that is not found in leiomyomas (Fig. 3B). These results suggest that while CD90 positivity may not be a mesenchymal stem cell marker in these tissues, CD90 expression patterns may indicate cells with alternate phenotypes. Additionally, the homogenous expression pattern of the leiomyomas indicates a clonogenic tissue. Analysis of human myometrial and leiomyoma tissues by immunohistochemistry with a CD90 antibody showed that while CD90 expression in normal myometrium was excluded from the cells in the large arteries, in leiomyoma tissue CD90 expression was observed throughout, including the cells in large vessels (Fig. 3C). Further analysis identified a significant number of CD90- cells as the smooth muscle and endothelial cells in the tunica media of large vessels of normal myometrium (Fig. 3D).

Side Population and CD90 Distribution of Human Myometrium

Since we saw consistent differences between the myometrium and leiomyomas in both CD90 and SP distribution, we examined whether or not these populations overlapped in the normal myometrium. Given that we saw only a single CD90 population in leiomyomas, we focused this analysis exclusively on the normal myometrium in an attempt to correlate both CD90+ and CD90- cells with SP and non-SP (NSP). After gating out the CD45+ hematopoietic cells, we found that the SP comprised a higher percentage of the CD90- cells than the CD90+ subpopulation. In fact, the SP percentage in the CD90- subset was 1.5-fold enriched over the unseparated neat SP percentage while the SP of CD90+ subset was only 21% of the neat SP percentage ($p < 0.0005$, $n=4$; Fig. 4A). Alternatively, an analysis of the SP as compared to the NSP in terms of CD90 distribution shows that the SP of normal myometrium is almost exclusively CD90- (91.4%) whereas the NSP shows a more heterogeneous CD90 distribution (NSP CD90- 61.8%, $p < 0.02$, $n=4$; Fig. 4B). This overlap between the SP and CD90- cells is consistent with our previous observation that the normal myometrium exhibits both a higher SP and more CD90- cells than leiomyoma tissue and suggests that the cells that may define the difference between normal myometrium and leiomyoma tissue may reside in CD90- population.

Growth Characteristics of CD90 Populations in Myometrium and Leiomyomas

Given the correlations between the CD90 distribution and the somatic stem cell-associated SP phenotype, we sought to examine whether there were functional differences between the CD90 populations in the two tissues. We sorted the myometrium and leiomyomas into their CD90 positive and negative populations and plated the cells at equal densities ($n=3$). As we found leiomyomas to be homogeneously CD90+, we set the CD90- gate based on the normal myometrium in an attempt to isolate the most likely CD90- population of leiomyomas. The cells were grown for three weeks and then re-analyzed for their CD90 expression. Both the CD90 positive and negative populations of the normal myometrium were able to repopulate a heterogeneous CD90 phenotype (Fig. 5, left). However, the leiomyoma populations could only recreate the original homogeneous CD90+ population regardless of whether CD90 positive or negative populations were plated (Fig. 5, right). These results suggest that while the cells of the myometrium can create a heterogeneous CD90 distribution regardless of the plated cell status, the cells of the leiomyoma may already be committed to a homogenous CD90+ phenotype, indicating terminal differentiation.

DISCUSSION

The increased colony formation of the myometrial cells (Fig 1), suggests an increased inherent growth potential of the cells of the myometrium when compared to the leiomyoma cell population. Given that leiomyomas have been shown to express higher levels of mitotic activity^{18, 19} and higher levels of the anti-apoptotic protein Bcl-2 *in vivo* than normal myometrium^{20–22}, one would have predicted more robust growth from leiomyoma cells. Our results showing otherwise, however, are corroborated by studies that indicate normal myometrial cells proliferate faster with shorter doubling times *in vitro* when compared to leiomyomas^{23, 24}. The discrepancy between the *in vitro* growth pattern and what is predicted based by *in vivo* molecular profiling probably reflects differences in inherent growth patterns between cell populations that make up the two tissues when removed from their normal environmental influences. As the niche microenvironment plays an important role in regulating and supporting somatic stem cell growth and division *in vivo*²⁵, the disruption of this environment and its signals would probably result in a lack of normal control and unmask the inherent growth potential of these cells. As a result, we believe that the enhanced colony formation of the normal myometrium reflects the enhanced growth potential of cells in this tissue when compared to leiomyoma cells.

