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Modulation of Stem Cells Differentiation and Myostatin as an
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14. ABSTRACT We have completed in Year 3 the in vitro characterization of the three muscle derived stem cells (MDSC) isolated from the wild type (WT), myostatin knock out (Mst KO), and mdx mice. We had shown in Years 1 and 2 that the in vitro myogenic differentiation and myotube formation by MDSC was refractory to modulation by myostatin, follistatin, myostatin antibodies and shRNA, hormones, and nitric oxide, and specifically that the Mst KO and mdx MDSC were totally unable to form myotubes.. We have now found out: a) that this inability of the Mst KO and mdx MDSC to form myotubes in vitro occurs despite they can differentiate into myofibroblasts and neural and smooth muscle cells, albeit not into adipocytes, b) is not overcome even in dual paracrine and juxtacrine cultures with WT MDSC and with C2C12 myoblasts, and c) is not accompanied by the silencing of stem cell genes, like Sca 1 and Oct-4. MDSC from a myostatin+/dystrophin+ transgenic mouse expressing gfp under the Oct-4 promoter, morphologically resemble very small embryonic-like (VSEL) stem cells. Although the three MDSC cultures showed similar levels of expression for 98% of the 260 stem cell and myogenic genes assayed, the genetic inactivation of myostatin or dystrophin was associated with a dramatic down-regulation of Actc1, Acta1, Spp1, MyoD, and notch 2, critical genes for the onset of myogenesis. We also have defined novel antifibrotic strategies to apply concurrently with MDSC implantation into mdx mice during Year 4. The in vivo studies to determine the capacity of WT MDSC alone to repair notexin-injured diaphragm and gastrocnemius in old mdx mice are now ongoing					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusion.....	9
References.....	10
Appendices.....	12

Introduction

The **overall objective** of this whole grant is:

To investigate in the mdx mouse a novel therapeutic approach for Duchenne's muscular dystrophy (**DMD**) based on the inhibition of myostatin (**Mst**) expression and/or activity, for the alleviation of fibrotic and fatty degeneration of the muscle, that would also facilitate the differentiation of transplanted dystrophin+ (**D+**) muscle-derived stem cells (**MDSC**), in order to ameliorate disease progression.

This will be achieved by: a) comparing the in vitro myogenic and fibrogenic/adipogenic potential of MDSC from D-/Mst+, D+/Mst+ or D+/Mst- mice; b) blocking myostatin expression by gene transfer of myostatin short hairpin RNA (Mst shRNA), or transplantation of D+ MDSC engineered with Mst shRNA, and measuring the myogenic/fibro-adipogenic balance, dystrophin expression, and muscle function; and c) combining this with the inhibition of myostatin activity by follistatin.

The specific objectives to be fulfilled in Years 2 and 3 were:

Year 2: Task 3 and part of 4. Milestones: A) association of myostatin/follistatin expression in vivo with the fibro-adipogenic degeneration in mdx muscle with and without injury; B) start effects on mdx muscle repair by Mst siRNA and stem cells programmed or not with this construct;.

Year 3: Part of task 4, and part of 5: Milestones: A) Complete Task 4; B) determination of the effects on the mdx gastrocnemius contractile function by selected approaches.

Body. Description of research accomplishments

The initiation of the proposed in vivo studies in the mdx mice during Year 3 was partially achieved, because the project had to focus in finding out the reasons for a series of unexpected in vitro results with the modulation of MDSC myogenesis. We had shown in Years 1 and 2 that in vitro myogenic differentiation and myotube formation by MDSC were refractory to modulation by myostatin, follistatin, myostatin antibodies and shRNA, hormones, and nitric oxide, and specifically that the Mst KO and mdx MDSC were totally unable to form myotubes.

We have now found out that this inability of the Mst KO and mdx MDSC to form myotubes in vitro occurs despite they can differentiate into myofibroblasts and neural and smooth muscle cells (albeit not into adipocytes), is not overcome even in dual paracrine and juxtacrine cultures with WT MDSC and with C2C12 myoblasts, and is not connected to silencing of stem cell genes like Sca 1 and Oct-4. We characterized the MDSC isolated from a myostatin+/dystrophin+ transgenic mouse expressing gfp under the Oct-4 promoter, and showed that they morphologically resemble very small embryonic-like (**VSEL**) stem cells. Although the three MDSC cultures had similar levels of expression for 98% of the 260 stem cell and myogenic genes assayed, the genetic inactivation of myostatin or dystrophin was associated with a dramatic down-regulation of Actc1, Acta1, Spp1, MyoD, and notch 2, critical genes for the onset of myogenesis. We also defined novel antifibrotic strategies to apply concurrently with MDSC implantation in mdx mice during Year 4. The in vivo studies to determine the capacity of WT MDSC alone to repair notexin-injured diaphragm and gastrocnemius in old mdx mice are ongoing.

The essential details for the in vitro studies are presented in the scientific communication B-1 and the paper A-1 (see appendix) whose abstracts are reproduced below

B-1. Gonzalez-Cadavid NF, Tsao J, Vernet D, Gelfand R, Nolzco G (2009) Modulation of cell lineage commitment by skeletal muscle derived stem cells, MDSC, from mdx and myostatin knockout mice. Military Health Research Forum 2009, Kansas City,

Background. Muscle lipofibrotic degeneration characterizes Duchenne muscular dystrophy (DMD), hampers cell therapy in the muscle, and is a feasible therapeutic target. Myostatin (Mst), a negative regulator of muscle mass, is antimyogenic and stimulates fibrogenic and adipogenic differentiation of stem cells. Inhibiting myostatin in the DMD model, the mdx mouse, improves myogenesis and reduces fibrosis.

Goals. To investigate in the mdx mouse a novel therapy for DMD based on the inhibition of myostatin, for the alleviation of muscle lipofibrotic degeneration, and stimulation of myogenesis by implanted muscle-derived stem cells from wild type mice (Wt MDSC). This is achieved by: a) comparing the in vitro myogenic and fibrogenic/adipogenic potential of Wt, Mst ko, and mdx MDSC; b) blocking myostatin by follistatin, myostatin short hairpin RNA (Mst shRNA), or implantation of MDSC engineered with Mst shRNA, and measuring the myogenic/fibro-adipogenic balance and muscle function.

Brief description of methodologies. MDSC isolated from the three mouse strains (pP6 pre-plate fraction) were tested for myogenesis, fibrogenesis, and adipogenesis, in the presence of anti-myostatin antibody, follistatin, or myostatin, or by transfection with AdV-Mst shRNA or AdV-Mst cDNA, comparing with TGF β 1, T3, and a nitric oxide donor. Wt MDSC were implanted into various tissues in the rat to assess their tissue repair capacity. MDSC isolated from a transgenic mouse expressing gfp under the embryonic Oct-4 gene promoter were implanted into mdx and Wt mice. Tissue fibrosis and cell death in intact and injured mdx muscles are being studied at several periods. Cell markers were compared by quantitative immunocytochemistry dual fluorescence, and/or quantitative western blot and/or real time RT/PCR.

Results to date. Non-confluent Wt pP6 underwent fibrogenic, osteogenic, cardio-myogenic, and SMC differentiation from early passages, but skeletal myotubes were only detected in confluent cultures from passages 10 through 30, when pP6 started to lose this differentiation ability. However, they converted into other lineages for over 50 passages, but did not undergo adipogenesis. In contrast, confluent Mst ko and mdx pP6 were unable to generate myotubes, and the same occurred with pP1-pP5 fractions in all mouse strains. pP6 myogenesis could not be modulated by any of the tested factors, with the exception of testosterone and SNAP, even if TGF β and myostatin stimulated fibrogenesis of a multipotent cell line. Wt pP6 implanted in several organs of the rat generated skeletal myofibers, smooth muscle tissue and other cell types, and stimulated tissue repair and recovery of function. A fraction of Wt pP6 from the Oct-4 Pr-gfp express the Oct-4 embryonic stem cell marker, visualized by green fluorescence, and these cells were localized in the muscle.

Conclusions. Myogenic differentiation of MDSC in vitro is passage-dependent and refractory to myostatin modulators, despite MDSC undergo myogenesis in vivo, but are moderately responsive to nitric oxide. The resistance of the Mst ko and mdx MDSC to in vitro myogenesis is so far unexplained. Dual cultures with Wt MDSC are ongoing to determine whether myogenesis may be awakened by paracrine or juxtacrine clues. Expression of Oct-4 in MDSC may explain their myogenic activity.

A-1. Tsao J, Vernet D, Gelfand R, Kovanecz I, Nolzco G, Gonzalez-Cadavid NF. The genetic inactivation of myostatin or dystrophin expression inhibits the myogenic differentiation of a population of stem cells from the mouse skeletal muscle. J Endocrinol, to be submitted

The loss of myofibers in Duchenne muscular dystrophy (DMD) caused by the absence of dystrophin is aggravated by lipofibrotic degeneration. A promising therapeutic strategy is based

on implanting dystrophin + muscle derived stem cells (MDSC) engineered or modulated to commit into myogenic rather than lipofibrogenic lineages. Inhibiting or counteracting myostatin (a negative regulator of muscle mass that stimulates fibrogenic and adipogenic differentiation of stem cells), in implanted MDSC or in the host endogenous MDSC, may improve muscle repair in DMD. As an initial approach we aimed to compare in vitro the balance of myogenic, fibrogenic, and adipogenic commitments of MDSC lacking myostatin (myostatin knockout mice; Mst KO) or dystrophin (mdx mice) in comparison to MDSC from wild type (WT) mice, under various potential modulators, and to investigate whether differences in the differentiation response of MDSC were due to key stem cell and myogenic genes. Surprisingly, myogenesis by the WT MDSC was refractory to myostatin, follistatin, myostatin antibodies and shRNA, hormones, and nitric oxide, and the Mst KO and mdx MDSC were totally unable to form myotubes, even in dual cultures with WT MDSC. However, all MDSC differentiated into myofibroblasts, and neural and smooth muscle cells, but not into adipocytes, and expressed Sca 1 and Oct-4, as well as other stem cell genes. MDSC from a myostatin+/dystrophin+ transgenic mouse expressing gfp under the Oct-4 promoter, morphologically resembled very small embryonic-like (VSEL) stem cells. The Mst KO MDSC, but not the mdx MDSC, inhibited myogenesis by the WT MDSC under cell-to-cell contact. Although the three MDSC cultures showed similar levels of expression for 98% of the 260 stem cell and myogenic genes assayed, the genetic inactivation of myostatin or dystrophin was associated with a dramatic down-regulation of Actc1, Acta1, Spp1, MyoD, and notch 2, critical genes for the onset of myogenesis. Whether MDSC subjected to the in vivo paracrine and juxtacrine modulation of the muscle tissue will respond to myostatin differentiation signals, and whether the mdx MDSC may be activated by this modulation, needs to be determined.

In addition to these reported results, we have shown that C2C12 myoblasts were unable to stimulate myogenesis in dual cultures with WT MDSC or induce the formation of myotubes in dual juxtacrine and paracrine cultures, as investigated by immunocytochemistry and western blot for MHC II.

In parallel studies we have applied WT MDSC alone and concurrently with a continuous long-term administration of a PDE 5 inhibitor to elevate in a sustained way the cGMP levels in the heart, in order to counteract fibrosis and stimulate cardiomyocyte regeneration in a rat model of myocardial infarction. This is described in a manuscript in preparation, also acknowledging this grant **(A-2)**. Although not directly applicable to the current experimental design, this study has supported a pharmacological antifibrotic approach that we intend to explore in Year 4 as an adjuvant to the effects of the WT MDSC implanted into the skeletal muscle of the mdx mouse. A somewhat similar approach based on investigating the mechanisms of the antifibrotic effects of PDE 5 inhibitors was also conceptually related to the current grant in a review of our related contributions **(A-3)**, and thus this was acknowledged in the paper (although quoting the alternative grant number given initially by DOD). Finally, additional manuscripts from our group have supported the use of inhibitors of TGF β 1 activity (decorin), and xanthine oxidoreductase (allopurinol) as antifibrotic and anti-oxidative stress approaches, and of PPAR γ agonists as an antifibrotic, antioxidant and anti-inflammatory agent (References **1-3**), to be applied concurrently with MDSC in the mdx mouse.

The in vivo studies implanting MDSC in the mdx mice were slowed down by the previous studies, but are ongoing. Our original project design differs from other approaches of several groups on stem cell therapy for muscular dystrophy in the mdx mouse (see selected references), by four main features:

- 1) It compares the effects of MDSC on the diaphragm, which is the most affected muscle in both human Duchenne and in the mdx mouse, and that so far has not been studied using cell implants for tissue repair, with the effects of MDSC on the

- gastrocnemius, a muscle that because of its mass and easy access, has been the preferred target together with the tibialis and soleus for this type of therapy.
- 2) It focuses on the use of 10-month old mdx mice, an age where the gastrocnemius, as well as the diaphragm, are severely affected in comparison to the mild dystrophy seen in the gastrocnemius of younger animals (2-4 months old) that does not resemble the situation in DMD.
 - 3) It is based on injuring the muscles with a very small dose of notexin prior to the MDSC implants, in order to maximize their uptake and differentiation into myofibers
 - 4) It proposes adjuvant interventions to reduce lipofibrotic degeneration in the host muscle, mainly the in vitro or in vivo modulation to counteract myofibroblast and adipogenic differentiation, by inhibiting myostatin expression genetically in the implanted MDSC, or by treatments in the host mdx mouse with shRNA, or by blocking its activity with follistatin or antibodies

With this purpose, we aged mdx mice (purchased from Jackson labs at 8 weeks of age) in our vivarium to reach 10 months of age, and tested the notexin protocol we had proposed in the grant **(5-7)**. Although the WT mice survived, the mdx mice injected in the gastrocnemius with a higher dose **(7)** died on the following day. After a series of further tests with separate injections in the diaphragm and gastrocnemius and alternative ways of injection, under the surveillance and approval of our IACUC, we realized that the problem was probably due to a certain level of systemic leak of the notexin from the gastrocnemius to the general circulation that did not affect the WT mice, but compromised the more sensitive old mdx.

We therefore adopted a protocol based on a total 0.04 ug notexin injection (2 ul) into the ventral side of one half of the diaphragm in one series of mdx mice, and two 0.2 ug (in total) injections (5 ul each) in two extremes of the gastrocnemius in another series of mdx mice, in both cases in anesthetized animals subjected to surgery to expose the muscles and subsequent suture. The MDSC were injected 4 days later with a total of 0.5×10^6 DAPI-labeled MDSC (twice, 5 ul in each site) in both the gastrocnemius and diaphragm, again after exposing the muscles.

This protocol was applied to a series of 24 mice separated in 4 groups as follows:

	Notexin	MDSC	Coll 1 Pr	Specimens
Gr 1: WT MDSC gastro (5)				
Left gastrocnemius	5	5	5	5
Right gastrocnemius	-	-	5	5
Gr 2: Mst KO MDSC gastro (5)				
Left gastrocnemius	5	5	5	5
Right gastrocnemius	-	-	-	-
Gr 3: control injury gastro (5)				
Left gastrocnemius	5	-	5	5
Gr 4: WT MDSC diaphr (4)				
Half diaphragm	5	2	-	2
Half diaphragm	4	-	-	2

All the animals have survived and appear to be in excellent health, have received the injection of the collagen I promoter-beta galactosidase construct as scheduled, and will be sacrificed at 3 weeks to examine the histological and biochemical parameters described in the grant.

We have also ordered 60 additional mdx mice to age them in our vivarium to reach 10 months of age for the future experiments (see program for Year 4).

Bulleted list of key research accomplishments

- To our knowledge the attached paper **A-1** is the first report on the in vitro myogenic capacity of the pP6 pre-plating fractions identified as MDSC isolated from skeletal muscles from transgenic mice with independent genetic inactivation of the myostatin (Mst KO MDSC) and dystrophin genes (mdx MDSC), in comparison to the extensively described WT MDSC.
- In addition to our previously reported findings during Year 1 and 2, we have now found that a fundamental difference among the three types of MDSC is that the Mst KO and mdx MDSC express negligible amounts of mRNAs from genes that are key regulators of myogenesis, specifically spp1, actc1, acta1, notch 2, and myoD, in comparison to WT MDSC.
- The Mst KO MDSC inhibits the formation of myotubes by the WT MDSC under cell to cell contact, through an as yet unknown mechanism, whereas this was not observed in paracrine interactions.
- Our overall in vitro results suggest that the putative role of MDSC vis-à-vis the progenitor satellite cells in myofiber repair, and the generally accepted view of the roles of myostatin and dystrophin in myogenesis, may have to be reexamined once the in vivo results are completed during year 4

Reportable outcomes

A. Papers acknowledging this grant (see Appendix)

A-1. Tsao J, Vernet D, Gelfand R, Kovanecz I, Nolzco G, Gonzalez-Cadavid NF. The genetic inactivation of myostatin or dystrophin expression inhibits the myogenic differentiation of a population of stem cells from the mouse skeletal muscle. J Endocrinol, to be submitted

A-2. Gonzalez-Cadavid NF, Rajfer J. Treatment of Peyronie's disease with PDE5 inhibitors: an antifibrotic strategy. Nat Rev Urol. 2010 Mar 9. [Epub ahead of print]

A-3. Wang J S-C, Nolzco G, Kovanecz I, Vernet D, Kopchok GE, Chow S, Keyhani A, White RA, Gonzalez-Cadavid NF (2010) Effects of muscle derived stem cells and long-term treatment with a PDE5 inhibitor on myocardial infarction in a rat model. Cardiovascular Res, to be submitted

B. Abstracts and presentations related to results in the current grant

B-1. Gonzalez-Cadavid NF, Tsao J, Vernet D, Gelfand R, Nolzco G (2009) Modulation of cell lineage commitment by skeletal muscle derived stem cells, MDSC, from mdx and myostatin knockout mice. Military Health Research Forum 2009, Kansas City,

C. New applications for funding

The following grant application, which is still pending has been submitted using in part results obtained during Year 3 of this grant:

C1. Gonzalez-Cadavid NF (PI) NIH 1R21DK089996-01 Human iPS in erectile dysfunction after radical prostatectomy in rat models 07/01/10-06/30/12. Score: 30

This grant explores the preconditioning of iPS (induced pluripotent stem cells) and endogenous stem cells with modulators of the nitric oxide pathway such as molsidomine, PDE 5 inhibitors and sGC stimulators

Conclusions

Program for Year 4

Having finished the in vitro characterization of the MDSC from the three mouse strains (WT, Mst KO and mdx), and resolved the problems encountered with the unexpected notexin toxicity and the technically difficult diaphragm intervention, we are ready to intensify the in vivo studies. We plan to complete the schedule below during Year 4, but if needed we will request a no-cost extension with institutional funding support to be able to finish these important experiments.

