Published in final edited form as:

Obstet Gynecol. 2009 August ; 114(2 Pt 1): 300–309. doi:10.1097/AOG.0b013e3181af6abd.

Stimulating Vaginal Repair in Rats Through Skeletal Muscle–Derived Stem Cells Seeded on Small Intestinal Submucosal Scaffolds

Matthew H. Ho, MD, PhD, Sanaz Heydarkhan, MSc, Dolores Vernet, PhD, Istvan Kovanecz, PhD, Monica G. Ferrini, PhD, Narender N. Bhatia, MD, and Nestor F. Gonzalez-Cadavid, PhD

Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Urology Research Laboratory, Torrance, California; the Division of Female Pelvic Medicine and Reconstructive Surgery, Department of Obstetrics and Gynecology, Harbor-UCLA Medical Center, David Geffen School of Medicine at UCLA, University of California, Los Angeles, California; the Division of Endocrinology and Molecular Medicine, Department of Internal Medicine, College of Medicine, Charles Drew University of Medicine and Science, Los Angeles, California; and the Department of Urology, David Geffen School of Medicine at UCLA, University of California, Los Angeles, California

Abstract

OBJECTIVES—Grafts are used for vaginal repair after prolapse, but their use to carry stem cells to regenerate vaginal tissue has not been reported. In this study, we investigated whether 1) muscle-derived stem cells (MDSC) grown on small intestinal submucosa (SIS) generate smooth-muscle cells (SMC) in vitro and upon implantation in a rat model of vaginal defects; 2) express markers applicable to the in-vivo detection of vaginal endogenous stem cells; and 3) stimulate the repair of the vagina.

METHODS—Mouse MDSC grown on monolayer, SIS, or polymeric mesh, were tested for cell differentiation by immunocytochemistry, Western blot and real-time polymerase chain reaction (PCR). Stem cell markers were screened by DNA microarrays followed by real-time PCR, immunocytochemistry, and Western blot. Rats that underwent hysterectomy and partial vaginectomy were left as such or implanted in the vagina with 4',6-Diamidino-2-Phenylindole (DAPI)-labeled MDSC on SIS, or SIS without MDSC, immunosuppressed, and killed at 2–8 weeks. Immunofluorescence, hematoxylin-eosin, and Masson trichrome were applied to tissue sections.

RESULTS—Muscle-derived stem cell cultures on monolayer and on scaffolds differentiate into SMC, as shown by α -smooth muscle actin (ASMA), calponin, and smoothelin markers. Muscle-derived stem cells express embryonic stem cell markers Oct-4 and nanog. Dual DAPI/ASMA fluorescence indicated MDSC conversion to SMC. Muscle-derived stem cells/SIS stimulated vaginal tissue repair, including keratin-5 positive epithelium formation and prevented fibrosis at 4 and 8 weeks. Oct-4+ putative endogenous stem cells were identified.

© 2009 by The American College of Obstetricians and Gynecologists. Published by Lippincott Williams & Wilkins

Corresponding author: Nestor F. Gonzalez-Cadavid, PhD, LABioMed at Harbor-UCLA Medical Center, Urology Research Laboratory, Bldg. F-6, 1124 West Carson Street, Torrance, CA 90502; ncadavid@ucla.edu.

Financial Disclosure

The authors did not report any potential conflicts of interest.

CONCLUSION—Muscle-derived stem cells/SIS implants stimulate vaginal tissue repair in the rat, thus autologous MDSC on scaffolds may be a promising approach for the treatment of vaginal repair.

Pelvic organ prolapse, and specifically vaginal prolapse, is highly prevalent and may occur in up to 50% of parous women.^{1,2} Surgical cure rates vary, and recurrences are common in primary ungrafted methods of anterior vaginal repair. This has led to the use of synthetic mesh or biological grafts to provide support for the weakened fascia and musculature.¹⁻⁴ Mesh and graft reduced objective prolapse recurrence rates compared with ungrafted methods.³⁻⁵ However, complications are frequent, particularly vaginal fibrosis, inflammation, and epithelial erosion, and the long-term durability and safety of these devices are unknown.^{5,6}

In other areas of urogenital tissue repair, stem cell therapy is being investigated, particularly in the bladder and urethra for the treatment of stress urinary incontinence,⁷ and in the penile corpora cavernosa, for erectile dysfunction.^{8,9} Stem cells from accessible adult tissues, such as the bone marrow or skeletal muscle, pose lower risks of carcinogenesis and immunorejection than embryonic stem cells^{10,11} and are also being applied for tissue repair in clinical trials of some nonurogenital conditions (<http://clinicaltrials.gov>). Differentiated vaginal epithelial and smooth muscle cells have been used to create a neovagina in a rabbit model.¹²

Muscle biopsies allow the isolation for autografts of muscle-derived stem cells (MDSC), which have been characterized in rodents and isolated from human muscle^{13,14} and are different from the myogenically committed satellite cells studied for stress urinary incontinence.¹⁵ Muscle-derived stem cells differentiate in vitro and in vivo into skeletal myotubes, osteoblasts, chondrocytes, and neural cells^{9,13} and when implanted into the rat penile corpora cavernosa originate smooth muscle cells (SMC) replacing the cells lost during aging-related tissue fibrosis and correcting erectile dysfunction.⁹ Stem cell therapy may also aim for the pharmacologic activation of “dormant” endogenous stem cells that have been found in most organs, including the penis,^{9,16} ovary,¹⁷ and testis¹⁸ and which may express the embryonic stem cell markers Oct-4 (octamer binding transcription factor) or nanog.¹⁹

The purpose of this study was to investigate whether implantation in the rat vagina of MDSC grown on small intestinal submucosa (SIS) promotes tissue repair without inducing fibrosis and whether endogenous stem cells present in the vagina can induce vaginal repair.