The Hoechst 33342 dye-excluding SP has been associated with stem/progenitor cell phenotypes in a variety of tissues and malignancies^{5, 26–28}. We have previously shown that the SP in mouse myometrium is enriched for undifferentiated cells that exhibit the ability to differentiate into myometrial cells *in vitro*¹⁶. The SP cells of the human endometrium have been identified as a subset in G0 arrest, enriched for colony formation, capable of generating both glandular and stromal phenotypes^{29, 30}. More recently, the SP cells of the human myometrium has been shown to exhibit characteristics of mesenchymal stem cells including the ability to differentiate into osteocytes and adipocytes and the ability to regenerate functional human myometrial tissue when xenotransplanted into mice¹⁷. We found that the normal myometrium consistently exhibited a significantly higher SP than leiomyomas (Fig 2A,B), suggesting that the myometrium contains a higher mesenchymal stem/progenitor cell population than leiomyomas, which could potentially account for the differences in colony formation we observed.

As CD90 has been shown to identify subsets of fibroblasts with different functional properties^{14,31} in the human uterus, we believe the differences in CD90 expression patterns between the myometrium and leiomyomas points to functional differences in differentiation between the two tissues. The homogenous CD90 expression pattern of the leiomyomas reflects the clonogenic and homogenous cell population of the tissue while the heterogeneous CD90 expression pattern of the myometrium reflects the heterogeneity of the myometrial cell population (Fig 3). Furthermore, the ubiquitous CD90 expression that results when leiomyoma cells of varying CD90 expression levels are plated is an indication of the terminal differentiation of these cells -- even the cells that appear CD90 negative or low on flow cytometry when plated are committed to becoming a CD90 positive leiomyoma cell when expanded *in vitro* (Fig 5, right). More telling, the ability of the myometrial cells to produce a heterogeneous CD90 expression pattern regardless of the CD90 status of the cells plated reveals the inherent differentiation ability of the myometrial population (Fig 5, left). While we do believe CD90 expression to be indicative of functional differences between cells and tissues, we do not believe that it is an independent marker of stem or progenitor cells and that further work to confirm the presence and elucidate the identity of these cells in both the myometrium and leiomyomas is needed.

In addition to a marker of phenotypic sub-populations, our identification and characterization of CD90 expression as a differential marker in uterine leiomyomas may shed some light to

their pathogenesis. In human myometrium, CD90+ fibroblasts preferentially differentiate into alpha-smooth muscle actin-expressing myofibroblasts in response to TGF- β whereas the CD90- fibroblasts differentiate into lipofibroblasts in response to peroxisome proliferator-activator receptor-gamma (PPAR- γ) but not when CD90+ fibroblasts are exposed to PPAR- γ or CD90- fibroblasts are exposed to TGF- β 14. Furthermore, CD90 expression identifies subsets of fibroblasts with different roles in the inflammatory cascade with CD90+ fibroblasts expressing high levels of COX-1, producing prostaglandin E2 (PGE2) and regulating the inflammatory response in the myometrium while CD90- fibroblasts express high levels of COX-2 and produced low levels of PGE2 11, 31. TGF- β in leiomyomas has been shown to induce extracellular matrix (ECM) production and reduce levels of proteolytic enzymes 32–34.