A thorough review of the literature up to date has revealed a recent increased number of studies on cell therapy of muscle dystrophy, mainly Duchenne in the mdx mouse, as detailed in the selected list of references **(13-37)** and the additional ones detailed on our manuscript. A similar situation has occurred with the counteraction of myostatin expression or activity as a way to prevent lipofibrotic degeneration and increase myofiber repair in the dystrophic mouse, again as in the selected references **(8-12)** and the additional ones on our manuscript. These strategies are in general neither combined to stimulate MDSC differentiation nor focus on the diaphragm or the aged gastrocnemius in old mdx mice.

However, since translation of the current cell therapy approaches from the mdx to the DMD has not been successful so far, we will focus on additional strategies that may be more clinically adequate. This will be in lieu of the sophisticated gene therapy against myostatin, but pursuing those more practical aspects of counteracting myostatin activity, and particularly aiming to oppose lipofibrotic degeneration with novel pharmacological interventions. All of these will be applied in combination with the MDSC approach. In addition, we will simplify our experimental design, leaving the labor intensive functional studies to just the treatment that histologically and biochemically is more effective in the studies below. The planned schedule is as follows:

1. Follow up on the in vitro studies reported in the attached paper, by:
 - a. continuing the attempts to mimic in vitro the paracrine and juxtacrine environment of the MDSC implanted in the muscle, such as with C2C12 myoblasts in dual cultures under different conditions including pharmacological modulation, to determine whether the balance of myogenic/lipofibrogenic differentiation can be improved by these interactions;
 - b. exploring the mechanism of the Mst KO MDSC inhibition of myogenesis by WT MDSC in dual juxtacrine cultures, to find out which are the genes down-regulated by this interaction in the WT MDSC;
 - c. conducting gain-of-function experiments for each one of the genes that we found to be considerably down-regulated by myostatin or dystrophin genetic inactivation in the Mst KO and mdx MDSC, to try to find a way to activate myogenesis in the endogenous MDSC of the mdx mouse
2. Add more mdx mice to reach an n=6 in the ongoing in vivo experiment, including Mst KO MDSC implant in the diaphragm and complete all the scheduled histological and biochemical determinations, to conclude whether MDSC can correct the severe dystrophy in the aged gastrocnemius and in the diaphragm, and myostatin inactivation improves this process
3. Conduct new studies with all the planned outcomes and procedures as in #2, but implanting only WT MDSC into the mdx mouse, under the following simultaneous

long-term continuous treatments acting as adjuvant of MDSC proliferation, differentiation, and terrain conditioning:

- a. Antibodies against myostatin, as the more translatable approach to counteract myostatin in vivo, as planned in the grant.
 - b. Decorin, as an antifibrotic approach to counteract the effects of members of the TGF β family, as shown by us **(1)**
 - c. Allopurinol, as an antioxidant approach, to counteract oxidative stress as a profibrotic effector, as shown by us **(1)**.
 - d. Pioglitazone, as a PPAR γ agonist with antifibrotic, anti-inflammatory and antioxidant action, as shown by us **(2,3)**
 - e. Molsidomine, as a nitric oxide donor with potential antifibrotic effects, as shown by us **(1)**.
 - f. PDE 5 inhibitor, as a sustained stimulator of cGMP levels in an antifibrotic strategy, as shown by us **(A-2, A-3)**
4. Perform the scheduled functional measurements of muscle strength only in control and one treated group from above

References

They are listed in paper A-1 enclosed in the Appendix, as well as in the list below:

a. Some relevant papers from our group:

1. Ferrini MG, Moon J, Rivera S, Vernet D, Rajfer J, Gonzalez-Cadavid NF (2009) Antifibrotic and antioxidant therapies prevent the development of fibrosis in the penile corpora cavernosa associated with type 1 diabetes in the iNOS knock out mouse. Am Urol Assoc Meet, Chicago, IL, J Urol, BJU Int, to be submitted (2010)

2. Toblli JE; Ferrini MG; Cao G; Vernet D; Angerosa M; Gonzalez-Cadavid NF (2009) Antifibrotic effects of pioglitazone on the kidney in a rat model of type 2 diabetes mellitus. Nephrol Dial Transpl, Aug;24(8):2384-91..

3. Toblli JE, Cao G, Angerosa M, Gonzalez-Cadavid NF (2009) The antifibrotic effects of pioglitazone at low doses on the diabetic rat kidney are associated with the improvement of markers of cell turnover, angiogenesis and endothelial dysfunction. Am J Physiol (Integr Physiol), submitted

b. Some papers for notexin in mdx mice

4. Matecki S, Guibinga GH, Petrof BJ. Regenerative capacity of the dystrophic (mdx) diaphragm after induced injury. Am J Physiol Regul Integr Comp Physiol. 2004 Oct;287(4):R961-8.

5. Guibinga GH, Ebihara S, Nalbantoglu J, Holland P, Karpati G, Petrof BJ. Forced myofiber regeneration promotes dystrophin gene transfer and improved muscle function despite advanced disease in old dystrophic mice. Mol Ther. 2001 Nov;4(5):499-507..

6. McCroskery S, Thomas M, Platt L, Hennebry A, Nishimura T, McLeay L, Sharma M, Kambadur R. Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice. J Cell Sci. 2005 Aug 1;118(Pt 15):3531-41.

7. Yokota T, Lu QL, Morgan JE, Davies KE, Fisher R, Takeda S, Partridge TA. Expansion of revertant fibers in dystrophic mdx muscles reflects activity of muscle precursor cells and serves as an index of muscle regeneration. J Cell Sci. 2006 Jul 1;119(Pt 13):2679-87.

c. Some papers for myostatin modulation in mdx mice

8. Morine KJ, Bish LT, Pendrak K, Sleeper MM, Barton ER, Sweeney HL. Systemic myostatin inhibition via liver-targeted gene transfer in normal and dystrophic mice. *PLoS One*. 2010 Feb 11;5(2):e9176.

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10. Abe S, Hirose D, Kado S, Iwanuma O, Saka H, Yanagisawa N, Ide Y. Increased expression of decorin during the regeneration stage of mdx mouse. *Anat Sci Int*. 2009 84(4):305-11.

11. Tsuchida K. Myostatin inhibition by a follistatin-derived peptide ameliorates the pathophysiology of muscular dystrophy model mice. *Acta Myol*. 2008 27:14-8.

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D. Some papers for myostatin modulation in mdx mice

13. Mizuno Y, Chang H, Umeda K, Niwa A, Iwasa T, Awaya T, Fukada SI, Yamamoto H, Yamanaka S, Nakahata T, Heike T. Generation of skeletal muscle stem/progenitor cells from murine induced pluripotent stem cells. *FASEB J*. 2010 Feb 24. [Epub ahead of print]

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Appendices

They include the publications/manuscripts for papers A1-A3, and the PI biosketch

**THE GENETIC INACTIVATION OF MYOSTATIN OR DYSTROPHIN EXPRESSION INHIBITS
THE MYOGENIC DIFFERENTIATION OF A POPULATION OF STEM CELLS FROM THE
MOUSE SKELETAL MUSCLE**

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Running Title: Myostatin or dystrophin silencing and muscle stem cells

Key words: mdx mouse, Duchenne, fibrosis, dystrophy, muscle repair, muscle derived stem cells,

Abbreviations: **ASMA:** α -smooth muscle actin; **Mst KO:** myostatin knock out mouse; **QIA:** quantitative image analysis; **TGF β 1:** transforming growth factor β 1. **VSEL:** very small embryonic-like stem cells. **WT:** wild type mouse.

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ABSTRACT

The loss of myofibers in Duchenne muscular dystrophy (**DMD**) caused by the absence of dystrophin is aggravated by lipofibrotic degeneration. A promising therapeutic strategy is based on implanting dystrophin + muscle derived stem cells (**MDSC**) engineered or modulated to commit into myogenic rather than lipofibrogenic lineages. Inhibiting or counteracting myostatin (a negative regulator of muscle mass that stimulates fibrogenic and adipogenic differentiation of stem cells), in implanted MDSC or in the host endogenous MDSC, may improve muscle repair in DMD. As an initial approach we aimed to compare in vitro the balance of myogenic, fibrogenic, and adipogenic commitments of MDSC lacking myostatin (myostatin knockout mice; **Mst KO**) or dystrophin (mdx mice) in comparison to MDSC from wild type (**WT**) mice, under various potential modulators, and to investigate whether differences in the differentiation response of MDSC were due to key stem cell and myogenic genes. Surprisingly, myogenesis by the WT MDSC was refractory to myostatin, follistatin, myostatin antibodies and shRNA, hormones, and nitric oxide, and the Mst KO and mdx MDSC were totally unable to form myotubes, even in dual cultures with WT MDSC. However, all MDSC differentiated into myofibroblasts, and neural and smooth muscle cells, but not into adipocytes, and expressed Sca 1 and Oct-4, as well as other stem cell genes. MDSC from a myostatin+/dystrophin+ transgenic mouse expressing gfp under the Oct-4 promoter, morphologically resembled very small embryonic-like (**VSEL**) stem cells. The Mst KO MDSC, but not the mdx MDSC, inhibited myogenesis by the WT MDSC under cell-to-cell contact. Although the three MDSC cultures showed similar levels of expression for 98% of the 260 stem cell and myogenic genes assayed, the genetic inactivation of myostatin or dystrophin was associated with a dramatic down-regulation of Actc1, Acta1, Spp1, MyoD, and notch 2, critical genes for the onset of myogenesis. Whether MDSC subjected to the in vivo paracrine and juxtacrine modulation of the muscle tissue will respond to myostatin differentiation signals, and whether the mdx MDSC may be activated by this modulation, needs to be determined.

INTRODUCTION

The lipo-fibrotic degeneration of skeletal muscle, i.e. excessive deposition of endomysial collagen, other extracellular matrix, and fat, is a feature that characterizes muscle dystrophy in general, and Duchenne muscular dystrophy (**DMD**), a lethal X-linked disease (**1-3**), and in its animal models such as the mdx mouse for DMD (**4-7**). This process, associated with inflammation and oxidative stress (**8**), is partially responsible for the severe contractile dysfunction due primarily to the bouts of myofiber necrosis caused by dystrophin genetic inactivation in DMD and the mdx mouse, and is a major cause of mortality in DMD. Dystrophic muscle fibrosis also hampers the uptake and survival of cells implanted for potential therapeutic approaches (**9**) and not only results from myoblast accumulation but from a putative reprogramming of myoblasts to a fibrotic phenotype (**5**). Therefore, trying to ameliorate this process while stimulating myogenesis constitutes an ancillary strategy to favor repair and regeneration of dystrophic muscle tissue in the absence of dystrophin replacement.

Although there are several pharmacological alternatives to combat muscle lipo-fibrotic degeneration and the chronic inflammation that usually elicits it, biological factors such as myostatin (Mst), a member of the TGF β family that is the most studied negative regulator of muscle mass (**10**), are becoming potential key targets. In the case of myostatin, this is because of its noxious pleiotropic effects in muscle dystrophy not only as an antimyogenic agent, but also as a pro-fibrotic and adipogenic factor (**11-17**). Inhibiting myostatin by the respective antibodies or shRNA, or by the myostatin propeptide, in the most widely used DMD animal model, the mdx mouse, improves myogenesis and reduces fibrosis, and the same occurs by genetic deletion of myostatin in the myostatin knock-out mouse where myofiber hypertrophy is associated with less fat and fibrosis (**18-23**). The effects of myostatin on myofiber size and on myogenesis in vitro are counteracted by testosterone in part by reducing myostatin levels (**24-26**), and, indirectly, by nitric oxide by stimulating myoblast replication, differentiation, and fusion into myotubes (**27,28**).

It is assumed that in the dystrophic or injured muscle, tissue repair and the opposite process of lipofibrotic degeneration involve not only the differentiation of progenitor satellite cells, fibroblasts, and pre-adipocytes into myofibers, myofibroblasts, and adipocytes, respectively, but also the modulation of lineage commitment by stem cells present in the adult muscle **(29-32)**. These stem cells have been isolated from the rodent and human skeletal muscle and named in general as muscle-derived stem cells **(MDSC)**, because they have the ability to differentiate in vitro into multiple cell lines, and generate myofibers, osteoblasts, or smooth muscle cells after implantation into the skeletal muscle, bone, or corpora cavernosa and vagina, respectively **(29-34)**. In some cases, they act mainly by secreting paracrine growth factors that are believed to modulate the differentiation of endogenous stem cells or survival of differentiated cells in the tissue. However, the roles of MDSC in the normal biology of the skeletal muscle and in its pathophysiological processes are largely unknown.

Myostatin modulates the in vitro differentiation of pluripotent cells, albeit sometimes with conflicting results **(17,35-38)**. It also inhibits the proliferation and early differentiation of satellite cells from the skeletal muscle and of cultured myoblasts, and improves the success of their in vivo transplantation **(39-41)**. To our knowledge, no reports are available on myostatin effects on the differentiation of MDSC.

MDSC obtained from wild type **(WT)** mice, that express both myostatin and dystrophin, have been used experimentally to trigger repair of the mdx muscle with variable results **(42-46)**, but they appear to be superior in this respect to myoblasts or satellite cells **(47)**. However, some of the main limitations of myoblast therapy when translated from the murine models into DMD and other muscle dystrophies in humans may also affect the MDSC and other types of stem cells **(48)**. Therefore, it is important to elucidate how to enhance the repair capacity of WT MDSC by in vitro or in vivo modulation of their multilineage potential, and how to stimulate or even awake endogenous stem cells of dystrophic muscle to regenerate myofibers while avoiding differentiation into cells responsible for lipofibrotic degeneration. However, no reports

are available on this potential in vitro modulation of MDSC, or the effects that myostatin or dystrophin gene inactivation exerts on this balance.

The aim of the present study was to investigate the in vitro myogenic versus fibrogenic and adipogenic differentiation of MDSC isolated from the Mst KO and mdx skeletal muscle vis-à-vis the WT counterpart, and the potential manipulation of these processes by modulating myostatin expression or activity, and by other putative regulators of muscle mass and fibrosis. The paracrine and juxtacrine cross-talk of the three types of MDSC on their respective myogenic commitment was also studied in dual cultures. The ultimate goal is to gain an initial insight on how in vitro preconditioning of MDSC may modulate their capacity to repair dystrophic skeletal muscle, and to design in vivo pharmacological interventions that may mimic these processes, and even activate dystrophin negative MDSC. .

METHODS

Animals

Only male mice were used throughout, and unless stated they were 12-15 weeks old. Mdx mice (C57BL/6/10ScSn-Dmd^{mdx}), named here as “mdx”, the model for X-linked muscular dystrophy caused by dystrophin inactivation, were obtained from Jackson Laboratories (Bar Harbor, ME). At this age the first round of muscle necrosis and regeneration has already subsided (“stable phase”). In one experiment mice were allowed to reach 6 months of age, when fibrotic degeneration is evident, not only in the diaphragm but in the gastrocnemius. Mst knock-out (Mst^{-/-}) mice (C57BL/6J/Mst^{-/-}), named here as “Mst KO”, are regularly maintained and bred in our vivarium on a BL/6 background **(49)**, derived from the original strain on a BalBc background. Aged-matched wild type control mice (C57BL/6J), named here as “WT”, were also from Jackson Laboratories. The Oct-4 Pr-gfp transgenic mouse **(50)**, named here as Oct-4,

expresses the green fluorescent protein Egfp under the control of the gene enhancers and promoter of the embryonic stem cell gene Oct-4. Animals are available in our vivarium.

MDSC isolation from different strains of mice

Hind limb muscles from the WT, Mst KO, and mdx mice were subjected to the preplating procedure to isolate MDSC **(7,33,34,47)**. 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 (FBS). Cells were plated onto collagen I-coated flasks for 1 hr (preplate 1 or pP1), and 2 hrs (preplate 2 for pP2), followed by sequential daily transfers of non-adherent cells and re-platings for 2 to 6 days, until preplate 6 (**pP6**). The latter is the cell population containing MDSC. Cells were maintained in GM-20 on regular culture flasks (no coating) and used in the 14th-28th passage, since WT MDSC have been maintained in our laboratory for at least 40 generations with the same, or even increasing, growth rate. In the case of the Oct-4 Pr gfp MDSC ("Oct-4 MDSC") the same procedure was applied. Green fluorescent single cells or clusters/spheroids were monitored, as well as their morphological features (large nucleus, easily detachable, <10 μ m). In some cases, Sca1⁺ cells were selected with immunobeads (Milteny) coated with antibody against the selected antigen (Sca1) **(33)**.

Stem cell characterization, differentiation, and modulation

MDSC cultures from the three mouse strains were analyzed for the expression of stem cell markers below, on collagen-coated 6-well plates and 8-removable chamber plates. Multipotency was analyzed in 2-week incubations with GM-20 or GM-10 (GM with 10% fetal bovine serum) supplemented or not with 10 nM DMSO or 5 ng/ml TGF β 1, or, to induce myofiber formation, after reaching confluence, for 2-3 weeks with Hedrick's medium **(59)**, or as described.