MATERIALS AND METHODS

Hind limb muscles of C57BL/6 mice were subjected to the preplating procedure to isolate MDSC.^{9,20} Mouse MDSC are the only ones prepared by this method that have been characterized as stem cells.¹³ Tissues were dissociated using sequentially collagenase XI, dispase II, and trypsin, and after filtration through 60 nylon mesh and pelleting, the cells were suspended in GM-20 (Dulbecco's Modified Eagle's Medium [DMEM] with 20% fetal bovine serum). Cells were plated onto collagen I-coated flasks for 1 hour (preplate 1), and 2 hours (preplate 2), followed by sequential daily transfers of nonadherent cells and replatings for 2 to 6 days, until preplate 6. The latter is the cell population containing MDSC. Cells were maintained in GM-20 on regular culture flasks (no coating) and used in the 15th to the 25th passage, because mouse MDSC have been maintained in our laboratory for at least 40 generations with the same, or even increasing, growth rate, thus confirming their stem cell nature. The absence of SMC was verified at the initial passages by immunocytochemistry and Western blot for α -smooth muscle cell actin (ASMA) (see below).

Muscle-derived stem cells were grown also in two types of scaffolds: 1) porcine small intestinal submucosa (SIS, Cook Biotech Inc., West Lafayette, IN),²¹ and 2) polymeric mesh (Vicryl mesh, Ethicon Inc., Somerville, NJ).²² Both materials were cut in 0.5 cm² fragments, sterilized in alcohol and submerged in DMEM/20% fetal bovine serum. Muscle-derived stem cells were then seeded and allowed to grow until they reached more than 60% confluence on the SIS scaffold or they started to cover the small holes in the mesh of the polymeric scaffold.

Retired breeders female Fisher 344 rats (Harlan Sprague-Dawley Inc., San Diego, CA), were used. The animals were treated according to National Institutes of Health regulations with the LABioMed Institutional Animal Care and Use Committee–approved protocol. Anesthetized rats were divided in the following groups (n = 2/group/time period): 1) “intact controls,” not subjected to surgery; 2) “defected vagina, untreated,” subjected to hysterectomy and a partial vaginectomy to induce apical, anterior, and posterior defects. The vagina was closed with absorbable sutures; 3) “defected vagina, treated with SIS implantation,” as number 2, but implanted at the moment of surgery with the SIS scaffold. The SIS scaffold was sutured into the vaginal stump with absorbable sutures. Two nonabsorbable sutures (nylon) were used to mark the ends of the scaffold; 4) “defected vagina, treated with SIS/MDSC implantation,” as number 2, but implanted at the moment of surgery with the SIS scaffold that had been seeded with MDSC. In this case, the cells were grown in vitro and labeled with either the nuclear blue fluorescent stain 4',6-Diamidino-2-Phenylindole (DAPI), or with DAPI and the membrane/cytoplasmic red fluorescent stain PKH26, as detailed in each case. Tacrolimus was given daily to group 4 (1 mg/kg, subcutaneously) to avoid immunorejection of the mouse stem cells.⁹ At 2, 4, or 8 weeks after implantation (and in some cases at 6 weeks), rats were killed under anesthesia, the vagina was excised and divided in approximately 2–3-mm transversal sections numbered from the more distal from 1 to 6 (according to the overall length of the vagina). Sections were cryoprotected in 25% sucrose, immersed in optimal cutting temperature compound, and subjected to cryosectioning (5 micrometers for regular microscope, or 20–30 micrometers for confocal microscope) without fixation, unless stated.

The general morphology of vaginal frozen tissue sections was evaluated with hematoxylin-eosin. For immunocytochemistry, cells grown in triplicate on collagen-coated 8-well removable chambers, or in frozen tissue sections, were reacted^{9,23} with some of the primary antibodies against 1) human ASMA (mouse monoclonal in Sigma kit, 1/2, Sigma Chemical, St. Louis, MO) a marker for both SMC and myofibroblasts, or 2) Oct-4 (rabbit polyclonal, 1/500, BioVision, Mountain View, CA), a marker for embryonic stem cells; in this case frozen sections were fixed with 2% formaldehyde for 10 minutes. Immunohistochemical detection was performed by quenching in 0.3% H₂O₂-phosphate buffered saline, blocking with goat or corresponding serum, and incubating overnight at 4°C with the primary antibody. This was followed by biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA), respectively, for 30 minutes, the avidin-biotin-peroxidase complex containing avidin-linked horse radish peroxidase (1:100; Vector Laboratories), 3,3' diaminobenzidine, and counterstaining with hematoxylin, or no counterstaining, as indicated.

When DAPI and/or PKH26 fluorescence was detected, immunofluorescence was applied for ASMA (same antibody as above) or keratin 5, a squamous epithelial marker²⁴ (Monoclonal, 1/200, Vector Laboratories) using a secondary anti-mouse IgG antibody that was biotinylated (goat, 1/200, Vector Laboratories), and this complex was detected with streptavidin-Texas Red (red fluorescence), or in the case of Oct-4, also with streptavidin-FITC (green fluorescence). After washing with phosphate buffered saline, the sections were mounted with Prolong antifade (Molecular Probes, Carlsbad, CA, USA). Negative controls

for immunohistochemistry or immunofluorescence omitted the first antibodies or they were replaced by IgG isotype. Sections were viewed under an Olympus BH2 fluorescent microscope or in a confocal microscope, using regular light or blue and/or red filters and overlay.