Our results indicate that cells from leiomyomas exhibit less growth potential indicated by less colony formation, fewer markers of mesenchymal stem cells indicated by a lower SP, and are more clonogenic and homogenous as indicated by homogenous CD90 expression, when compared to the cell population of normal myometrium. We have also shown evidence by immunofluorescence that the CD90- cells in normal myometrium might be derived from vascular smooth muscle cells (Fig. 3). It will be interesting to determine whether these CD90- α -smooth muscle actin-positive cells are the CD90- cells with increased differentiation potential. Associating the expression of CD90 with fewer stem cell activities may seem counterintuitive since CD90 is a classical marker for mesenchymal stem cells¹⁰. However, taken together, our findings suggest that leiomyomas do exhibit fewer properties associated with mesenchymal stem cells even though nearly all leiomyoma cells appear to express CD90..

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Figure 1. Normal myometrium shows more robust growth *in vitro* than leiomyomas

After isolation, 2.5×10^3 normal myometrial and leiomyoma cells were plated in mesenchymal stem cell media. Normal myometrium shows more robust growth after 14 days of incubation compared to leiomyoma tissue as measured by well surface area coverage ($41.05\% \pm 11$ vs. $6.15\% \pm 2.8$, $p < 0.009$, $n = 5$); representative wells shown above their respective bar columns.

Figure 2. Normal myometrium has a higher percentage of Verapamil-sensitive side population than leiomyomas

Cells were isolated from normal myometrium and leiomyomas to compare their side population by flow cytometry with CD45+ hematopoietic cells excluded. (A) Representative analysis of normal myometrium (top) and leiomyomas (bottom) from one patient showing a higher Verapamil-sensitive SP in normal myometrium. (B) The statistically significant ($p < 0.05$) different average % SP populations of normal myometrium and leiomyomas of 7 different patients are shown plotted ($1.69\% \pm 0.75$ vs. $0.40\% \pm 0.11$). Error bars represent SEM.

Figure 3. Normal myometrium has a CD90⁻ population not found in leiomyomas

Cells were isolated from myometrium and leiomyomas and analyzed for their CD90 expression by flow cytometry after excluding hematopoietic cells. (A) Surface marker analysis shows that normal myometrium has a lower population of CD90⁺ cells compared to leiomyomas (41.5% ± 5.9 vs. 70.5% ± 2.3, $p < 0.003$, $n = 5$). (B) Representative histogram of one patient showing that rather than lacking a CD90⁺ population, normal myometrium has a CD90⁻ population which is not found in leiomyomas. (C) Immunohistochemistry confirms (left panel) heterogeneous CD90 expression in the myometrium and (right panel) ubiquitous CD90 expression in leiomyomas ($n = 3$). Bar = 100 μ m. (D) Immunofluorescence was performed to detect nuclei with DAPI (panel a), CD90 expression (panel b, *in green*) and α -smooth muscle actin (α SMA,

panel c, *in red*) colocalization in normal myometrial smooth muscles cells but not in the vascular smooth muscles cells (panel d). Bar =50 um.

Figure 4. The myometrial side population is enriched for CD90- cells

(A) Analysis of normal myometrial cells shows that the SP percentage of the CD90- subset is 1.5-fold enriched over the comparable neat SP whereas the CD90+ population is only one-fifth of the neat SP ($p < 0.0005$, $n = 4$). (B) Conversely, analysis of the SP and NSP of normal myometrium shows that the SP is almost exclusively comprised of CD90- (91.4% CD90- vs. 8.63% CD90+) cells whereas the NSP has significant CD90- (61.8%) and CD90+ (38.2%) populations.

Figure 5. Normal myometrial cells reconstitute a heterogeneous CD90+/- population whereas leiomyoma cells only produce a CD90+ population

When sorted into CD90+ and CD90- populations (outlined populations in top row FACS plots) and plated, after 3 weeks incubation, myometrial cells (left) creates heterogeneous CD90+ and CD90- populations (bottom row red histograms) whereas leiomyoma cells (right) only produces a homogeneous CD90+ population (bottom row red histograms) regardless of whether CD90+ or CD90- cells are plated initially (n=3).