In certain cases, cultures were treated with or without 20 μ M 5'-azacytidine (AZCT) in GM-20 for 3 days to induce multipotency, prior to switching them to the appropriate medium **(13,17)**.

For the tests on the modulation of MDSC skeletal myogenic differentiation by various factors, cells were allowed to reach confluence, switched to Hedrick's medium, and incubated for 2 weeks with 2 μ g/ml recombinant 113 amino acid myostatin protein (R-Mst), a recombinant 16 kDa protein containing 113 amino acid residues of the human myostatin protein (BioVendor Laboratory Medicine Inc., Palackeho, Czech Republic), or with a recombinant mouse follistatin protein (RD Systems, Minneapolis, Mn) at 0.2 μ g /ml **(13,17)**, changing medium twice a week. In other experiments, incubations with the monoclonal (Chemicon International, Temecula, Ca) and polyclonal (Millipore Corp, Billerica, Ma) antibodies against myostatin (1/20) were substituted for the previous treatments. Alternatively, the adenoviruses expressing the mouse myostatin full-length cDNA under the CMV promoter (AdV-CMV-Mst375) and an shRNA, which targets myostatin shRNA and inhibits more than 95% of myostatin gene expression **(13,17,20)** **(AdV-Mst shRNA)** were transduced into MDSC at 80% confluence, and then switched to Hedrick's medium as above. For a potential hormonal regulation of MDSC differentiation, confluent MDSC in Hedrick's medium were incubated with testosterone or dihydrotestosterone (Sigma Aldrich, St Louis, Mo) at 100 and 20 nM, respectively, or thyroid hormone (T3) (Sigma Aldrich) at 2.4 ng/ml as above. Finally, SNAP (Alexis Biochemicals, San Diego, CA) was used as a nitric oxide donor at 50 μ M on confluent MDSC.

Implantation of MDSC into skeletal muscle

Mice were treated according to National Institutes of Health (NIH) regulations with an Institutional Animal Care and Use Committee-approved protocol. The WT and mdx MDSCs ($0.5\text{--}1.0 \times 10^6$ cells/50 μ L Hanks) were labeled with the nuclear fluorescent stain 4',6-diamidino-2-phenylindole (DAPI) **(33,34)**, and implanted aseptically under anesthesia into the surgically exposed tibialis anterior of both 4 month old WT and 8 month old mdx mice. The muscle had

been cryoinjured by pinching it for 10 seconds with a forceps cooled in liquid N₂ immediately prior to implantation. Mice were euthanized, the tibialis excised from the limb and subjected to cryoprotection in 30% sucrose, embedding in OCT and cryosectioned. Similarly, MDSC isolated from the Oct-4 Pr gfp mouse were implanted into the WT and mdx tibialis as described.

Immunocytochemistry dual immunofluorescence

Cells on collagen-coated eight-well removable chambers and frozen tissue sections, were fixed in 2% p-formaldehyde reacted **(12,13,17,20,33,34,49)** with some of the following primary antibodies against: (1) human myosin heavy chain type II (MHC-II; monoclonal, 1:200 Vector Laboratories, Burlingame, CA, USA), a marker for skeletal myotubes; (2) human α SMA (mouse monoclonal in Sigma kit, 1:2, Sigma Chemical, St Louis, MO, USA), a marker for both SMC and myofibroblasts; (3) human calponin-1 (basic mouse monoclonal, 1:25, Novocastra, Burlingame, CA, USA) an exclusive markers for SMCs; (4) human CD34 (rabbit polyclonal, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), a stem cell marker; (5) mouse Sca1 (rat monoclonal, 1:200, BD Pharmingen, Franklin Lake, NJ, USA) another stem cell marker; (6) neurofilament 70 (NF70; mouse monoclonal, 1:10, Millipore, Bullerica, Massachusetts, USA) (7) Dystrophin (rabbit polyclonal, 1:200 Abcam, Cambridge, Massachusett, USA), and (8) Sca-1 (mouse monoclonal, 1:100, BD Pharmingen, San Jose, CA USA) and M.O.M blocking kit (Vector, Burlingame, CA). In cases where MDSC DAPI on 8-well chambers were not previously tagged with DAPI, all nuclei were stained with coverslips with DAPI anti-fading emulsion

For cells not previously labeled with DAPI, cultures or tissue sections were subjected to immunohistochemical detection by quenching in 0.3% H₂O₂, blocking with goat (or corresponding serum), and incubated overnight at 4 °C with the primary antibody. This was followed by biotinylated anti-mouse IgG (Vector Laboratories), respectively, for 30 min, the ABC complex containing avidin-linked horseradish peroxidase (1:100; Vector Laboratories), 3,3'-diaminobenzidine, and counterstaining with hematoxylin, or no counterstaining. For cells labeled

with DAPI, fluorescent detection techniques were used. The secondary anti-mouse IgG antibody was biotinylated (goat, 1:200, Vector Laboratories) and this complex was detected with streptavidin-Texas Red. For the CD34 detection we used a biotinylated anti-rabbit IgG (goat, 1:200, Vector Laboratories). After washing with PBS, the sections were mounted with Prolong antifade (Molecular Probes, Carlsbad, CA, USA). Negative controls in all cases omitted the first antibodies or were replaced by IgG isotype. In the case of Oct-4, streptavidin-FITC was used (green fluorescence).

In tissue cryosections (10 μ m), muscle fibers were either stained with hematoxylin/eosin, or by MHC-II, either by Texas red fluorescence as above, or with the diaminobenzidine tetrahydrochloride-based detection method (Vectastain-Elite ABC kit; Vector Labs), counterstaining with Harris hematoxylin. Tissue sections that were incubated with mouse IgG instead of the primary antibody served as negative controls. The sections were viewed under an Olympus BH2 fluorescent microscope, and cell cultures under an inverted microscope. In some cases, the cytochemical staining was quantitated by image analysis using ImagePro-Plus 5.1 software (Media Cybernetics, Silver Spring, MD, USA) coupled to a Leica digital microscope bright field light fluorescence microscope/VCC video camera. After images were calibrated for background lighting, wherein integrated optical density ($\text{IOD} = \text{area} \times \text{average intensity}$) was calculated.

Gene expression profiles

Pools of total cellular RNA from three T25 flasks for each MDSC that were incubated with DMEM supplemented with FBS at 20% were isolated with Trizol-Reagent (Invitrogen, Carlsbad, CA). RNA quality was assessed by agarose gel electrophoresis and subjected to cDNA gene microarrays (SuperArray BioScience Corp., Frederick, MD) **(13,33)**, using the mouse stem cell (OMM-405), Oligo GEArray microarray: 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, and then fold changes in relative gene expression were calculated

The expression of some of the down- or up-regulated genes detected above was examined on 0.5 ug RNA by reverse transcription (RT) using a 16-mer oligo(dT) primer, as previously described **(13,33)**, and the resulting cDNA was amplified using PCR in a total volume of 25 µl. The locations of the primers utilized for the quantitative estimation of mouse myostatin mRNA were nt 136–156 (forward) and 648–667 (reverse), numbering from the translation initiation codon (later called F2/R2) as previously described. For mouse GAPDH primer sequences were from the mRNA sequence NM_008084.2, using a forward primer spanning nts 778-797 and reverse primer spanning nts 875-852, with a product length of 98 nt. Additional primers were designed using the NCBI Primer Blast program applied to mRNA sequences and synthesized by Sigma-Aldrich. Numbering refers to the length in NT from the 5' end of the mRNA: Acta1 (skeletal muscle actin) NM_009606.2 (forward 501-520 and reverse 841-822, product length 341); Actc1 (cardiac actin) NM_009608.3 (forward 38-58 and reverse 554-530, product length 517); Bmp4 (Bone morphogenetic protein 4) NM_007554.2 (forward 326-345 and reverse 908-888, product length 583; 5) MyoD NM_010866.2 (forward 515-534 and reverse 1013-994, product length 499); 6) Spp1 NM_009263.2 (forward 133-153 and reverse 524-505, product length 392); 7) Pax3 NM_008781.4 (forward 1164-1183 and reverse 1893-1874, product length 730); and 8) Mef2a (Myocyte Enhancer Factor 2A) NM_001033713.1 (forward 12-30 and reverse 742-723, product length 731). The number of PCR cycles used for each primer set are stated in Fig. 6. All primers were designed to include an exon-exon junction in the forward primer except for Gapdh, Bmp4, and MyoD1.

Protein expression by western blots

Cell homogenates were obtained in boiling lysis buffer (1% SDS, 1mM sodium orthovanadate, 10 mM Tris pH 7.4 and protease inhibitors, and centrifuging at 16,000 *g* for 5 min **(12,13,17,20,33,34,49)**. 40 µg of protein were run on 7.5% or 10% polyacrylamide gels, and submitted to transfer and immunodetection with antibodies against: 1) human αSMA (monoclonal, 1/1000, Calbiochem, La Jolla, CA, USA); 2) human calponin-1 (basic) (mouse monoclonal, 1/25, Novocastra, Burlingame, CA, USA), as exclusive marker for SMC; 3) proliferating cellular nuclear antigen (**PCNA**) (mouse monoclonal, 1:100, Chemicon, Temecula, CA, USA) a marker for replicating cells; 4) Oct-4 (rabbit polyclonal, 1/500, BioVision, Mountain View, CA, USA), as stem cell marker; 5) MyoD (rabbit polyclonal 1/200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA USA), as muscle origin marker; 6) GAPDH (mouse monoclonal, 1/3000, Chemicon, Temecula, CA USA) as a housekeeping gene. Membranes were incubated with a secondary polyclonal horse anti-mouse IgG linked to horseradish peroxidase (1:2000; BD Transduction Laboratories, Franklin Lakes, NJ, USA, or 1:5000, Amersham GE, Pittsburgh, PA, USA) and bands were visualized with luminol (SuperSignal West Pico, Chemiluminescent, Pierce, Rockford, IL, USA). For the negative controls the primary antibody was omitted..

Myostatin, myosin heavy chain type II (MHCII), and GAPDH protein determinations were done by Western blotting. For myostatin, we utilized a monoclonal antibody for cell protein extracts and a polyclonal antibody for muscle tissue extracts based on our preliminary data examining which antibody yielded the most specific binding reaction (data not shown). For detecting mouse myostatin expressed from HEK293 cells transfected with pcDNA3.1-myostatin, 40 µg of protein extract was run on 12% gel electrophoresis, using a 1:1000 mouse monoclonal anti-myostatin primary antibody that was custom made against a peptide. The secondary anti-mouse antibody (BD Biosciences) was diluted 1:5000 and was linked to horseradish peroxidase. For muscle tissue extracts (50 µg), a custom polyclonal anti-myostatin antibody was

used at a dilution of 1:200. The antibody employed for myostatin was a polyclonal antibody elicited against a synthetic peptide within the sequence of the 110 carboxy terminus amino acids of human myostatin, which was named antibody B. This antibody has been extensively validated by its ability to detect the recombinant 375-amino acid myostatin precursor and the processed 110-amino acid protein **(13,17,49)** as well as a 30- to 32-kDa band in mouse, rat, and human skeletal extracts, which is considered to be the dimer of the 110-amino acid protein cleaved from the myostatin precursor.

For other proteins, 1:200 mouse monoclonal anti-MHC II antibody (Novocastra Laboratories, Newcastle, UK) or 1:10000 anti-GAPDH monoclonal antibody (Chemicon International, Temecula, CA, USA) was used. The washed membranes were incubated with 1:2000 dilution of secondary antibody linked to horseradish peroxidase. Immunoreactive bands were visualized by using the Supersignal West Pico chemiluminescence detection system (Pierce, Rockford, IL, USA). Band intensities were estimated by densitometry and corrected by the respective GAPDH band intensities.

Statistics

Values are expressed as the mean (SEM). The normality distribution of the data was established using the Wilk–Shapiro test. Multiple comparisons were analyzed by a single factor ANOVA, followed by *post hoc* comparisons with the Newman–Keuls test. Differences among groups were considered statistically significant at $P < 0.05$.

RESULTS

MDSC cultures from the Mst KO and mdx mice appear similar to their counterparts from WT mice in morphology, replication, cell markers and multipotent differentiation

MDSC, concentrated in the pre-plating pP6 fraction, were subjected to a comparative study once the WT cells were shown to form in vitro robust skeletal myotubes (see next section) at about passage 13. The morphology of the three cultures was essentially similar while proliferating, and the replication times for the WT and mdx MDSC were also similar (21.2 vs 19.8 hs, respectively). However, the Mst KO was surprisingly sluggish (27.0 hs), considering that inactivation of myostatin, a down-regulator of cell replication, should stimulate proliferation. This morphology and replication pattern continued throughout the 13-28 passages period where this comparison among the three strains was maintained.

Fig. 1 shows by immunofluorescence of frozen sections that the mdx skeletal myofibers lack dystrophin, as expected, whereas the protein is well expressed in the periphery of the myofibers in the WT muscle. In agreement, many of the MDSC from the WT express dystrophin, even if no myotubes were allowed to be formed in proliferation medium, whereas no dystrophin is detected in the mdx MDSC. As expected, dystrophin is expressed in the muscle and MDSC from the Mst KO mice, and the WT MDSC culture was previously shown to be Sca 1 + **(34)**, as expected. However, myostatin was not detected in any of the three MDSC, either by western blot or by RT/PCR (not shown).

The key embryonic stem cell marker, Oct-4, is also expressed in the three MDSC cultures growing in PM in sub-confluent cultures, and is mainly localized in the nuclei, where the transcriptionally active isoform, the Oct-4A, is expected to occur **(Fig. 2 top)**, accompanied by some cytoplasmic staining denoting the Oct-4B variant. This is confirmed by western blot **(middle left)**, showing an equal and considerable expression of the 45 kDa Oct-4A protein among the three lines, and a much lower content of the 33 kDa Oct-4B **(52)**. The embryonic stem cell features are also suggested from two additional findings. First, the Oct-4 promoter is activated, as denoted by green fluorescence in the cytoplasm (where gfp remains) of the Oct-4 MDSC, that was very intense in some of them and absent in cells from the WT mouse used as control (not shown). As expected, the nuclei were not fluorescent, and there were also gfp+ tiny

rounded cells indicated with arrows. The latter cells were also seen loosely attached to other types of cells in the pP2-pP5 cultures suggesting that they associate transiently with the other preplating fractions. Second, the alkaline phosphatase reaction, a feature of embryonic stem cells, was in general moderately positive, with more intense staining in some individual cells particularly in the Mst KO and mdx MDSC (**bottom**). However, partial osteogenic differentiation cannot be excluded.

The expression of other stem cell related genes in the three types of MDSC was studied in total RNA by DNA microarray analysis with a related panel of 260 genes. **Table 1** shows that there are not substantial differences in the expression of most well known embryonic stem cell genes such as c-Myc, Oct-4 (**Pou5**), alkaline phosphatase 2 and 5, telomerase reverse transcriptase, leukemia inhibitory factor (**LIF**), master mind like 1, and many other related genes. This agrees with the fact that the multilineage differentiation capacity of these MDSC seems to be qualitatively similar among the three types, as shown by the generation in neurogenic medium of cells expressing the neuronal marker NF70 (**Fig. 3 A**), and in fibrogenic medium of cells expressing alpha smooth muscle actin (**ASMA**) (**B**). The three MDSC cultures also differentiated into cells expressing calponin as smooth muscle cell marker and von Willebrandt as endothelial cell marker (not shown).

The lack of active myostatin or dystrophin does not allow MDSC to undergo in vitro skeletal myogenesis, and this is associated with down-regulation of some critical genes

The WT MDSC forms polynucleated myotubes expressing MHC II in confluent cultures upon incubation for 1 to 2 weeks in a special myogenic medium named here as “Hedrick’s” (**Fig. 4**), used to stimulate the less intense and slower myogenic differentiation of adipose tissue derived stem cells (**51**). However, remarkably neither the mdx MDSC nor the Mst KO MDSC were able to generate any myotube in this medium. This is also illustrated in the western blot analysis where the strong MHC-II 210 kDa band in the WT MDSC extract is not seen in the Mst

KO or mdx MDSC. However, in these fully confluent cultures in myogenic medium where Mst KO and mdx MDSC fail completely to form a single myotube, there is a considerable expression of both forms of Oct-4 with no apparent differences among the three cultures.

The robust MHC II+ myotube formation by confluent WT MDSC was manifested also in proliferation medium with very low FBS (2%) (not shown), and remarkably, maintained in the presence of 10% or 20% FBS (**Fig. 5 A, B**), although in Hedrick's myotubes appeared more robust and multinucleated (**C**), thus showing that cell to cell contact is sufficient to trigger MDSC myogenesis, rather than the need for growth factor depletion in the myoblast/myotube conversion. Again, mdx MDSC (**D**) and Mst KO MDSC (not shown) were completely unable to form myotubes. No adipogenesis could be detected with Oil red O in Hedrick's medium (not shown). The strong myogenic ability of WT MDSC was confirmed by western blot of parallel confluent cultures, where MHC-II was expressed in all media in triplicate cultures, although more intensively in Hedrick's (**E**). Remarkably, there was no difference in MyoD expression among the different media (**F**). ASMA, a marker of myofibroblast or smooth muscle formation, was also expressed similarly in the confluent cultures along the three media (**G**), suggesting that mononucleated cells were undergoing a conversion into these cells.

None of these media were able to trigger myotube formation or MHC-II expression in Mst KO or mdx MDSC (despite they continued proliferating normally), neither prolonging incubations to 4 weeks, or using earlier or late different passages, induced myogenesis. In contrast, WT MDSC cultures were active in formation of myotubes for up to 40 passages, although later on the size and number of the myotubes started to decline. Cultures of Mst KO or mdx pP5 or pP4 obtained during the pre-plating procedure also failed to generate skeletal myotubes.