Masson trichrome staining²³ was applied to tissue sections fixed overnight in Bouin's fixative (Sigma). For quantitative image analysis, staining intensity was determined by computerized densitometry using the ImagePro Plus 5.1 program (Media Cybernetics, Silver Spring, MD), coupled to the Olympus BH2 microscope with a Spot RT color digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). Results were expressed as the ratio between the areas stained in red comprising the epithelium, lamina propria, and muscularis, divided by the area occupied by collagen fibers and extracellular matrix stained in blue. Ten nonoverlapping fields were screened per section. Three sections per tissue specimen from the groups of two rats were then used to calculate the mean $\pm$ standard error of the mean, based on 30 separate measurements per rat.

Cell homogenates were obtained in boiling lysis buffer (1% SDS, 1 mm sodium orthovanadate, 10 mm Tris pH 7.4 and protease inhibitors), and centrifuging at 16,000 g for 5 minutes.^{9,23} Forty micrograms of protein were run on 7.5% or 10% polyacrylamide gels, and submitted to transfer and immunodetection with antibodies against 1) human ASMA (monoclonal, 1/1,000, Calbiochem, LA Jolla, CA); 2) human calponin-1 (basic) (mouse monoclonal, 1/25, Novocastra, Burlingame, CA), as exclusive marker for SMC; 3) proliferating cellular nuclear antigen (PCNA) (mouse monoclonal, 1:100, Chemicon, Temecula, CA) a marker for replicating cells; 4) Oct-4 (rabbit polyclonal, 1/500, BioVision, Mountain View, CA), as stem cell marker; 5) myoglobin (rabbit polyclonal 1/200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), as muscle origin marker; and 6) GAPDH (mouse monoclonal, 1/3,000, Chemicon, Temecula, CA) as a housekeeping gene.

Membranes were incubated with a secondary polyclonal horse anti-mouse IgG linked to horseradish peroxidase (1:2,000; BD Transduction Laboratories, Franklin Lakes, NJ, or 1:5,000, Amersham GE, Pittsburgh, PA), and bands were visualized with luminol (SuperSignal West Pico, Chemiluminescent, Pierce, Rockford, IL). For the negative controls, the primary antibody was omitted.

Pools of total cellular RNA from three T75 flasks for MDSC that were incubated with DMEM supplemented with fetal bovine serum at either 20% ("high serum") or 2.5% ("low serum") were isolated with Trizol-Reagent (Invitrogen, Carlsbad, CA). Quality of RNA was assessed by agarose gel electrophoresis and subjected to cDNA gene microarrays (SuperArray BioScience Corp., Frederick, MD),²⁵ using the following Oligo GEArray microarrays: 1) mouse stem cell (OMM-405); 2) mouse cell surface markers (OMM-055); and 3) mouse cardiovascular disease biomarkers (MM-037), which together covered potential markers for MDSC. Biotin-labeled cDNA probes were synthesized from total RNA, denatured, and hybridized overnight at 60°C in GEHybridization solution to these membranes. Chemiluminescent analysis was performed per the manufacturer's instructions. Raw data were analyzed using GEArray Expression Analysis Suite (SuperArray BioScience Corp., Frederick, MD). Expression values for each gene based on spot intensity were subjected to background correction and normalization with housekeeping genes.

Equal amounts (2 micrograms) of RNA were reverse-transcribed in duplicate using a RNA polymerase chain reaction (PCR) kit (Applied Biosystems, Foster City, CA). Forward/reverse PCR primers for real-time PCR for smoothelin and GAPDH are as follows: nt 575–597/626–641 on BC074818.2 (67bp) and 606–626/758–738 on BC023196. Mouse gene PCR primer sets (RT2) were purchased from SuperArray Bioscience. The QIAGEN Sybr

Green PCR kit with HotStar *Taq*DNA polymerase (QIAGEN, Valencia, CA) was used with i-Cycler PCR thermocycler and fluorescent detector lid (Bio-Rad, Hercules, CA).²⁵

Melting was for 15 minutes at 95°C, followed by 40 cycles of three-step PCR, including melting for 15 seconds at 95°C, annealing for 30 seconds at 58°C, elongation for 30 seconds at 72°C, with an additional detection step of 15 seconds at 81°C and a melting curve from 55–95°C (at 0.5°C per 10 seconds).²⁶ Inverse derivatives of melting curves showed sharp peaks for smoothelin and GAPDH at 81°C and 85°C indicating correct products. We analyzed cDNAs (25 ng) in quadruplicate in parallel with GAPDH controls; standard curves (threshold cycle compared with log pg cDNA) were generated by log dilutions of from 0.1 pg to 100 ng standard cDNA (reverse-transcribed mRNA from MDSC). The mRNA starting quantities were calculated from the standard curves and averaged using i-Cycler, iQ software. The ratios of smoothelin mRNA to GAPDH mRNA were computed.

For regular real-time PCR (25), 2 micrograms of RNA were reverse-transcribed, and cDNA was amplified for 38 cycles by PCR at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute. Polymerase chain reaction products were analyzed in 2% agarose. Forward/reverse PCR primers in 5′-3′ nucleotide positions were as follows: 1) Oct-4 (GenBank Accession No. NM_013633), forward: nt 830–850; and reverse: nt 1130–1150; 2) nanog (GenBank Accession No. XM_001471588) forward: nt 732–852; and reverse: nt 1032–1152; and 3) CD63 (GenBank Accession No. NM_001042580) forward: nt 478–898; and reverse: nt 778–798, 4) GAPDH (GenBank Accession No. BC059110) forward: nt 611–631; and reverse: nt 743–763.

Masson trichrome values are expressed as the mean $\pm$ standard error of the mean for 30 independent measurements per rat and 60 determinations per group. The normal distribution of the data was established using the Wilks-Shapiro test. Multiple comparisons were analyzed by a single-factor analysis of variance, followed by post-hoc comparisons with the Newman-Keuls test. Differences among groups were considered statistically significant at $P < .05$.

RESULTS

To show with specific differentiation markers that MDSC are indeed able to originate SMC *in vitro*, they were incubated for various periods of time (7–56 days) in DMEM with 20% fetal bovine serum. Figure 1A shows by immunocytochemistry that as early as 9 days, some of the cells express ASMA, a marker for both myofibroblasts and SMC, and that TGF β 1 (Fig. 1B) intensifies this expression. Successive periods showed similar results (not shown), and this was confirmed at 22 days by Western blot (Fig. 1C). The SMC generation was indicated with an antibody for the SMC marker calponin, not expressed in myofibroblasts, although in this case the increase of the intensity of the TGF β 1 band corrected by GAPDH was only moderate. The cells were actively replicating as shown by proliferating cell nuclear antigen (PCNA). The levels of smoothelin mRNA, another specific SMC marker, were elevated by TGF β 1, and this effect continued until at least 49 days, as shown by real-time PCR (Fig. 1D).

The MDSC may grow on scaffolds and differentiate into the desired lineage, as shown by hematoxylin-eosin staining on an epi-illumination stereomicroscope observation of the monolayer formed on the small intestinal submucosa (SIS) membrane (Fig. 1E). Because it is difficult to perform fluorescence or immunocytochemical observations on the totally opaque SIS scaffold, we opted to grow the MDSC prelabeled with the nuclear fluorescent stain DAPI onto a translucent polymeric scaffold (Vicryl mesh) and determine whether the cells transform into SMC. The MDSC rapidly proliferated onto the scaffold (Fig. 1F) and

presumably originated SMC, as detected by dual immunofluorescence for DAPI and ASMA (Fig. 1G).

Labeling with DAPI is relatively short-lived, and therefore we searched for potential markers of these cells that would not be expressed in the vagina. First, DNA microarrays representing several stem cell and related genes, as well as tissue markers, were applied to identify RNA transcripts in MDSC that were grown under either high serum (20% fetal bovine serum) to stimulate cell proliferation or low serum (2.5% fetal bovine serum) to arrest cell growth and to stimulate cell differentiation (Fig. 2A and B). Some embryonic stem cell mRNAs (nanog, Oct-4, CD63, Wnt1) and skeletal muscle or myogenic markers (myoglobin, muscle creatin kinase, notch 3) were identified, and the approximate intensity of expression was tabulated (Fig. 2C).

Real-time PCR for two of those genes, nanog and Oct-4, revealed a low expression in MDSC, as well as in the vaginal tissue, whereas CD63 was negligible in MDSC but well-expressed in the vagina. The Oct-4 and nanog were considerably expressed in the testis, as a source of germinal stem cells, and particularly in the case of nanog in the penis, as another urogenital organ. In the skeletal muscle (hind limb), where MDSC originated, Oct-4 expression was also high, whereas nanog was negligible (Fig. 3A and C, available online at <http://links.lww.com/A1488>). Western blotting with an Oct-4 antibody revealed a strong band in the MDSC that runs as Oct-3B and a very faint one as Oct-3A, whereas both bands were expressed similarly at high levels in the vagina, the skeletal muscle, and the penis. (Fig. 3B, available online at <http://links.lww.com/A1488>). Myoglobin, a skeletal muscle marker, was expressed in MDSC and the vagina, and as expected, very highly in the skeletal muscle, but not in the penis. The myoglobin band in the vaginal tissue possibly results from contamination with adjacent skeletal muscle tissue. Therefore, Oct-4, because of being an embryonic stem cell marker, was used to locate potential endogenous stem cells in the vagina, whereas due to its low expression in the MDSC, it was not useful as a tag to follow MDSC implantation in this tissue.

To study the effects of implanted MDSC on vaginal reconstruction, rats were either left intact and untreated, or were subjected to hysterectomy and partial vaginectomy to create the apical, anterior, and posterior vaginal defects. Muscle-derived stem cells grown on SIS were labeled with DAPI and implanted on the vagina at the site of vaginal defects, whereas other animal groups were implanted with SIS without cells or left untreated and killed at 2, 4, 6, and 8 weeks (n = 2/group). This time course was selected to follow up qualitatively in a preliminary “proof of concept” cell uptake, survival, differentiation, and effects on histology at several standard periods used in studies of stem cell implantation. The vaginal tissues were divided in regions numbered 1 through 6 from the one most distant to the site of implantation. The DAPI labeled MDSC on the SIS scaffolds were visualized at 4 weeks as numerous blue fluorescent nuclei in frozen unfixed tissue sections of the tissue around the site of implantation (regions 5 and 6), as shown on Figure 4A and B. These sections were immunostained with an antibody for ASMA and a red fluorescence Texas red–tagged secondary antibody that shows, in a merge of the blue and red fluorescence, that many of the implanted cells were positive for ASMA expression. Some few MDSC migrated away from the SIS implants to more distant regions (1 and 3), showing also differentiation into SMC (Fig 4C and D). Muscle-derived stem cells growing on SIS were also dually labeled with DAPI and a membrane red fluorescence tag, PKH26, and then implanted in the vagina as above. At 6 weeks (not shown) DAPI labeling became more diffuse and restricted to a small area of the field, whereas red fluorescence is still apparent and even advanced into the nuclei. This indicates that the MDSC survive for at least 6 weeks.