Despite the profound differences in formation of MHC II + myotubes in confluent Mst MDSC and mdx MDSC, versus WT MDSC, the transcriptional expression of myogenesis related genes in proliferating MDSC was, as in the case of the stem cell genes in Table 1, very similar.

For instance, expression of BMPRs (bone morphogenic protein receptors), the Wnt signaling receptors frizzled and jag, IGF1, Notch 1, and Notch 3, did not differ among the three types of MDSC (**Table 2**). However, six notable differences were noticed where each gene was considerably down-regulated to a similar extent in the Mst KO and mdx MDSC, versus a strong expression in the WT MDSC. They corresponded to Spp1 (secreted phosphoprotein 1, or osteopontin), Actc 1 (cardiac α -actin), MyoD1, cadherin 15, Myf 5, and Notch 2 (see discussion). In contrast, other cadherins (11 and 6), related to neuromuscular development, were up-regulated by 9 and 4-fold, respectively, in both the Mst KO and mdx MDSC. Other than this, there was a virtual 98% similarity among the three MDSC types in terms of the 260 genes investigated.

These results were corroborated by RT/PCR for some of the mRNAs depicted on the tables. **Fig. 6** shows the gel electrophoresis stained with ethidium bromide at the top, and the densitometric values of each band corrected by the housekeeping gene values at the bottom. These ratios are comparable among the three MDSC cultures for each gene, but not among the different genes for each culture, because of the different number of cycles applied for the respective transcript amplification. It is obvious that Spp1, Actc1, Acta1, MyoD, and Mef2a, are down-regulated in Mst KO and mdx MDSC as compared with WT MDSC, and that Pax 3 is overexpressed, while Bmp 4 levels are not changed, in agreement with the DNA microarrays. Interestingly, the Oct-4 MDSC resemble the WT MDSC but not for Spp1, myoD, and Bmp 4.

The WT MDSC are refractory to in vitro modulation of myogenic differentiation by a series of potential modulators, which are also unable to turn on myogenesis in Mst KO and mdx MDSC

Incubation of WT MDSC for 3 days with 5-azacytidine, a demethylating agent and potent inducer of myogenic capacity in pluripotent cell lines (**13,17**) prior to reach confluency and switching to myogenic medium, did not stimulate myotube formation, and failed completely to

induce it in Mst KO and mdx MDSC (not shown). This suggested that MDSC may turn to be very refractory to the usual modulators of myogenesis. The first ones tested with a similar paradigm were myostatin, that would be expected to down-regulate myotube formation in the WT MDSC, and follistatin, that should exert the opposite effect by binding myostatin. **Fig. 7 D** shows that the area occupied by MHC II + myotubes was not modified in the cultures treated with 2 ug/ml myostatin (**B**), or 0.5 ug/ml follistatin (**C**), as compared to untreated controls (**A**). Follistatin did not induce myogenesis on Mst KO and mdx MDSC (not shown). This failure of myostatin or follistatin to affect myogenesis in MDSC occurred despite these cells express ActRIIb, the myostatin receptor in the three cultures, as shown by western blot (**E**), indicating that they should be responsive to exogenous myostatin. Endogenous myostatin expression was not detected in any untreated culture (not shown), even if TGF β 1, another key member of the TGF β family was expressed (**F**). Finally, neither the monoclonal nor the polyclonal antibodies against myostatin (1/20) affected myogenesis in the WT MDSC or induced it in the Mst KO or mdx MDSC, as compared to the respective cultures incubated with control IgG (not shown).

In order to determine whether forced expression of myostatin in WT MDSC would induce in an autocrine fashion a receptor-independent inhibition of myogenesis, or viceversa blocking myostatin expression would stimulate it, we transfected the cultures with an adenovirus construct for a cDNA expressing the full length myostatin (**AdV Mst cDNA**) or alternatively with an AdV construct encoding an shRNA against myostatin that also expresses beta galactosidase (**AdV Mst shRNA**). **Fig. 8 A** shows that the AdV Mst cDNA transduced into HEK293 generates myostatin in some of the cells, as seen by immunocytochemistry. The same construct transduced into WT MDSC also produces the 45-50 kDa unprocessed full length myostatin protein, detected by western blot (**B**). The AdV Mst shRNA-beta gal construct that had been shown previously to inhibit myostatin expression was also efficiently transfected into MDSC, in this case detected by a general X-gal staining (**C**), absent in the untransfected control (**D**).

Despite this evidence, the AdV Mst cDNA did not reduce myotube formation or MHC II expression in the confluent WT MDSC, and the AdV Mst shRNA could not stimulate these processes in these cells or induce them in the Mst KO or mdx MDSC (not shown).

Since we had shown previously (50') that 100 nM testosterone or 25 nM DHT stimulated myogenesis in the multipotent C3H 10T(1/2) cells, confluent WT MDSC in Hedrick's medium from the three sources were incubated in triplicate with or without the male sex steroids, and MHC II expression in the pools was determined by western blot and myotube formation by immunohistochemistry for MHC II. No stimulation of these processes was found despite the fact that the androgen receptor 118 kDa protein was distinctively expressed by western blot, albeit only in WT MDSC (not shown). The unresponsiveness of the confluent WT MDSC exposed for 2 weeks to potential modulators of myogenesis replenished fresh with each change of Hedrick's medium, was extended towards TGF β 1 (5 ng/ml), thyroid hormone (TH/T3) (2.4 ng/ml), SNAP as NO donor (5 μ M), and SNAP and PDE5 inhibitor tadalafil (20 nM). None of them induced myogenesis in Mst KO or mdx MDSC (not shown).

Paracrine or juxtacrine modulation by WT MDSC in dual cultures fails to induce myotube formation in the Mst KO and mdx MDSC, and the latter are also unable to donate nuclei to injured mdx myofibers in vivo

The three types of MDSC were tested in dual cultures to ascertain whether they influence each other's myogenic ability. Paracrine effects were studied with one type of confluent MDSC seeded on a top compartment, separated by a 6 μ m permeable membrane from the second type of confluent MDSC plated on the bottom compartment, and incubated in Hedrick's medium for 2 weeks (**Fig. 9 A**). Analysis of the bottom MDSC for MHC II by western blot for indicated that WT MDSC at the top were unable to induce myogenesis of Mst KO or mdx MDSC at the bottom, and the Mst KO and mdx MDSC at the top did not seem to exert any

modulatory effects on this expression on the WT MDSC at the bottom. Immunohistochemistry for MHC II in parallel cultures confirmed these results (not shown).

Juxtacrine (cell to cell contact) effects were studied by mixing at confluence on the same well of a 6-well plate equal numbers of one type of cells labeled with DAPI with another type not subjected to nuclear labeling with DAPI (for immunocytochemistry) and following MHC II expression also by western blot in the cell mix after 2 weeks in Hedrick's medium **(B)**. The unlabeled Mst KO MDSC reduced MHC II expression in the labeled WT MDSC, in some cases quite drastically **(lanes 2 vs 1, and 4, 7 vs 3)**, and this was repeated with the reverse arrangement, labeled Mst KO with unlabeled WT MDSC **(lane 5 vs 3)**, thus excluding any effect of DAPI labeling on this process. The unlabeled mdx MDSC did not affect expression in the labeled WT MDSC **(lane 6 vs 3)**, and obviously the Mst KO and mdx MDSC did not affect each other for eliciting MHC II expression **(lane 8)**. Dual fluorescence immunocytochemistry for DAPI and Texas red labeled MHC-II confirmed these effects (not shown)

The failure of the Mst KO and mdx MDSC to form myotubes in vitro in any of the studied conditions is further supported by a comparison of their relative ability to incorporate nuclei into myofibers in vivo when implanted into the cryo-lacerated gastrocnemius of the mdx mouse excised after 2 weeks. **Fig. 10 top** shows that Sca 1+ cells are present in scattered spots within frozen sections of the intact WT gastrocnemius subjected to Sca 1 immunoassay and counterstained with DAPI around the periphery of myofibers in the vicinity of some nuclei (merge of red/blue fluorescence), but they are considerably reduced in the corresponding sections from the mdx gastrocnemius, at 4-5 months of age. These Sca 1+ cells are assumed to be endogenous MDSC-like cells, but this needs further confirmation. The bottom panels show that in the case of the DAPI-labeled WT MDSC implanted into the mdx muscle subjected to cryo-injury, the MDSC nuclei are not only detected in many of the myofibers, but many of these nuclei are central rather than peripheral, as may be expected from regenerating myofibers. Other nuclei are seen in the interspersed connective tissue among the fibers. In contrast, the

labeled nuclei from the mdx MDSC remain interstitial, and no intra-myofiber DAPI-labeled nuclei could be seen in a series of adjacent sections. The Mst KO MDSC were not tested.

DISCUSSION

To our knowledge this is the first report on the in vitro myogenic capacity of the pP6 pre-plating fractions identified as MDSC isolated from skeletal muscles from transgenic mice with independent genetic inactivation of the myostatin and dystrophin genes, in comparison to the extensively described wild type MDSC **(32,47)**. Our main findings were: a) in contrast to the WT MDSC, the Mst KO and mdx MDSC are unable to form myotubes in vitro, or incorporate their nuclei into the myofibers of mdx mouse injured skeletal muscle, despite there are no fundamental differences among the three MDSC cultures in terms of morphology, replication rates, expression of most members of a subset of key embryonic-like stem cell and other markers, and multi-lineage differentiation other than skeletal myogenic conversion; b) a fundamental difference was however, that Mst KO and mdx MDSC express much lower amounts, of key genes in myogenesis such as *spp1*, *actc1*, *acta1*, *notch 2*, and *myoD*, than WT MDSC; c) the three types of MDSC are refractory in vitro to modulation or induction of myotube formation by well known regulators of this process or of myofiber number, such as myostatin inhibition or overexpression, follistatin, androgens, nitric oxide, and others, despite some of the respective receptors are expressed in all MDSC cultures; d) the only effect on myotube formation we could detect is the inhibition exerted upon cell to cell contact by the Mst KO MDSC on the WT MDSC, whereas this was not observed in paracrine interactions. These somehow unexpected results in cell culture may not necessarily apply in vivo, but with this caveat are still difficult to reconcile with the putative role of MDSC vis-à-vis the progenitor satellite cells in myofiber repair, and with the generally accepted view of the roles of myostatin and dystrophin in myogenesis.

The WT MDSC used here as control, fulfill all the criteria that have been extensively defined over the last decade by the group that discovered them as potential tools for skeletal muscle, cardiac, and osteogenic repair upon implantation into the target organs **(37,42)**. They were isolated as the pP6 fraction using the preplating procedure on collagen-coated flasks, and shown to have the expected morphology, replicate fast and for at least 50 passages, express Sca1, and differentiate in vitro into multiple cell lineages. The latter includes a robust formation of multinucleated and branched myotubes, that translates in vivo into their ability to donate their nuclei to injured skeletal myofibers in regeneration, as evidenced by the central location of the donor nuclei. In previous studies we had shown that WT MDSC generate at least smooth muscle cells and epithelial cells when implanted into urogenital tissues **(33,34)**, adding up to the extensive demonstration of their stem cell nature by Huard's group **(37,42)**. One of our novel findings here is that WT MDSC have some embryonic-like stem cell features, mainly the expression of Oct-4, myc, LIF, and other embryonic stem cell genes, and that they contain both tiny rounded cells similar to the VSEL described in many adult organs **(53)**, and other larger ones that also express gfp in the case of the myostatin + and dystrophin + Oct-4 MDSC.

Another finding is the unexpected observation that myotube formation by the WT MDSC in vitro is completely refractory to modulation by agents that are well known to affect this process, or skeletal muscle mass in vivo. The fact that myotube formation by WT MDSC was not influenced by: a) downregulation or overexpression of myostatin, despite the detectable expression of its receptor (Act11b); b) counteracting myostatin activity by the respective antibodies or follistatin, that in vivo stimulate myofiber growth **(21-23)**; c) incubating with androgens that induce myofiber growth **(24-26)**, or nitric oxide that stimulates satellite cell fusion in vitro **(27,28)**, poses questions related to the role of MDSC in vivo during normal myogenesis. The first one is whether WT MDSC have a direct significant role in this process or act only during tissue repair after injury, and this occurs via conversion into satellite cells, that would then be more susceptible to myostatin, androgens, and other potential modulators of myoblast

to myotube formation. The latter effect would then resemble what we found out in vitro in the pluripotent C3H 10T1/2 cells, where myostatin did not significantly affect their differentiation into myofibroblasts, but did induce a switch to a fibrotic phenotype in the already differentiated myofibroblasts **(13)**. A study showing that myostatin stimulated fibroblast proliferation in vitro and induced its differentiation into myofibroblasts, and increased TGF β 1 expression in C2C12 myoblasts, did not examine MDSC differentiation **(14)**. The claim of a small inhibitory effect of myostatin on the fusion index in MDSC **(54)** may represent less fusion efficiency but not reflect the actual effects on the number and size of myotubes, as determined here, so that this requires further clarification in terms on the actual modulation of MDSC differentiation.

The second question is whether satellite cells per se, not MDSC, are the only myogenic progenitors during normal myofiber growth, as opposed to repair of damaged fibers **(55)**. Therefore the selected in vitro conditions will not mimic the repair process, or alternatively unknown in vivo paracrine or juxtacrine modulators may modify the response of MDSC to the better characterized agents tested in this work. A third question is whether myostatin and other modulators investigated in this work would simply stimulate in vivo satellite cell replication and fusion to the adjacent myofibers to induce hypertrophy, without truly affecting differentiation. The

We have been unable to find any report on the isolation or characterization of MDSC from the Mst KO or the mdx mice, and therefore it is also both novel and unexpected to find that these cells obtained from the same skeletal muscles, using identical procedures, and displaying rather similar non-myogenic pluripotency and stem cell marker features, are however completely unable to form myotubes in vitro or to fuse with myofibers in vivo. In fact, our prediction was that both types of cells should be more myogenic than the WT MDSC, in the case of Mst KO MDSC because of the absence of myostatin, and in the case of mdx MDSC because they originate from muscles with a considerable regeneration ability after the spontaneous bouts of necrosis that characterize this strain. Remarkably, bone marrow stem

cells isolated from the mdx mouse are unable to undergo myogenesis, despite their wild type counterparts are **(56)**

The fact that Mst replenishment, either as recombinant protein or as cDNA does not counteract the unexpected myogenic inhibition found in the Mst KO MDSC, suggests that these MDSC are not actively involved in the skeletal muscle hypertrophy that characterizes the Mst KO mouse **(49)**. The inability of undergoing myogenesis is not due to myostatin depletion per se, but rather to unknown effects of this gene inactivation on other myogenic pathways that may be defective. Although in the case of mdx MDSC we did not perform a gain of function experiment with dystrophin or its DNA, we may speculate that, considering the high regenerating ability of mdx satellite cells **(57)**, it is likely that this gene inactivation that leads to necrosis does not however impair myogenesis in vivo, and that the mdx MDSC would not participate in this process. This is also supported by the fact that, unlike the WT MDSC, the mdx MDSC implanted into the injured gastrocnemius of the mdx mouse did not fuse with preexisting myofibers or originate centrally located nuclei. However, validation of both assumptions requires further tests with other approaches. As opposed to the Mst KO MDSC, we cannot speculate whether is the absence of dystrophin or the down regulation of its down-stream pathways during gestation what inhibits myogenesis.

The juxtacrine inhibitory activity of the Mst KO MDSC on myotube formation by WT MDSC is intriguing because it would imply that a stem cell line isolated from the skeletal muscle of a mouse where myostatin is inactivated is exerting the opposite effects to what could be expected from the postulated role of myostatin in myogenesis **(10,49)**. Also, the apparent need for a cell to cell contact for Mst KO MDSC to reduce myogenesis in the WT MDSC suggests that this is a more complex process than a mere paracrine type of inhibition, and that the Mst KO MDSC are already “pre-programmed” for this effect by the genetic inactivation of myostatin. This observation, not found with the mdx MDSC, requires further investigation to exclude the

possibility that the Mst KO MDSC would overgrow the WT MDSC, although the respective proliferation rates as single cultures do not support it.

The reason why neither the Wt KO MDSC nor the mdx MDSC can undergo skeletal myogenesis may lie on “imprinted” genetic modifications on the expression of certain key genes for skeletal muscle differentiation caused by the absence of myostatin and dystrophin expression that so far had not been characterized. One such a gene is cardiac α -actin (Actc) the major striated actin in fetal skeletal muscle and in adult cardiomyocytes, but strongly down-regulated in adult skeletal muscle to 5% of the total striated actin **(58)**, whose mRNA is highly expressed in the proliferating (non-differentiating) WT MDSC but very little in the Mst KO and mdx MDSC. The same applies to the α 1-actin (Acta1) mRNA, the adult protein encoding thin filaments **(59)**. Since actins are so crucial for cell division, motility, cytoskeleton, and contraction, and mutations are associated with severe myopathies, it would not be surprising that their downregulation could cause to the lack of myogenic commitment in the Mst KO and mdx.

Similarly, the striking transcriptional down-regulation of myoD, a critical early gene in skeletal myogenesis **(60)**, and of secreted phosphoprotein 1, or osteopontin, a gene mostly involved in ossification, inflammation, and fibrosis, but postulated recently to participate in early myogenesis and skeletal muscle regeneration **(61)**, may also be related to the absence of myogenic capacity in the Mst KO and mdx MDSC. Interestingly, the fact that Pax 3 mRNA, upstream of MyoD in the myogenic signaling **(62)** is expressed to the same levels in Mst KO and mdx MDSC as in WT MDSC, but Pax 7, downstream in the signaling and related to satellite cell activation, is only expressed in WT MDSC, suggests that the myogenic commitment of Mst KO and mdx MDSC is arrested in a very early stage. Since a critical regulator of skeletal muscle development, Mef2a (Myocyte enhancer factor 2a) **(63)**, is expressed similarly in the three MDSC (as Pax 3 is), albeit at very low levels, the silencing of Pax 7 expression may be one of the earliest stages in the loss of myogenic commitment by Mst KO and mdx MDSC. Therefore, it

is not surprising that a member of the cadherin family (cadherin-15) that is involved in later stages such as myoblast differentiation and fusion **(64)** is obliterated in these MDSC.