The other marker identified in the in vitro experiments for MDSC, the Oct-4 gene, detects the endogenous vaginal stem cells and the implanted MDSC. The vaginal tissue from intact rats (no surgery) within a region equivalent to the site of MDSC implantation displays some discrete staining in isolated regions of the muscularis, apparently in the longitudinal bundles, as well as in the stratified squamous epithelium (not shown). Staining is rather similar in hysterectomized animals with partial vaginectomy after 4 weeks of surgery and no treatment (Fig. 5A–C, available online at <http://links.lww.com/A1489>). Four weeks after implantation of SIS only without MDSC, there was a more extended staining, with some spreading to both longitudinal and transversal bundles in the muscularis (Fig. 5D–F, available online at <http://links.lww.com/A1489>). This is likely due to “activation” of endogenous stem cell replication by the combined SIS implant/tissue repair reaction. The SIS/MDSC intensified these changes with even more dissemination of Oct-4+ cells, particularly in a thicker epithelium, suggesting that many of them are derived from the implanted MDSC and others from endogenous stem cell activation (Fig. 5G–I, available online at <http://links.lww.com/A1489>).

The stimulatory effects of MDSC/SIS on the regeneration of vaginal tissue are seen clearly 4 weeks after surgery on hematoxylin-eosin staining of the vaginal tissue sections, where there is an apparent increase in the muscularis after receiving the implant (Fig. 6C and D) as compared with the intact vagina (no surgery) (Fig. 6A) or the untreated hysterectomized/partially vaginectomized tissue (Fig. 6B). What is remarkable is the considerably stratified squamous epithelium that developed upon treatment with SIS and particularly with the MDSC on the SIS (Fig. 6C and D).

The stimulatory effect of MDSC on the growth of the epithelium was confirmed by immunohistodetection with Texas red fluorescence of keratin 5, a marker of squamous epithelium, compared with the respective hematoxylin-eosin staining at 4 weeks (Fig. 7). In this case, DAPI was applied after the sections were obtained as a nuclear fluorescent counterstain, not to detect the implanted MDSC. The untreated vagina from hysterectomized rats showed a disorganized and rather thin epithelium, with virtually no indentation with connective tissue papillae (Fig. 7B). Implantation of SIS without cells restored some papillae and made the epithelium thicker (Fig. 7D), but only the SIS holding the MDSC that normalized the epithelium thickness and appearance (Fig. 7F). To determine whether the MDSC themselves differentiated into epithelial cells, sections obtained from the vagina of hysterectomized/partially vaginectomized rats where the vagina had been implanted with red fluorescence PKH26-labeled MDSC growing on SIS were stained for keratin 5 but using FITC green fluorescence, and no MDSC staining was applied (Fig. 7A, C, E). Although red fluorescent implanted cells were visible in the vicinity of the green fluorescent keratin-5 positive area, there was no convincing overlapping in the overlay (not shown) that would suggest a direct conversion, thus implying that the MDSC exert a trophic effect on the epithelium more than a true differentiation.

Masson trichrome staining was applied to determine the extent of fibrosis in the tissue sections upon the different treatments at 8 weeks. Figure 8A shows in the vagina from intact animals not subjected to surgery the layer of longitudinal smooth muscle bundles in the muscularis underneath the lamina propria and the stratified squamous epithelium, stained in red, whereas virtually all the lamina propria and interstitial connective tissue in the smooth muscle is stained in blue, indicating collagen fibers. The tissue from hysterectomized and partially vaginectomized animals had a thinner but organized layer of smooth muscle (Fig. 8B). In contrast, after SIS implantation (Fig. 8C), there was very little smooth muscle and a thin epithelium, as if the scaffold would induce mainly connective tissue formation, probably from fibrosis and scar tissue formation. This may also be due, in part, to the wound healing process caused by surgery and regeneration process at longer time points. However,

when SIS with MDSC was implanted (Fig. 8D), there was a remarkably healthy smooth muscle and thicker epithelium. These results suggest that the use of MDSC on SIS scaffold regenerated vaginal smooth muscle and epithelium much faster than SIS alone. Quantitative image analysis (Fig. 8E) of the ratio between the red compared with blue areas, as a representation of the cellular/extracellular matrix ratio, was applied to 30 fields per rat. This analysis, limited by a small n imposed by the study design, indicated that MDSC seeded on the SIS generated a significantly higher amount of cellular components.

DISCUSSION

Based on a PubMed search (1980 to March 2009; key words: vagina, stem cells; all languages), this is the first article on the use of stem cells of any origin to repair defects or injury to the vagina in women or in animal models. We have shown that MDSC mounted on a biodegradable SIS scaffold implanted in the defected vagina (apical, anterior, and posterior defects) of rats survive for extended periods, differentiate into SMC in the muscularis, and promote epithelium regeneration, presumably by a trophic effect. This exploratory “proof of concept” approach was supported by finding a reduction in fibrosis and stimulation of the vaginal repair process, which seems to occur more efficiently than with SIS implants alone without cells or in the spontaneous regeneration occurring in the absence of any treatment. The current study establishes the foundation for future experimental paradigms to evaluate more precisely with a larger number of animals the effects of MDSC/SIS on vaginal tissue fibrosis at even more prolonged periods after implantation. Although we did not perform the functional study of the vaginal defects and this surgically injured vagina model is not the same as pelvic organ prolapse that has developed with time, our results show that implanted MDSC in the surgically injured vagina can survive, differentiate, and regenerate/repair the injured vagina.