In conclusion, our results show that MDSC obtained from wild type and transgenic mice lacking either myostatin or dystrophin express Oct-4 and other embryonic like stem cell genes and appear similar in most features, except for the null or poor expression in the Mst KO and mdx MDSC of some critical early genes in myogenesis and of others maintaining the integrity of myotubes and myofibers, thus leading to their inability to form myotubes in vitro or donate their nuclei in vivo. This would imply in the case of mdx muscle that satellite cells, that are key players in the bouts of muscle repair following necrosis in, are not affected, but it cannot be speculated to what extent the inhibition of MDSC myogenesis programmed by the absence of dystrophin may affect this repair. In turn, the genetic inactivation of myostatin not only induces similar, albeit counterintuitive, effects, but also appear to partially inhibit myotube formation by the WT MDS under cell to cell contact, thus implying a more profound modification of downstream gene cascades.

Although our results disprove the initial working hypothesis that myostatin inactivation would enhance in vitro the myogenic capacity of MDSC, and therefore turns unlikely that the KO MDSC would be more effective than WT MDSC in stimulating the repair of dystrophic muscle, this possibility needs further in vivo testing. The paracrine and juxtacrine modulation exerted in vivo during dystrophic muscle regeneration is much more complex than the one occurring in vitro and one cannot discard the possibility that genes like MyoD or Actc may be turned on in MDSC lacking myostatin and in this setting they would behave as expected and undergo a more potent myogenesis than the WT MDSC. This should be explored, as well as a similar prediction for their effects on turning on myogenically dormant MDSC in the mdx mice, so that the role of myostatin and dystrophin in the lineage commitment of MDSC, that remains elusive in vitro, could be better defined in vivo.

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LEGENDS TO FIGURES

Figure 1. Lack of dystrophin expression in the MDSC isolated from the mdx mouse. Top: representative pictures of unfixed cryosections from the gastrocnemius of the wild type (WT) and mdx mice that were immunostained for dystrophin using a secondary antibody linked to Texas red and examined for red fluorescence (....X). Bottom: representative pictures of the respective nearly confluent MDSC cultures in proliferation medium that were subjected to immunocytochemistry for dystrophin and counterstained with hematoxylin (...X).

Figure 2. A key embryonic stem cell marker (Oct-4) is expressed in MDSC irrespective of whether myostatin or dystrophin is genetically inactivated. Top: representative pictures of proliferating MDSC that were subjected to immunocytochemistry for Oct-4 (....X). Middle: left: homogenates from the same cell cultures were subjected to western blot for Oct-4; right: the pP6 cell fraction from the muscle of the Oct-4 Pr-gfp mouse (Oct-4 MDSC) was examined for green fluorescence (....X). Bottom: proliferating MDSC were subjected to cytochemistry for alkaline phosphatase (....X).

Figure 3. Myostatin or dystrophin genetic inactivation does not block the multipotent non-myogenic differentiation capacity of MDSC. Representative pictures of proliferating MDSC treated for 2 weeks in differentiation media and subjected to immuno-cytochemistry for NF-70 and ASMA to detect differentiation into neural cells and myofibroblasts (....X).

Figure 4. Myostatin or dystrophin genetic inactivation blocks the myogenic differentiation capacity of MDSC, but does not suppress nuclear Oct-4 expression. Micrographs: representative pictures of confluent MDSC from the three mouse strains maintained for 2 weeks in myogenic media and subjected to immunocytochemistry for MHC II to detect differentiation into polynucleated myotubes (....X). Bottom right: western blot for MHC II (210 kDa), nuclear Oct-4 (45 kDa), and cytoplasmic Oct-4 (33 kDa).

Figure 5. The potent myotube forming capacity of WT MDSC in myogenic medium is lowered but still maintained under high serum concentrations, in the presence of steady MyoD and ASMA expression. A: representative micrographs of myotubes generated in confluent WT MDSC maintained for 2 weeks in myogenic medium, and of confluent mdx MDSC that fail to originate myotubes, as evidenced by immunocytochemistry for MHC II. B: similar micrographs for WT MDSC in the above cultures in the three types of media subjected to immunodetection for MHC II, MyoD (...kDa), and ASMA (...kDa).

Figure 6. Confirmation by RT/PCR of changes in transcriptional expression detected by DNA microarrays. RNAs used for DNA microarrays of proliferating MDSC on Tables 1 and 2 were subjected to RT/PCR with specific primers for the number of PCR cycles stated in parenthesis, as follows: Spp1 (24), Actc1 (30), Acta1 (30), Pax3 (24), Bmp4 (30), MyoD1 (35), Mef2a (35), and GAPDH (26). Top: ethidium bromide-stained agarose gels; Bottom: densitometry of relative band intensities referred to housekeeping gene for the indicated number of PCR cycles.

Figure 7. Exogenous myostatin and follistatin fail to inhibit or stimulate myogenic differentiation of MDSC, despite the cells express the myostatin receptor and another member of the TGF β family. A-D: confluent WT MDSC in myogenic medium were incubated for 1 week with recombinant myostatin (B) or follistatin (C) or with no addition (A), subjected to immunocytochemistry for MHC II, and the relative area occupied by the myotubes was estimated by quantitative image analysis (D). Magnification: 40X. No myotubes were formed in confluent Mst KO and mdx MDSC in any treatment (not shown). E-F: western blot detection in confluent MDSC from the three mice strains of the expression of the ActRIIb (E) and TGF β 1. Myostatin was not detected (not shown).

Figure 8. Adenoviral constructs for myostatin and its shRNA are transduced and expressed in WT MDSC. A: immunocytochemistry for myostatin in HEK293 cells transduced with the AdV Mst cDNA expressing the full myostatin protein. B: western blot for myostatin in

WT MDSC transduced at 60% confluency with the same construct, either by itself or with the addition of the Gen-Jammer reagent (Stratagene, Cedar Creek, TX) (GJ); C: cytochemistry for β -galactosidase in WT MDSC transduced with the AdV Mst shRNA- β -gal expressing in tandem the shRNA against myostatin and β -galactosidase; D: control for C, with no virus. Magnification: 200X.

Figure 9. The WT MDSC do not induce myogenic differentiation in cocultures with MDSC lacking myostatin or dystrophin expression, but Mst KO MDSC reduces myotube formation by WT under cell to cell contact conditions. A: Western blot for MHC II in homogenates from MDSC grown in dual cultures on the bottom compartment under the paracrine influence of MDSC grown on the insert top compartment; B: similar western blots but for the mixes of MDSC subjected to juxtacrine cell to cell contact.

Figure 10. The in vivo repair capacity of WT MDSC on injured myofibers when transplanted to the muscle of the mdx mouse is lost in the mdx MDSC lacking dystrophin. A, B: merge of blue and red fluorescence in frozen sections of the gastrocnemius from intact WT (A) and mdx (B) gastrocnemius after Sca 1 immunostaining (Texas red/streptavidin) and counterstaining of all nuclei with DAPI (100X). C, D: merge of blue and red fluorescence for only DAPI labeled MDSC nuclei against the Texas red/streptavidin background staining for MHC II in myofibers. Frozen sections of the gastrocnemius were obtained from mdx mice subjected to cryo-injury in the muscle, implanted with DAPI-labeled WT (C) or mdx (D) MDSC, and allowed to undergo repair for 10 days (200X). MDSC nuclei centrally located within myofibers are indicated with yellow arrows.

TABLE 1**Some stem cell-related genes are transcribed similarly in MDSC irrespective of myostatin or dystrophin genetic inactivation**

RNAs were isolated from proliferating MDSC and subjected to DNA microarrays. The expression of some key genes that did not change significantly among the three MDSC is tabulated, selected from 98% of the other genes whose expression remained unchanged

		WT	KO	mdx
Myc	Myelocytomatosis oncogene	12.4	18.1	15.0
Pou 5M	Pou doman (Oct4)	10.1	16.7	11.5
Akp 2	Alkaline phosphatase 2	6.4	6.9	6.3
Akp 5	Alkaline phosphatase 5	1.2	1.6	1.2
Tert	Telomerase reverse transcriptase	1.0	1.0	0.7
Utf 1	Undifferentiated embryonic cell TP1	1.0	0.8	1.0
Man 1	Mastermind like 1	13.1	16.7	12.8
Lif	Leukemia inhibitory factor	1.5	0.9	0.9
PPARγ	Peroxisome proliferating ARγ	1.1	1.8	1.5

TABLE 2

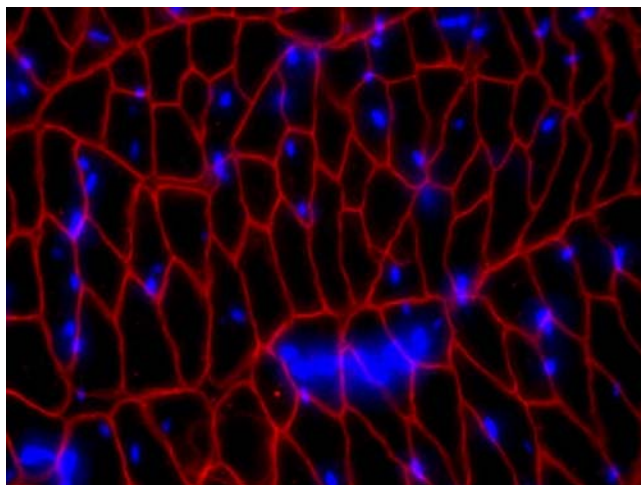
Some skeletal myogenesis-related genes are considerably down-regulated in MDSC by myostatin or dystrophin generic inactivation, where others remain unchanged

Results are from the same DNA microarrays on Table 1, with the top six rows corresponding to mRNA considerably reduced in Mst KO and mdx MDSC as compared with WT MDSC

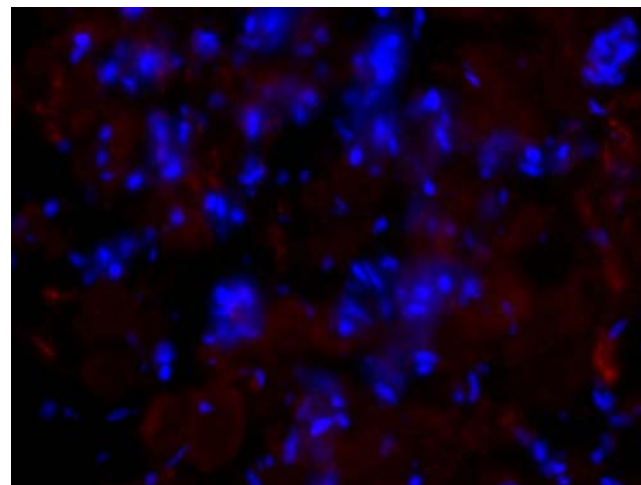
		WT	KO	mdx
SPP 1	Secreted phosphoprotein 1 (osteopontin)	70.8	20.3	7.0
Actc 1	α actin (cardiac)	39.9	6.5	4.9
Myo D1	Myogenic differentiation 1	17.5	2.7	2.7
Cadherin 15	Cadherin 15	8.7	1.7	1.3
Myf 5	Myogenic factor 5	4.2	2.7	2.7
Notch 2	Notch gene homolog 2	4.2	2.8	2.6
BMPR 2	Bone morphogenic receptor 2	23.3	20.3	19.5
BMPR 1a	Bone morphogenic receptor 1a	8.1	10.4	8.3
BMPR 1b	Bone morphogenic receptor 1b	0.8	0.8	1.0
BMPR 4	Bone morphogenic protein 4	2.7	2.7	2.8
IGF 1	Insulin-like growth factor 1	5.1	4.2	5.3
Jag 1	Jagged 1	2.8	3.4	5.1
Fzd 1	Frazzled homolog 1	2.7	2.8	3.4
Notch 1	Notch gene homolog 1	2.6	2.7	1.2
Notch 3	Notch gene homolog 3	2.8	2.6	1.3

**SKELETAL
MUSCLE**

WT



mdx



MDSC

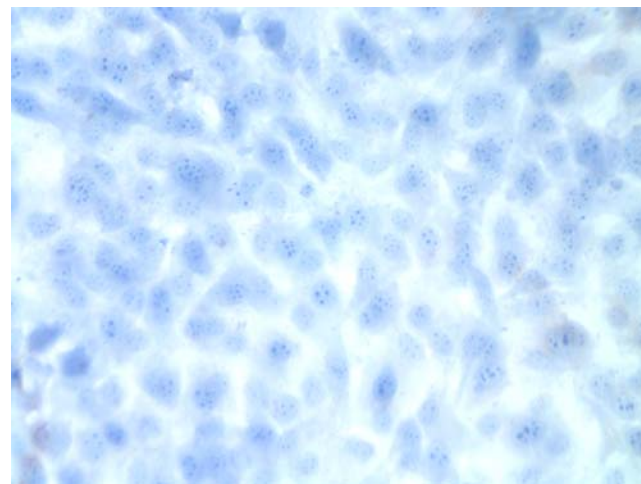
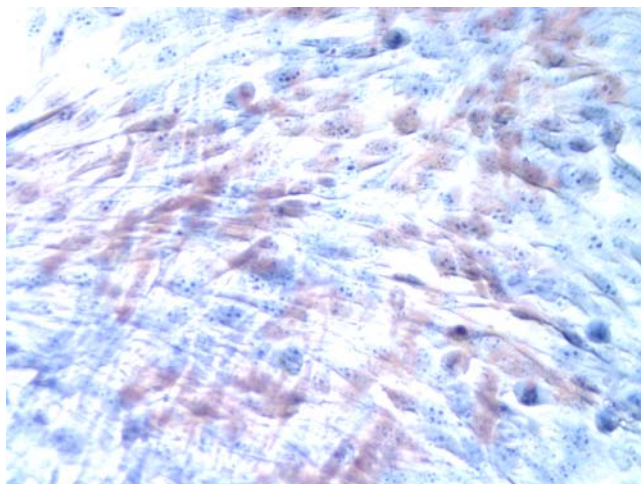


Fig 1

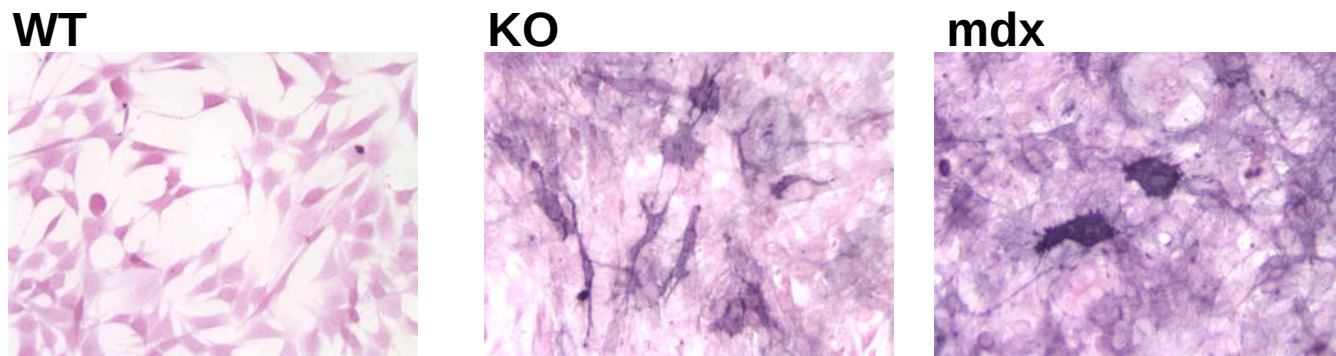
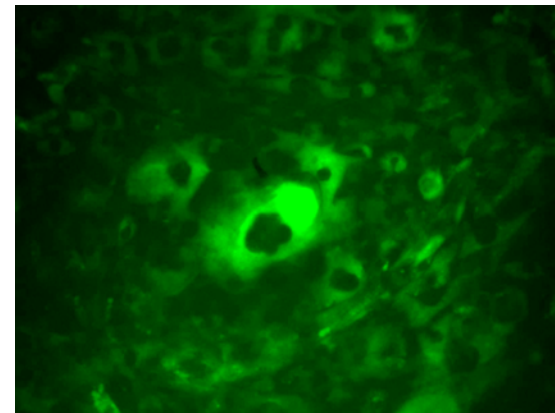
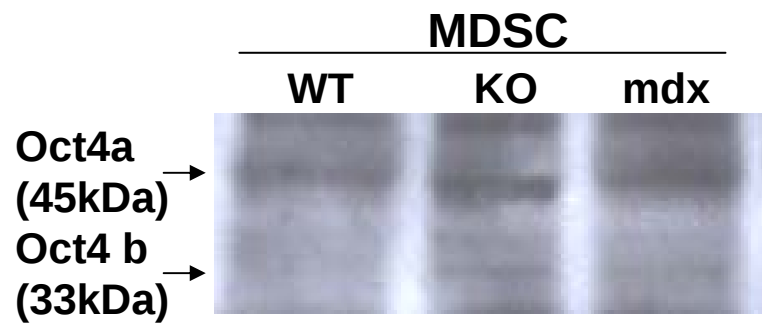
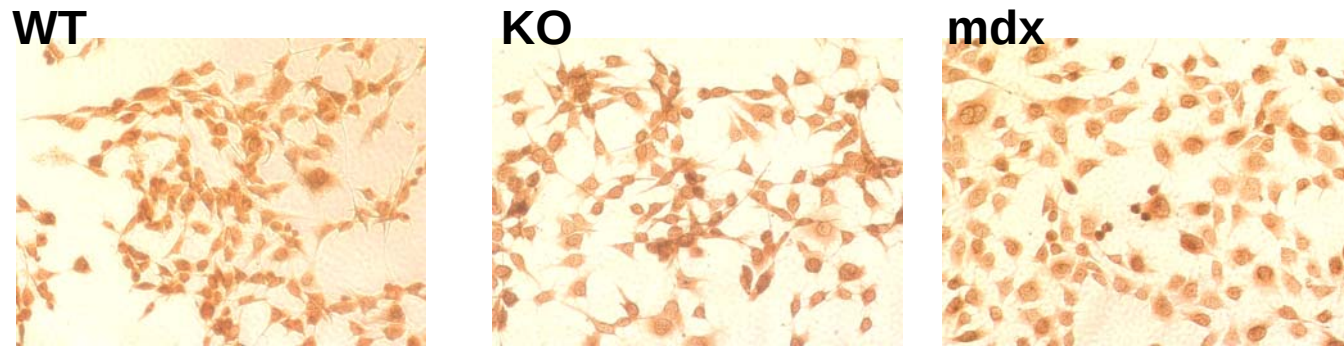


Fig 2

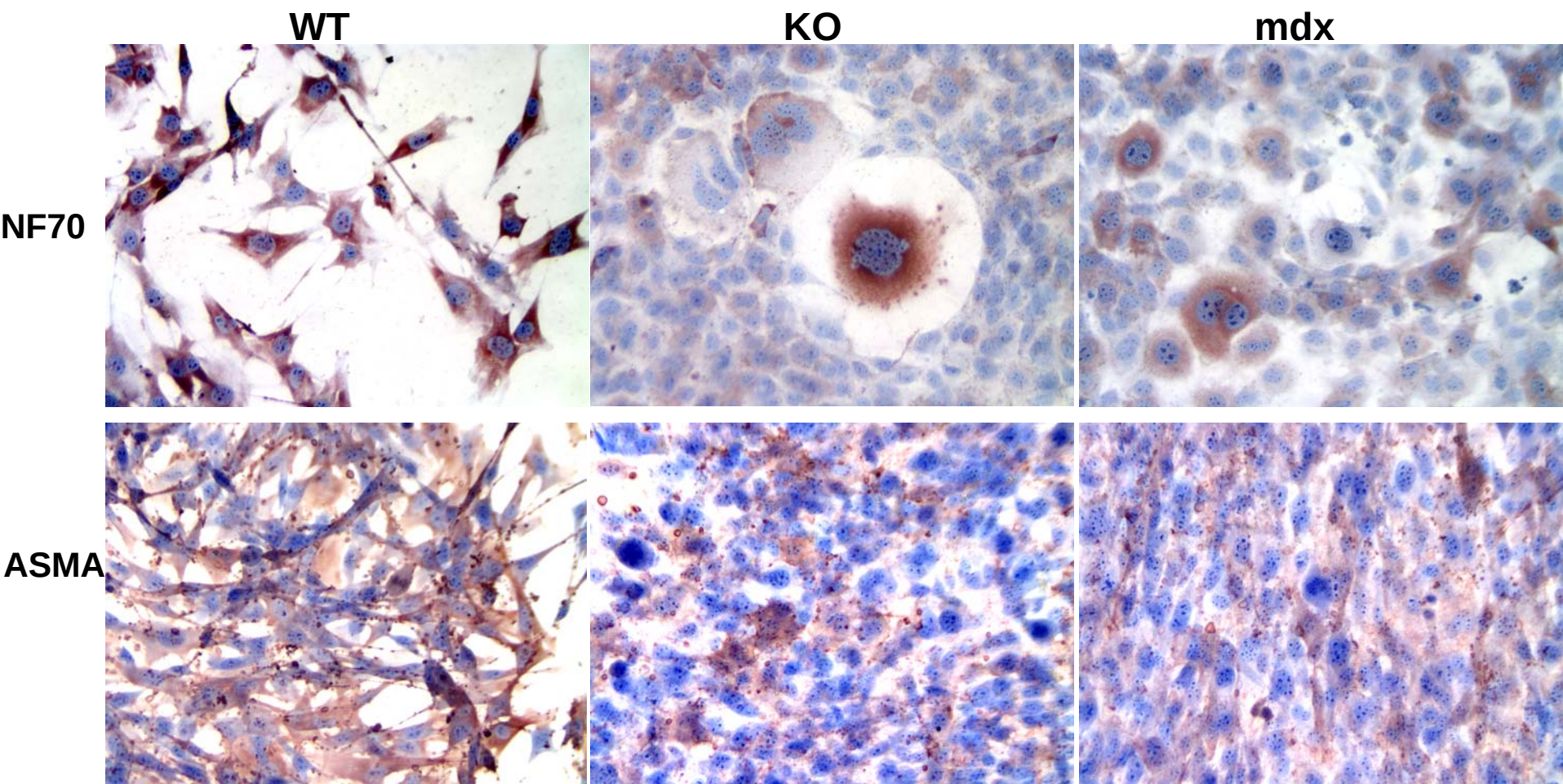


Fig 3

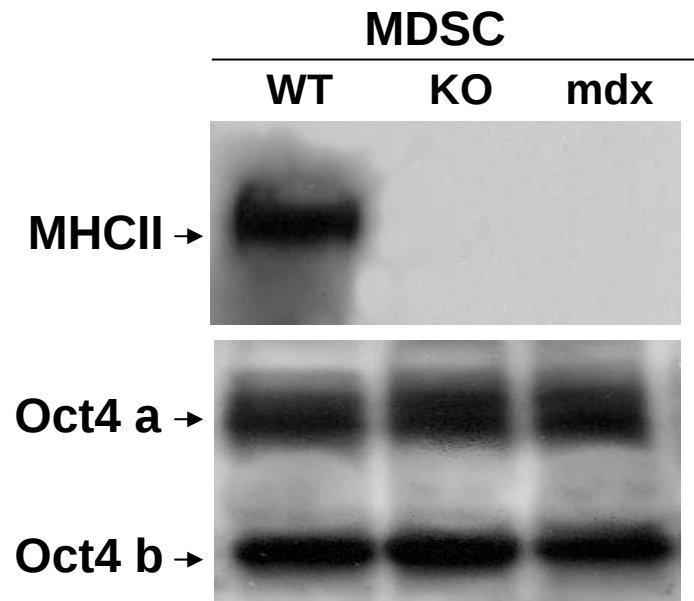
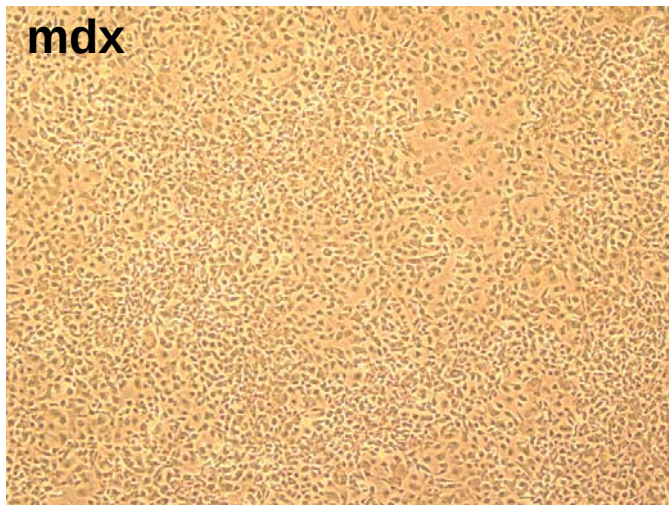
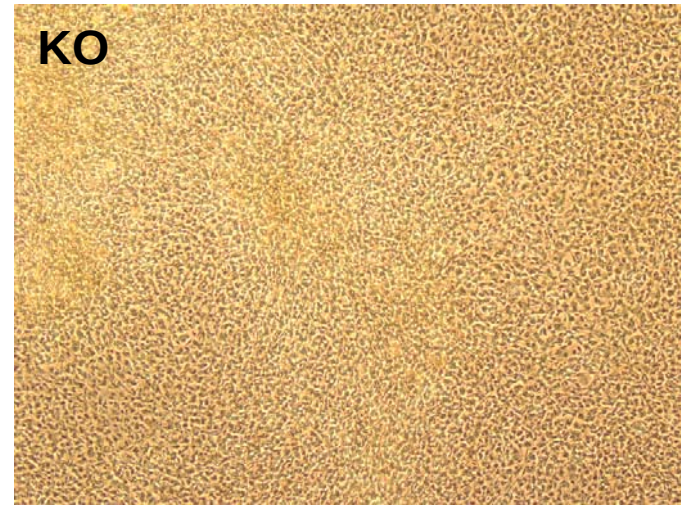
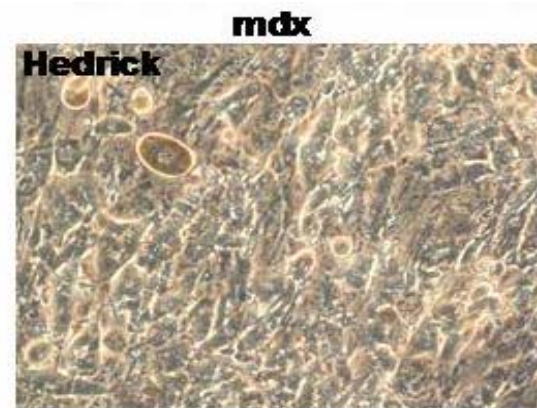
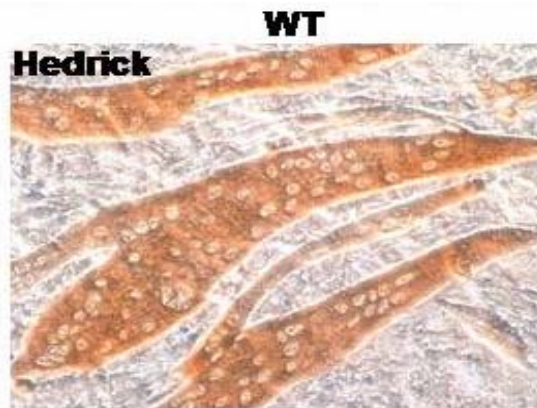
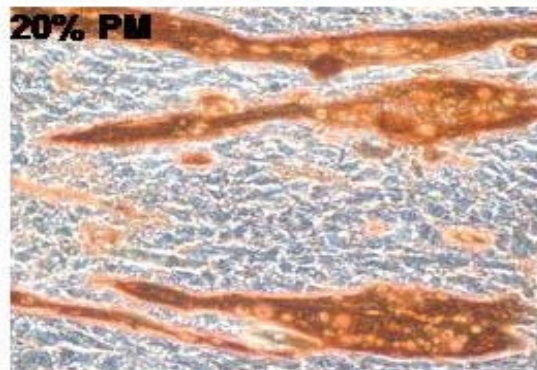


Fig 4

A



B



E MHC II



F MyoD



G ASMA



Fig 5

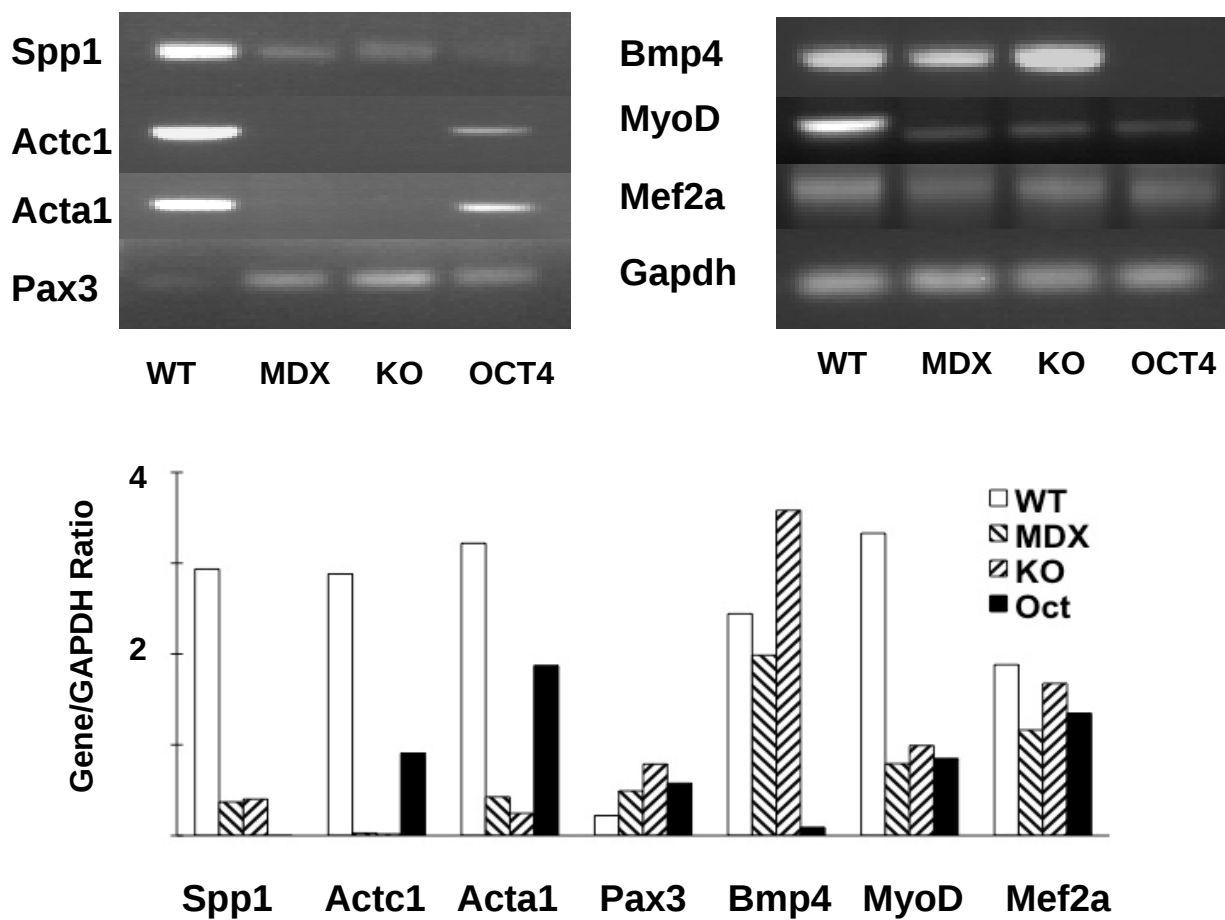
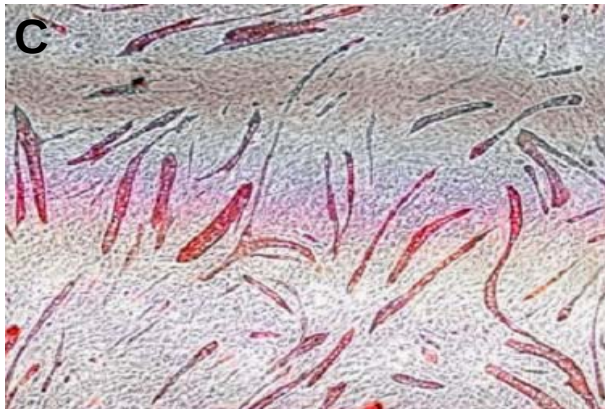
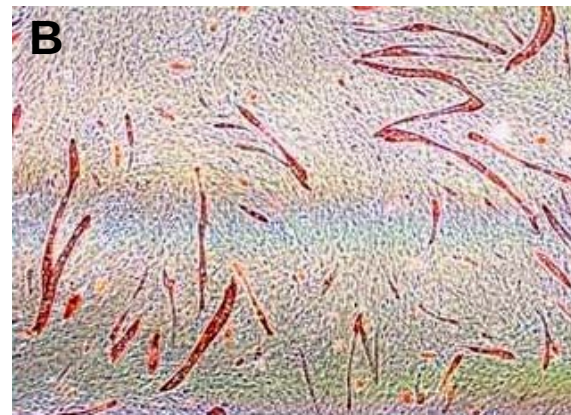
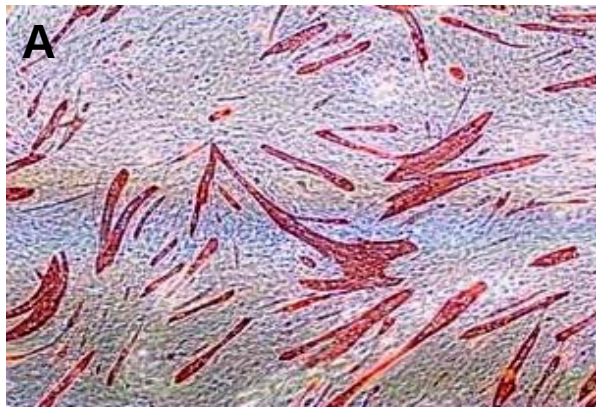
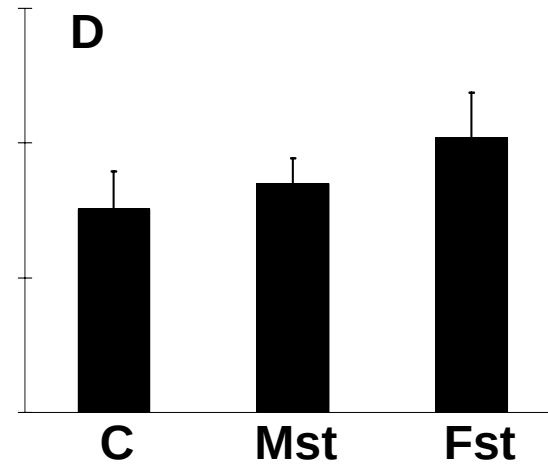