In addition, we have defined the expression in MDSC of certain genes, such as Oct-4, that is a well-recognized marker of embryonic stem cells. This gene served also to identify potential endogenous stem cells in the tissue that may constitute a target for repair therapy based on their potential activation by pharmacologic interventions. Muscle-derived stem cells per se, without scaffolds, were not tested, because we considered that the physical restriction imposed by the SIS support would on one side facilitate closure of the wound and on the other side help to maintain the implanted cells around the site of repair.

One of the main limitations of the current work is that it was difficult to assess quantitatively the extent of the putative improvement of vaginal regeneration by MDSC/SIS over SIS alone or in the absence of treatment, because with the exception of the effect on fibrosis, we have based our interpretation on qualitative comparisons between vaginal tissues from each experimental group. This was prompted by the difficulty of defining markers that single-handedly or in combination represent the complexity of the coordinated repair of the different cellular compartments in the vagina and their histologic and anatomic arrangement to restore the normal tissue function. In the case of the correction of the well-defined SMC loss in the penile corpora cavernosa occurring during aging, we employed ASMA estimated by quantitative Western blot,⁹ but for vaginal repair, the possibility that ASMA may also indicate generation of myofibroblasts involved in a fibrotic process²³ may complicate the interpretation. In addition, the balance between the muscularis and the squamous epithelium and lamina propria may not be well-represented by isolated markers for each tissue.

Within these constraints, the in situ estimation in tissue sections of the cellular compartment in relation to collagen and extracellular matrix is a reasonable approximation to evaluate the efficacy of MDSC/SIS, because it measures the functionally desirable restoration of the SMC/keratinocytes content compared with the noxious complication of excessive collagen

deposition, namely fibrosis. A caveat is that myofibroblasts, the main cell type in fibrosis,²⁷ may also be counted among the red staining area. However, the main reason for our experimental design was that as first stage, this work aimed to define the proof of concept of implanted cells surviving in the injured vagina, converting into one of the desired cell types (SMC), and stimulating the formation of the epithelium, while maintaining the proper histologic appearance. Further studies will be conducted to identify the most suitable markers for quantitative Western blots to provide a precise evaluation of the extent of repair. This would facilitate determination of the role of variables such as multiple compared with single injection sites, cell loads, pharmacologic adjuvants, influence of scaffold types, or ranges of persistence of implanted MDSC on the relative efficacy of each treatment and particularly on vaginal function.

Although in the present work we employed immunosuppression with tacrolimus because of the interspecies implantation we used (mouse MDSC into rat vagina), we envisage that this will not be needed in further animal studies or in clinical applications. First, in both cases, allografts, eg, rat MDSC donor in rat recipient and human MDSC in women, may be used, because stem cells are known to be considerably hypoimmunogenic as compared with differentiated cells.²⁸ Moreover, in the case of humans, autografts can easily be obtained from biopsies of the skeletal muscle that in a few weeks can generate cell cultures for reimplantation.¹³ An even less invasive approach would be based on pharmacologic awakening of potential dormant endogenous stem cells from vaginal tissues similar to the Oct-4 cells identified here, which would be the ultimate goal of stem cell therapy.²⁷ The endogenous stem cells, which in some cases may be rudimentary embryonic stem cells,^{18,19} are being increasingly identified in adult tissues as populations different from other stem cells already characterized, and in the vagina they may even be paracrinely stimulated by the implanted MDSC. In conclusion, MDSC seeded onto degradable scaffolds may constitute a promising approach for the treatment of vaginal prolapse.

Acknowledgments

Supported by an internal grant from the Division of Female Pelvic Medicine and Reconstructive Surgery of Harbor-UCLA Medical Center, University of California at Los Angeles (NGC/MH), and in part by grants NIH R21DK-070003 (NGC), DOD W81XWH-07-1-0181 (NGC), NIH 5SC2DK082388-02 MH), the Dennis W. Jahnigen Career Development Scholars Award from American Geriatrics Association (MH), the Astellas Award (MH), the June Allyson Foundation Award from the American Urogynecologic Society (MH), and the Research Grant from International Urogynecological Association (MH).

REFERENCES

1. South M, Amundsen CL. Pelvic organ prolapse: a review of the current literature. *Minerva Ginecol.* 2007; 59:601–612. [PubMed: 18043574]
2. Maher C, Baessler K, Glazener CM, Adams EJ, Hagen S. Surgical management of pelvic organ prolapse in women: a short version Cochrane review. *Neurourol Urodyn.* 2008; 27:3–12. [PubMed: 18092333]
3. Nguyen JN. The use of grafts for anterior vaginal prolapse repair: pros and cons. *Curr Opin Obstet Gynecol.* 2008; 20:501–505. [PubMed: 18797276]
4. Jia X, Glazener C, Mowatt G, MacLennan G, Bain C, Fraser C, et al. Efficacy and safety of using mesh or grafts in surgery for anterior and/or posterior vaginal wall prolapse: systematic review and meta-analysis. *BJOG.* 2008; 115:1350–1361. [PubMed: 18715243]
5. Bako A, Dhar R. Review of synthetic mesh-related complications in pelvic floor reconstructive surgery. *Int Urogynecol J Pelvic Floor Dysfunct.* 2008; 20:103–111. [PubMed: 18779916]
6. Huffaker RK, Muir TW, Rao A, Baumann SS, Kuehl TJ, Pierce LM. Histologic response of porcine collagen-coated and uncoated polypropylene grafts in a rabbit vagina model. *Am J Obstet Gynecol.* 2008; 198:582.e1–582.e7. [PubMed: 18295174]