Fig 6



Percent Area MHC II



E

Activin receptor
type IIB

58 kDa →



F

TGF β 1

12.5 kDa →



Fig 7

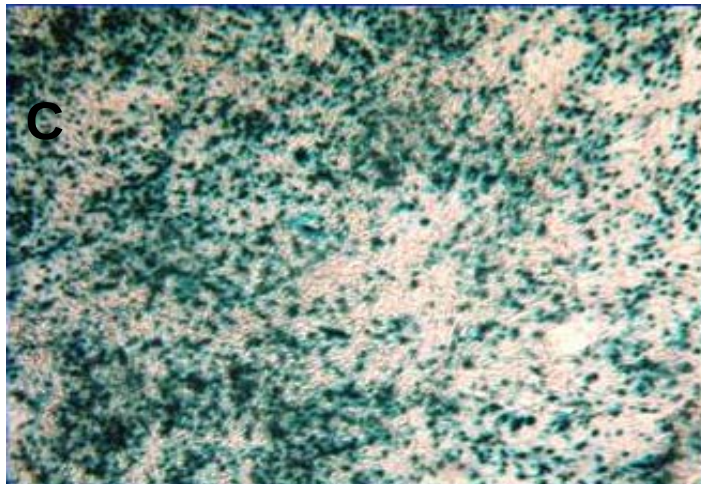
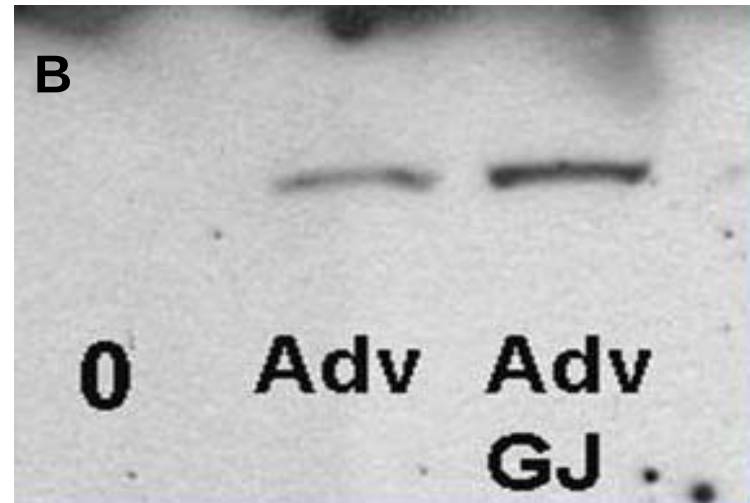
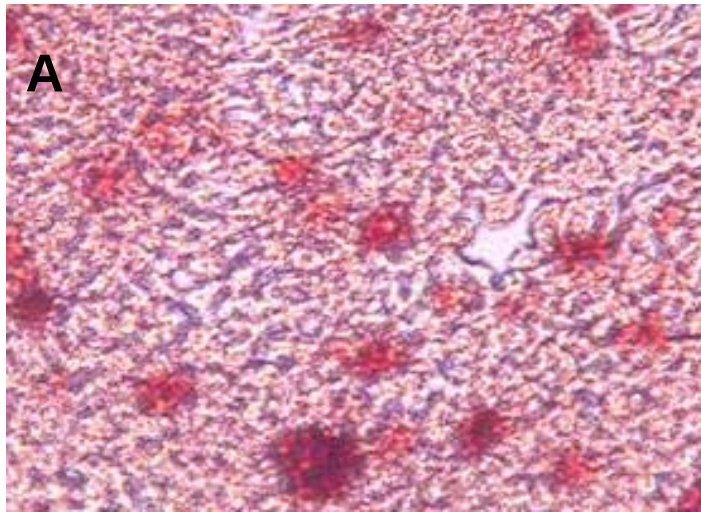
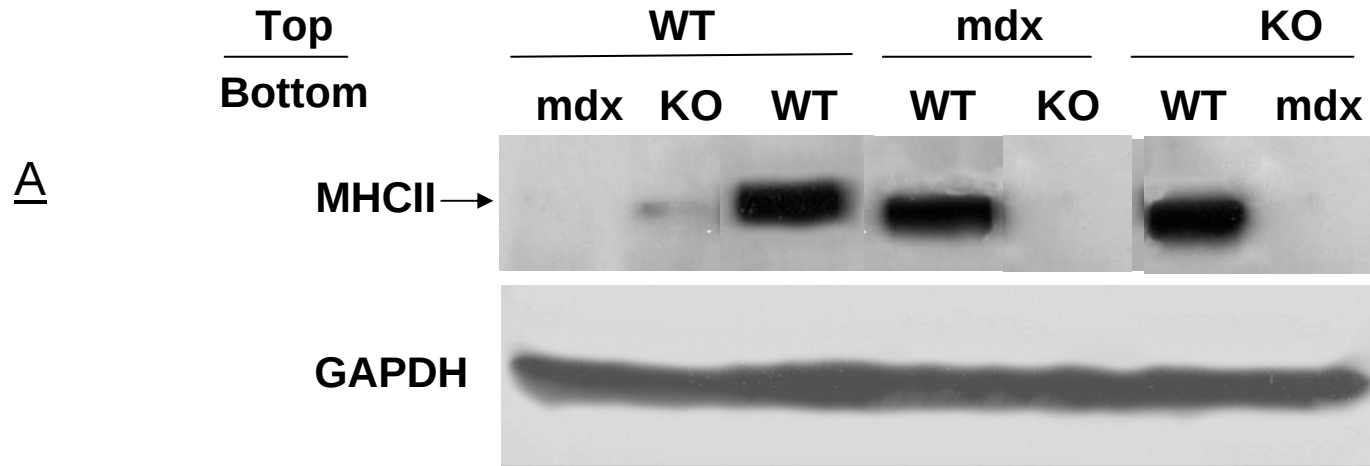


Fig 8

PARACRINE EFFECTS



JUXTACRINE EFFECTS

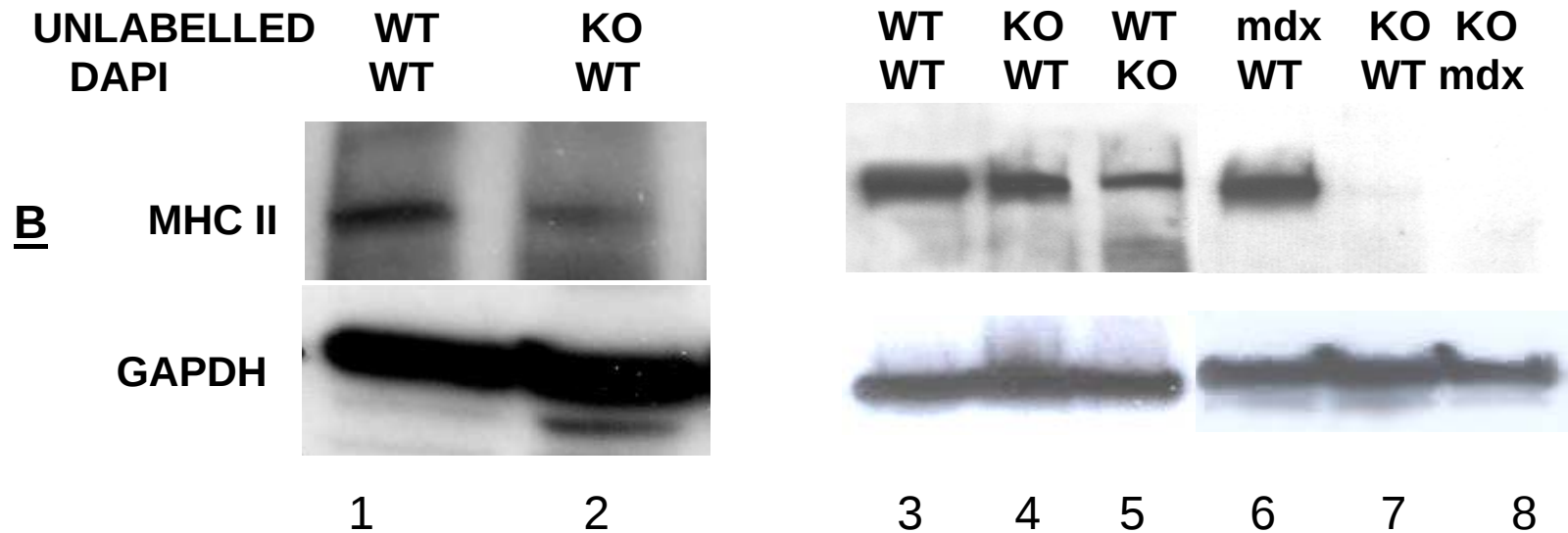


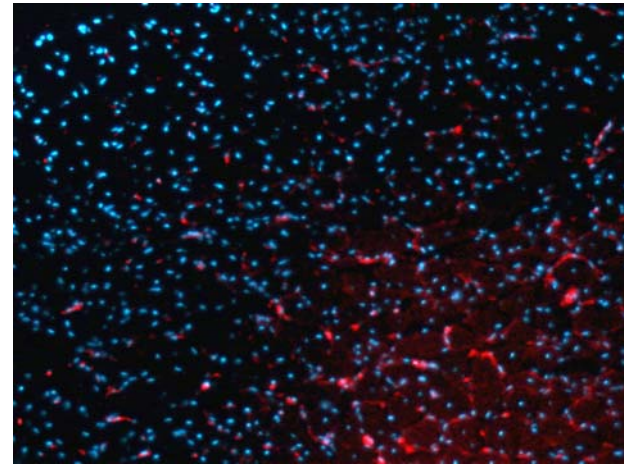
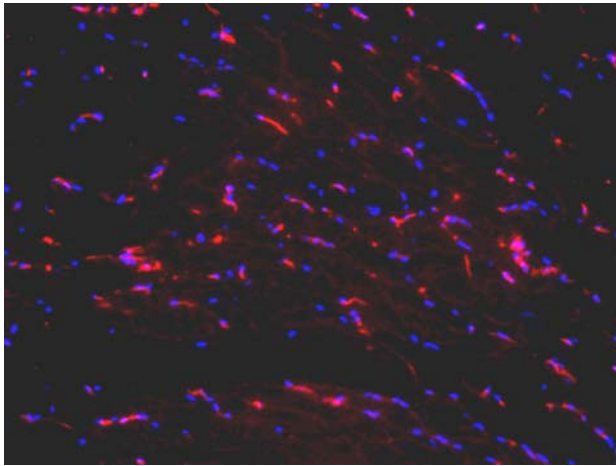
Fig 9

WT

mdx

Intact

SCA1



Injured
+ MDSC

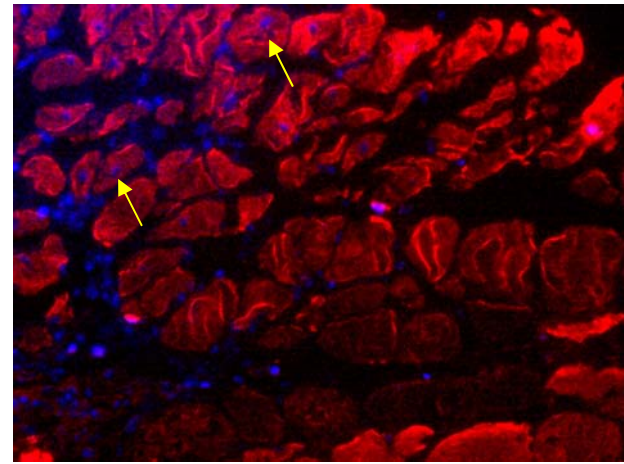
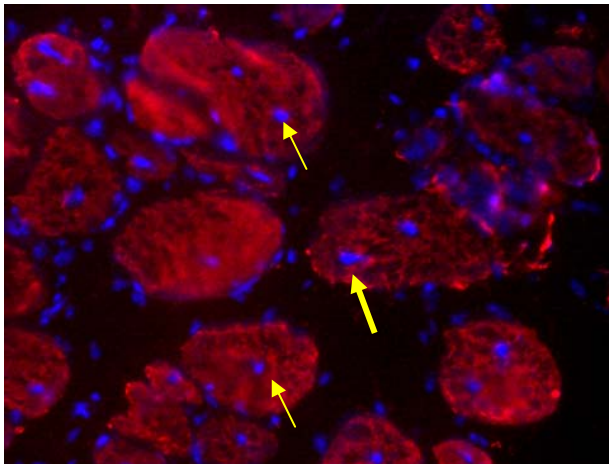


Fig 10

Treatment of Peyronie's disease with PDE5 inhibitors: an antifibrotic strategy

Nestor F. Gonzalez-Cadavid and Jacob Rajfer

Abstract | Peyronie's disease (PD) is a localized fibrotic condition of the tunica albuginea that is associated with risk factors for corpora cavernosa fibrosis (such as advanced age and diabetes) and Dupuytren contracture, another localized fibrotic process. Most of the current pharmacological treatments for PD are not based on antifibrotic approaches that have shown promising results in animal models and clinical efficacy in other fibrotic conditions, which may explain why they are generally unsuccessful. Evidence gathered in human specimens and animal models of PD have elucidated aspects of its etiology and histopathology, showing that overexpression of transforming growth factor β 1, plasminogen activator inhibitor 1, reactive oxygen species and other profibrotic factors, which are, in most cases, assumed to be induced by trauma to the tunica albuginea, leads to myofibroblast accumulation and excessive deposition of collagen. At the same time, a steady overexpression of inducible nitric oxide synthase, leading to increased nitric oxide and cGMP levels, seems to act as an endogenous antifibrotic mechanism. This process has also been reported in corporal and cardiovascular fibrosis, and has led to the demonstration that long-term continuous administration of phosphodiesterase type 5 inhibitors counteracts the development of a PD-like fibrotic plaque in a rat model, and later extended to the prevention of corporal fibrosis in animal models of erectile dysfunction.

Gonzalez-Cadavid, N. F. & Rajfer, J. *Nat. Rev. Urol.* advance online publication 9 March 2010; doi:10.1038/nrurol.2010.24

Introduction

Peyronie's disease (PD) is somewhat of a misnomer, as it is actually restricted to a small localized fibrotic plaque of the tunica albuginea of the penis.^{1,2} Epidemiologic studies suggest that the disease may be present in up to 10% of all men, but primarily affects those in their sixties and seventies.^{1–6} The reason PD attracts attention is that many men with the disease have some form of erectile dysfunction, and in the erect state the afflicted organ tends to curve and may be painful during intercourse. Despite its typical fibrotic histopathology, this condition is not associated with other localized or diffuse fibrotic processes, with the single exception of Dupuytren contraction, with which it shares a similar histopathology.^{7–9} No satisfactory medical treatments for PD are currently available; however, experimental models have provided new insights into its pathophysiology and etiology, which have facilitated the investigation of alternative therapeutic approaches, including long-term continuous administration of phosphodiesterase type 5 (PDE5) inhibitors¹⁰ as an antifibrotic modality. In this Review we examine the experimental evidence that forms the basis for this treatment strategy. No reports of the clinical efficacy of long-term PDE5 inhibition in patients with PD have been published, and, although the first preliminary animal study dates back to 2003,¹¹ this should still be considered as a novel management approach that requires future clinical validation.

Competing interests

The authors declare no competing interests.

Pathophysiology

A widely accepted hypothesis on the etiology of the PD plaque is that it originates from trauma or microtrauma to the erect penis, primarily during different types of sexual activities.¹² This hypothesis is based mainly on the demonstration of fibrin staining or immunodetection in tissue sections of human PD plaques,^{13–15} findings that have been corroborated in an animal model of PD.¹⁴ A plausible interpretation of this hypothesis is that the fibrin originates from fibrinogen that has extravasated into the interstices of the tunica albuginea during a traumatic sexual episode. Inhibition of the fibrinolytic system or an inability to degrade the intravasated fibrin would then lead to its persistence in the tunica, which initially leads to an acute inflammatory response. Because the fibrin is not degraded, the protein continues to exert a proinflammatory response, which ultimately leads to an abnormal healing process. The end result is the formation of a 'scar' that at some time evolves into a palpable plaque.¹⁶ Indeed, injection of fibrin directly into the tunica albuginea of the rat penis elicits a PD-like plaque, resembling in many aspects the histology and evolution of the human PD plaque.^{14,15,17}

The epidemiological association of PD with a history of sexually elicited trauma of the penile or pelvic surgery, which may affect the homeostasis of tissues in the penis, supports the trauma-related hypothesis for at least part of the patient population,^{8,18,19} despite some contradictory evidence.^{5,8,20} Considering the association of PD with Dupuytren contracture, genetic or immune-related predisposition to PD may modulate the tunical healing

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Key points

- Rodent models of Peyronie's disease (PD) are representative of most of the main histological and biochemical features present in human specimens
- Cell cultures obtained from the human PD plaque and its rat counterpart have added to the experimental evidence acquired in the human and in animal models
- Endogenous mechanisms of defense against tunical tissue inflammation, oxidative stress and fibrosis have been detected in the PD plaque and the rat PD-like lesion, and may be mimicked pharmacologically for treatment
- Endogenously elicited inducible nitric oxide synthase leads to sustained production of nitric oxide and cGMP, which counteract myofibroblast differentiation, accumulation of reactive oxygen species, cytokine release, and collagen deposition
- Continuous long-term administration of nitric oxide donors and phosphodiesterase 5 inhibitors has shown preventive and corrective effects in a rat model of PD: studies in patients with PD are now needed

reaction following any type of trauma to the penis, but this possibility has not been studied as intensively as is needed.^{21–23}

The pathology of the PD plaque has been investigated in a variety of studies in human and animal model specimens and in related cell cultures.^{1,10} Based on the results of histochemistry, immunohistochemistry and other assays performed on the human PD plaque tissues, it is clear that fibrosis—the excessive deposition of collagen and extracellular matrix (ECM) with disorganization of collagen fibers and loss of elastic fibers—is the main pathological process, combined in most cases with fibrin accumulation and different degrees of inflammation.^{12–14,24,25}

Myofibroblasts (cells that share the fibroblast and smooth muscle phenotypes^{26,27}) are not normally present in the tunica albuginea of the penis, but have been identified as the cells responsible for the disarrangement of the ECM in the PD plaque.^{28–32} The normal process of apoptosis that eliminates myofibroblasts after they have fulfilled their role in wound healing is somehow inhibited in PD, thus leading to their persistence in the tunica albuginea. This myofibroblast accumulation is common not just to scar formation in the skin or the infarcted heart, but to most other types of fibrosis.^{26,27} The PD plaque becomes harder by progressing through an intensification of fibrosis (with or without the persistence of inflammation) and, in at least 15% of the patients, through an advanced stage of calcification and ossification involving osteoblasts.^{33,34} Spontaneous regression of the plaque after its initial formation occurs in rare cases.^{9,34}

The scattered evidence regarding human PD plaque tissues has been considerably expanded by systematic approaches in experimental animal models, mainly in the widely used rat model of PD induced by transforming growth factor $\beta 1$ (TGF- $\beta 1$).^{1,10} This key profibrotic factor, present in multiple tissues³⁵ and produced in the human PD plaque, is found at increased levels in the blood of PD patients.³⁶ Similarly, when a peptide derived from the TGF- $\beta 1$ sequence is injected into the rat tunica, a plaque resembling that seen in human PD is found around

45 days later^{11,37–41} at the injection site. Other less frequently employed but nonetheless useful animal models are based on either the successive injections of an adenoviral construct expressing a constitutively active TGF- $\beta 1$ protein, leading to penile curvature during the erect state and, at times, calcification within the plaque,⁴² or on a single fibrin injection that mimics the extravasation of fibrinogen, initiating acute inflammation followed by the rapid development of the PD-like plaque.^{14,15,17} Both TGF- $\beta 1$ and myostatin, another profibrotic factor within the TGF- β family, are involved in this process,⁴¹ and it is quite likely that the key downstream signaling occurs via the Smad pathway, which is the mechanism common to most factors in this family.⁴³ A tight skin (Tsk) mouse model has been described that develops a spontaneous PD-like plaque with penile bending and areas of chondroid metaplasia with heterotypic ossification.⁴⁴

These animal models, therefore, represent most of the histologic and biochemical features of the human PD plaque, including inflammation, myofibroblast accumulation, collagen deposition, oxidative stress, calcification, ossification and penile bending, among others. Thus, we believe that the PD-like lesion, either elicited experimentally or by spontaneous mutations in the rodent tunica, is more complex than a mere tunical fibrosis event, and, imperfect as most disease models in laboratory animals are, this experimental plaque is adequate for preclinical testing of various therapeutic strategies for PD.