7. Smaldone MC, Chancellor MB. Muscle derived stem cell therapy for stress urinary incontinence. *World J Urol.* 2008; 26:327–332. [PubMed: 18470515]
8. Bivalacqua TJ, Deng W, Kendirci M, Usta MF, Robinson C, Taylor BK, et al. Mesenchymal stem cells alone or ex vivo gene modified with endothelial nitric oxide synthase reverse age-associated erectile dysfunction. *Am J Physiol Heart Circ Physiol.* 2007; 292:H1278–H1290. [PubMed: 17071732]
9. Nolzco G, Kovanecz I, Vernet D, Gelfand RA, Tsao J, Ferrini MG, et al. Effect of muscle-derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. *BJU Int.* 2008; 101:1156–1164. [PubMed: 18294308]
10. Blum B, Benvenisty N. The tumorigenicity of human embryonic stem cells. *Adv Cancer Res.* 2008; 100:133–158. [PubMed: 18620095]
11. Grinnemo KH, Sylvién C, Hovatta O, Dellgren G, Corbascio M. Immunogenicity of human embryonic stem cells. *Cell Tissue Res.* 2008; 331:67–78. [PubMed: 17846795]
12. De Philippo RE, Bishop CE, Filho LF, Yoo JJ, Atala A. Tissue engineering a complete vaginal replacement from a small biopsy of autologous tissue. *Transplantation.* 2008; 86:208–214. [PubMed: 18645481]
13. Urish K, Kanda Y, Huard J. Initial failure in myoblast transplantation therapy has led the way toward the isolation of muscle stem cells: potential for tissue regeneration. *Curr Top Dev Biol.* 2005; 68:263–280. [PubMed: 16125002]
14. Zheng B, Cao B, Crisan M, Sun B, Li G, Logar A, et al. Prospective identification of myogenic endothelial cells in human skeletal muscle. *Nat Biotechnol.* 2007; 25:1025–1034. [PubMed: 17767154]
15. Mitterberger M, Marksteiner R, Margreiter E, Pinggera GM, Frauscher F, Ulmer H, et al. Myoblast and fibroblast therapy for post-prostatectomy urinary incontinence: 1-year followup of 63 patients. *J Urol.* 2008; 179:226–231. [PubMed: 18001790]
16. Vernet D, Qian A, Nolzco G, Cantini L, Magee TR, Ferrini MG, et al. Evidence that osteogenic progenitor cells in the human tunica albuginea may originate from stem cells. Implications for Peyronie's disease. *Biol Reprod.* 2005; 73:1199–1210. [PubMed: 16093362]
17. Virant-Klun I, Zech N, Rozman P, Vogler A, Cvjeticanin B, Klemenc P, et al. Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes. *Differentiation.* 2008; 76:843–856. [PubMed: 18452550]
18. Zovoilis A, Nolte J, Drusenheimer N, Zechner U, Hada H, Guan K, et al. Multipotent adult germline stem cells and embryonic stem cells have similar microRNA profiles. *Mol Hum Reprod.* 2008; 14:521–529. [PubMed: 18697907]
19. Ratajczak MZ, Zuba-Surma EK, Shin DM, Ratajczak J, Kucia M. Very small embryonic-like (VSEL) stem cells in adult organs and their potential role in rejuvenation of tissues and longevity. *Exp Gerontol.* 2008; 43:1009–1017. [PubMed: 18601995]
20. Lee JY, Qu-Petersen Z, Cao B, Kimura S, Jankowski R, Cummins J, et al. Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. *J Cell Biol.* 2000; 150:1085–1100. [PubMed: 10973997]
21. Chaliha C, Khalid U, Campagna L, Digesu GA, Ajay B, Khullar V. SIS graft for anterior vaginal wall prolapse repair—a case-controlled study. *Int Urogynecol J Pelvic Floor Dysfunct.* 2006; 17:492–497. [PubMed: 16733627]
22. Allahdin S, Glazener C, Bain C. A randomised controlled trial evaluating the use of polyglactin mesh, polydioxanone and polyglactin sutures for pelvic organ prolapse surgery. *J Obstet Gynaecol.* 2008; 28:427–431. [PubMed: 18604681]
23. Cantini LP, Ferrini MG, Vernet D, Magee TR, Qian A, Gelfand RA, et al. Profibrotic role of myostatin in Peyronie's disease. *J Sex Med.* 2008; 5:1607–1622. [PubMed: 18422491]
24. Lloyd C, Yu QC, Cheng J, Turksen K, Degenstein L, Hutton E, et al. The basal keratin network of stratified squamous epithelia: defining K15 function in the absence of K14. *J Cell Biol.* 1995; 129:1329–1344. [PubMed: 7539810]
25. Artaza JN, Singh R, Ferrini MG, Braga M, Tsao J, Gonzalez-Cadavid NF. Myostatin promotes a fibrotic phenotypic switch in multipotent C3H 10T1/2 cells without affecting their differentiation into myofibroblasts. *J Endocrinol.* 2008; 196:235–249. [PubMed: 18252947]

26. Magee TR, Kovanecz I, Davila HH, Ferrini MG, Cantini L, Vernet D, et al. Antisense and short hairpin RNA (shRNA) constructs targeting PIN (Protein Inhibitor of NOS) ameliorate aging-related erectile dysfunction in the rat. *J Sex Med.* 2007; 4:633–643. [PubMed: 17433082]
27. Romagnani P, Lasagni L, Mazzinghi B, Lazzeri E, Romagnani S. Pharmacological modulation of stem cell function. *Curr Med Chem.* 2007; 14:1129–1139. [PubMed: 17456026]
28. Le Blanc K, Ringdén O. Immunomodulation by mesenchymal stem cells and clinical experience. *J Intern Med.* 2007; 262:509–525. [PubMed: 17949362]

Fig. 1.

Skeletal muscle-derived stem cells (MDSC) grow in vitro on biological and synthetic scaffolds and differentiate into cells that express smooth muscle cells markers. MDSC were incubated in triplicate for up to 56 days on 6-well plates in Dulbecco's Modified Eagle's Medium (DMEM)/2.5% serum and immunostained for α -smooth muscle actin (ASMA). Panels show an early incubation (9 days) at 200X. **A.** Control; no addition made. **B.** Transforming growth factor-beta 1 (TGF- β) added; 5 ng/mL. **C.** Expression of ASMA, smooth muscle cells (SMC) marker (calponin), and cell proliferation marker (proliferating cell nuclear antigen [PCNA]) at 22 days by Western blot. **D.** Expression of messenger RNA (mRNA) for SMC marker smoothelin at 21 and 49 days by reverse transcription/real time polymerase chain reaction. Values are expressed as ratios against the respective control. **E.** MDSC were seeded on small intestinal submucosa (SIS, Cook Biotech Inc., West Lafayette, IN) on 12-well plates in DMEM/2.5% serum (with TGF- β), grown for 2 weeks, stained with hematoxylin, and examined under a reverse phase microscope (100X). **F.** MDSC were seeded on Vicryl polymeric mesh as for SIS, stained with 4',6-diamidino-2-phenylindole, and examined under a fluorescent microscope using blue fluorescence (200X). **G.** Blue/red fluorescent overlay of cells subjected to immunostaining for ASMA with a Texas red secondary antibody (200X). CALP, calponin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; C, control.

Fig. 2. Skeletal muscle-derived stem cells (MDSC) express messenger RNAs (mRNAs) for embryonic stem cell markers and for muscle-specific markers as detected by DNA microarrays. **A.** RNA was isolated from MDSC cultured in duplicate under low (2.5%) or **B.** high (20%) fetal bovine serum for 3 weeks and hybridized against the stem cell superarray panel (OMM-405). **C.** The mRNA levels in this specific experiment were calculated for some embryonic stem cell markers and also for other selected genes identified with two other panels (OMM-37 and OMM-055), just to confirm a given gene expression.

Fig. 4. Skeletal muscle-derived stem cells (MDSC) growing on small intestinal submucosa (SIS) scaffolds differentiate into smooth muscle cells when implanted into injured vaginal tissue in the rat and spread away from the site of injection. Blue/red fluorescence overlay of vaginal tissue implanted with 4',6-diamidino-2-phenylindole (DAPI)-tagged MDSC/SIS after hysterectomy and partial vaginectomy and subjected to α -smooth muscle actin (ASMA) red-fluorescence immunodetection, focusing in the muscularis (200X). Frozen tissue sections (10% formalin-fixed) were obtained at 4 weeks after implantation. Region 5 (**A**) is immediately distal to the site of implantation, and region 6 (**B**) is directly in the site of implantation (suture site around orifice). **C and D.** Blue/red fluorescence overlay of vaginal tissue implanted with DAPI-tagged MDSC/SIS after hysterectomy and partial vaginectomy and subjected to ASMA red-fluorescence immunodetection, focusing in the muscularis (200X) but in more distal sections 1 (**C**) and 3 (**D**).

Fig. 6. Implanted skeletal muscle-derived stem cells (MDSC)/small intestinal submucosa (SIS) induce regeneration of the stratified squamous epithelium in the vagina around the site of implantation. Frozen sections for region 5 (not fixed) were stained with hematoxylin eosin. **A.** Intact controls, 40X. **B.** Hysterectomized rats, 40X; proximal. **C and D.** Hysterectomized rats with MDSC/SIS, 200X, at two areas of the tissue section.

Fig. 7.

The effect exerted by muscle-derived stem cells (MDSCs) on the stratified squamous epithelium is confirmed by immunohistochemistry for keratin 5. **A and B.** Untreated. **C and D.** Small intestinal submucosa (SIS). **E and F.** Small intestinal submucosa/muscle-derived stem cells. Frozen sections for region 5 (not fixed) were reacted against an antibody for keratin 5 that was detected by Texas red immunofluorescence, and nuclei were counterstained with 4',6-diamidino-2-phenylindole (200×) (**A, C, E**). Adjacent sections were stained with hematoxylin-eosin (200×) (**B, D, F**).

Fig. 8. Implantation of small intestinal submucosa (SIS) with skeletal muscle-derived stem cells (MDSC) increases the cellular:extracellular ratio during vaginal regeneration. **A–D.** Representative pictures of frozen tissue sections for region 5 (fixed or not) at 8 weeks that were subjected to Masson-trichrome histochemistry (100X). **A.** Intact. **B.** Hysterectomy untreated. **C.** SIS. **D.** SIS/MDSC. **E.** Quantitative image analysis for 10 fields per specimen, three specimens per rat, and two rats per group.