Finally, the use of cell cultures from the normal, myofibroblast-free tunica albuginea or from the human PD plaque or the induced PD-like plaque from a rat, which are enriched in myofibroblasts, has allowed us to more precisely define the role of myofibroblasts in the pathophysiology of PD.^{28–32,45,46} These cells have been shown to be responsible for the excessive collagen deposition seen in PD, and have even been postulated to cause penile bending by their contractile features. Moreover, pluripotent stem cells have been identified in the PD cultures. This may explain the fibrotic and osteogenic progression of the PD plaque upon the release of cytokines following microtrauma to the penis, which would stimulate stem cell commitment to this cell lineage.^{31,45} PD fibroblasts are also potentially tumorigenic, or acquire this trait upon culture, but it is not known whether this is related to the presence of stem cells.⁴⁷

There is no doubt that cell cultures derived from the human PD plaque and normal tunica albuginea closely represent their respective histologic features, notwithstanding the obvious shortcomings of any type of cell culture compared to the *in vivo* tissue. This has been tested with a multiplicity of immunocytochemical and western blot markers, as well as DNA microarrays and reverse transcription polymerase chain reaction procedures for the detection of fibroblasts, myofibroblasts and stem cells, and their respective differentiation and roles in inflammatory and fibrotic processes. The situation is similar regarding cell cultures obtained from the rat PD-like plaque, which have been shown to mimic their human counterparts. All these cultures have been useful

tools for defining therapeutic targets at the cellular and molecular level.

Cellular and molecular mechanisms

Results from experimental studies that have employed a variety of cellular and molecular biology techniques in the PD models described above, combined with the information obtained from the analysis of the human PD plaque, have made it possible to define an overall mechanistic picture of the initiation and progression of the PD plaque.⁴⁸ The mechanism resembles that seen in some other localized fibroses, including the more gradual and diffuse type that occurs in the penile corpora cavernosa of men with erectile dysfunction and many animal models of this disorder.⁴⁹ The main features of PD fibrosis are described below.

Fibrinogen extravasated into the tunica albuginea of the penis accumulates at the site of the future PD plaque owing to inhibition of the fibrinolytic and other proteolytic systems, primarily due to overexpression of plasminogen activator inhibitor 1 (PAI-1). The resulting fibrin formation, and possibly with the assistance of immunoglobulins and other extravasated proteins, triggers the release and/or activation of TGF- β 1, PAI-1, and reactive oxygen species (ROS), which are recognized as key profibrotic factors in many tissues, including the kidney and vascular system. Concurrent expression of other cytokines, including monocyte chemoattractant protein 1 (MCP-1; also known as CC-chemokine ligand 2 [CCL-2]), which is associated with acute inflammation that often progresses to a chronic phase, overexpression of other members of the TGF- β 1 family (such as myostatin) and components of their common Smad signaling pathway, and other unknown agents combine to elicit the fibrotic process. The PD plaque then develops through excessive collagen deposition, elastin degradation, myofibroblast differentiation from fibroblasts or stem cells in the tunica, oxidative stress, and eventually calcification (Figure 1).^{10,48,50,51}

The accumulation of tissue inhibitors of metalloproteinases (TIMPs) and the relative inhibition of collagenases (and/or a possible downregulation of their expression), which interferes with the normal breakdown of the accumulated collagen—and potentially, in the case of TIMPs, with therapeutic collagenase delivered to the plaque—contribute to the maintenance of the fibrotic process.⁵²

One of the main findings stemming from DNA microarray analysis of the molecular profile of the PD plaque is the recognition that this tissue may be undergoing constant cellular and molecular turnover, and that spontaneous development of defense mechanisms to counteract fibrosis and oxidative stress might occur.^{53,54} This transcriptional analysis detected overproduction of matrix metalloproteinases (MMPs) 2 and 9 (which contribute to collagen breakdown), decorin (which binds and neutralizes TGF- β 1), and thymosins (which activate MMPs) in both PD plaques and Dupuytren nodules, as well as in cell cultures of these tissues. All these proteins seem to act as antifibrotic agents, either by combating

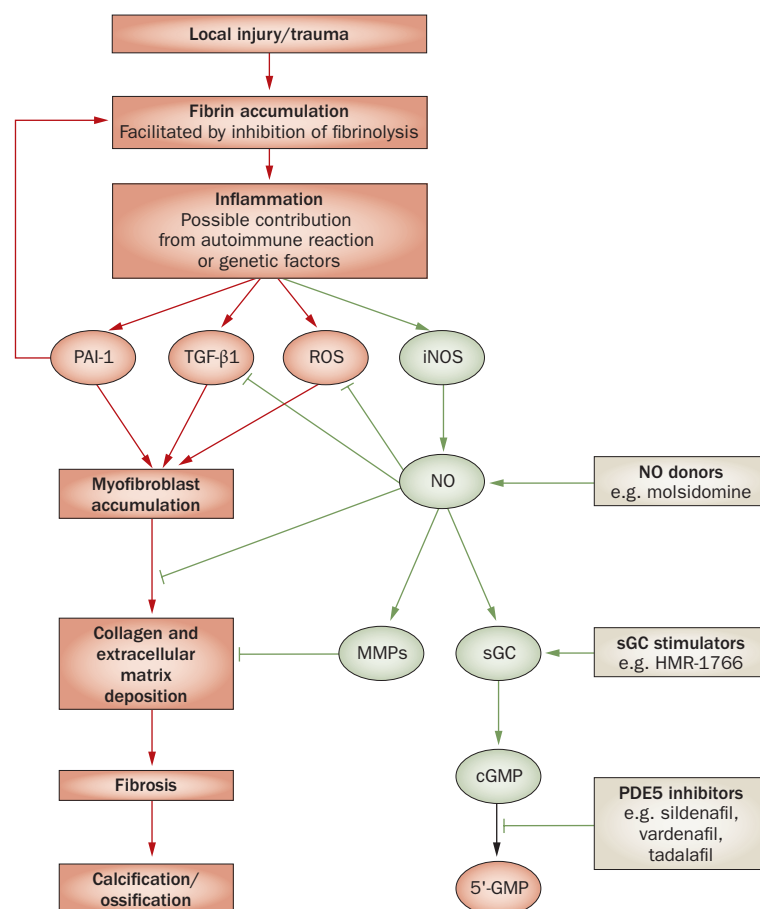


Figure 1 | Schematic representation of the etiology and pathophysiology of the fibrotic process leading to the Peyronie disease plaque (red), and the antifibrotic effects (green) of endogenous iNOS. Fibrin extravasated after injury or trauma to the penis accumulates in the tunica albuginea, which induces a local proinflammatory response. This leads to increased production of several key profibrotic factors, including PAI-1 (which inhibits fibrinolysis), TGF- β 1 and ROS, which contribute to the accumulation of myofibroblasts. Myofibroblasts are responsible for the increased collagen and extracellular matrix deposition leading to fibrosis, and, owing to inhibited apoptosis of these cells, persist in the tunica albuginea. The production of iNOS seems to be an endogenous antifibrotic factor, leading to increased levels of NO in the affected tissue. NO inhibits collagen synthesis and myofibroblast differentiation and reduces oxidative stress by quenching ROS. In addition, NO increases the activity of sGC, which in turn increases levels of cGMP which has further antifibrotic effects. Potential pharmacological interventions (gold) that might prevent or partially reverse plaque formation include NO donors, sGC stimulators and PDE5 inhibitors. Abbreviations: iNOS, inducible nitric oxide synthase; MMPs, matrix metalloproteinases; NO, nitric oxide; PAI-1, plasminogen activator inhibitor 1; PDE5, phosphodiesterase type 5; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; TGF- β 1, transforming growth factor β 1.

collagen deposition or promoting its breakdown; therefore, it is plausible to postulate that they are produced in response to the fibrotic processes and that progression depends on the balance between the noxious and protective mechanisms, which in some cases may lead to spontaneous regression of the plaque.

Role of inducible nitric oxide synthase

Despite the experimental evidence outlined above, the current pharmacological management of PD is mostly

empirical, as it is generally based on the use of drugs targeting nonspecific or ancillary aspects of PD, such as inflammation or cell replication.^{2,55} Virtually nothing has been translated from the abundant pharmacological studies in other types of fibrosis, or from pre-clinical studies in animal or cell culture models of PD. This renders PD a sort of orphan disease in terms of a scientifically rational approach to therapy, in contrast to the other types of fibrosis where, in general, clinical use is supported by promising preclinical studies.^{48,56}

However, among the putative endogenous mechanisms of defense against fibrosis that are postulated to operate in the PD plaque, the most intensively studied and highly promising in terms of therapeutic potential is the spontaneous induction of inducible nitric oxide synthase (iNOS), a NOS isoform that is not expressed in normal penile tissue.⁵⁷ Whereas in the past it was assumed that the presence of iNOS portended a deleterious outcome to a tissue, it is now believed to in fact be a protective mechanism against tissue fibrosis in certain settings. iNOS expression produces a steady output of nitric oxide, a compound that directly inhibits collagen synthesis and myofibroblast differentiation, quenches ROS via the production of peroxynitrite, and inhibits the TGF- β /Smad signaling pathway, thus counteracting fibrosis.^{11,17,25,30,41} The pro-apoptotic effects of nitric oxide might also contribute to reducing the myofibroblast population. Remarkably, iNOS production in the penis is not restricted to the tunica albuginea or to PD. For example, iNOS is detectable in the corpora cavernosa of patients with diabetes, advanced age, and even following radical prostatectomy, where fibrosis of the corpora leads to the development of corporal veno-occlusive dysfunction (CVOD). iNOS production may also been seen in the penile arteries in disease states where arteriosclerosis or arterial stiffness is present.^{57–64} In all these scenarios, iNOS production, resulting in some cases from an inflammatory process, is presumed to be an antifibrotic response to the development of fibrosis within these individual tissues.

Collectively, several lines of evidence in rodent models support the antifibrotic role of iNOS in the PD plaque and in corporal and vascular tissue. Gene transfer of iNOS cDNA, which then becomes constitutively expressed, reduces fibrosis in the tunica albuginea and corpora of the penis, whereas long-term, specific inhibition of iNOS activity (by the iNOS inhibitor L-NIL) counteracts this process in both tissues, as well as in the arterial wall.^{17,25,58,60} Furthermore, genetic inactivation of the iNOS gene in the iNOS knockout mouse intensifies collagen deposition in the corpora in a process exacerbated by diabetes.⁶⁴ This agrees with what has been shown in other tissues in the iNOS knockout mouse model, in which the absence of iNOS increases interstitial fibrosis after unilateral ureteral obstruction and hepatic fibrosis in animals fed a high-fat diet and in those with streptozotocin-induced diabetic nephropathy.^{65–70} iNOS also has a cardioprotective role in preconditioning during ischemia reperfusion injury in mouse kidney and in granulomatous disease.^{71,72}

Some evidence suggests that iNOS deletion seems to be protective rather than detrimental in certain types of fibrosis,^{73–76} and that iNOS overexpression is associated with increased fibrosis, particularly in the diabetic kidney.⁷⁷ However, nitric oxide is known to inhibit myofibroblast differentiation via inhibition of the TGF- β /Smad pathway, and to have general antifibrotic effects via inhibition of collagen synthesis and ROS quenching.^{78–82} The protective effects of iNOS depend, therefore, on the specific tissue type and the pathological conditions under which it is induced. In the case of the penile tissues, including the PD plaque, all the evidence so far obtained supports an antifibrotic role for iNOS.

Treatment with PDE5 inhibitors

Although some of the beneficial, antifibrotic effects of iNOS are directly attributable to nitric oxide, others may result from the increased levels of cGMP produced following stimulation of guanylate cyclase by nitric oxide, which subsequently leads to protein kinase G activation. cGMP, and in some cases PDE5 inhibitors, have been shown to inhibit myofibroblast formation in cell cultures of human and rat PD plaques^{30,32} and in lung fibroblasts.⁸³ These antifibrotic effects are also exercised by guanylate cyclase stimulators via protein kinase G stimulation and inhibition of fibrotic mediators such as angiotensin II, or by TGF- β or Rho activation.^{83–87}

An early preliminary study in the rat model of TGF- β 1-induced PD demonstrated that both oral sildenafil, a PDE5 inhibitor that protects cGMP from breakdown, and oral pentoxifylline, a predominantly PDE4 inhibitor that increases cAMP synthesis, counteract the development of the PD-like plaque.¹¹ In the case of pentoxifylline, it was proposed that the well-known cAMP–cGMP signaling crosstalk may be responsible for its antifibrotic effects, although direct effects of cAMP or the involvement of alternative pathways modulated by pentoxifylline can not be excluded. This study revealed a completely new mechanism of action for PDE5 inhibitors, in contrast to their standard on-demand clinical administration to facilitate penile erection upon sexual stimulation, which is mediated by their short-term relaxant effect on the corporal and arterial smooth muscle produced by a transient elevation of cGMP levels. The novel concept is that PDE5 inhibitors given for a sufficiently long time can induce a sustained elevation of nitric oxide and cGMP levels that, independently of their vasorelaxant effects, which would show only during sexual stimulation, act as antifibrotic agents by reducing collagen deposition, profibrotic factor release, oxidative stress and myofibroblast numbers.

In a subsequent study in the same rat model, it was shown that another PDE5 inhibitor, vardenafil, given orally and in different dosing regimens, not only prevented but partially reversed the formation of the PD-like plaque.⁴⁰ To test the early preventive effects of vardenafil, the drug was administered to male rats either in their drinking water or as a once-daily oral instillation at either 1 or 3 mg/kg per day for 45 days following a single injection of TGF- β 1 into the tunica albuginea

to induce the PD-like plaque. Other animals, in which a PD-like plaque had already been formed, received either dose of vardenafil in their drinking water for 42 days (late, therapeutic administration). Preventive treatment at the higher dose (both continuous and once-daily treatments) reduced the overall collagen content, collagen III/I ratio and the number of myofibroblasts and TGF- β 1-positive cells, and selectively increased the apoptotic index of cells (presumably including myofibroblasts), in the PD-like plaque. The lower dose was less effective. When vardenafil was given continuously in the drinking water for 42 days after the PD-like plaque was formed, a partial reduction in plaque size was observed. From these two studies,^{11,40} it was concluded that long-term oral treatment with a PDE5 inhibitor slows and reverses the early stages of an experimental PD-like plaque in the rat, and might ameliorate a more advanced plaque.

The optimal therapeutic regimen for discontinuous oral administration of PDE5 inhibitors was not assessed in these studies, so whether oral instillation, perhaps at a higher dose, can regress an already formed plaque is not known. However, the authors discussed the possibility of testing combinations of PDE5 inhibitors and other compounds used for the treatment of PD, such as verapamil (a calcium channel blocker), vitamin E (an antioxidant) and collagenase. An important point that was made was that, owing to the multifactorial nature of fibrosis and the difficulty of reversing established collagen crosslinking, combination therapy might be more effective than a single agent when a well-formed PD plaque is present.⁴⁰

This first demonstration of the antifibrotic effects of long-term, continuous administration of a PDE5 inhibitor was later extended to the corpora cavernosal fibrosis that underlies CVOD, caused either by aging or by neuropraxia secondary to cavernosal nerve resection, mimicking the post-radical-prostatectomy state.^{59–62} In these cases, the effects of the three PDE5 inhibitors (sildenafil, vardenafil and tadalafil) on collagen deposition in the rat corpora were similar to those seen in the PD-like plaque; however, they also seemed to provide protection against the loss of smooth muscle cells, which are responsible for normal corporal compliance and their ability to relax and achieve normal veno-occlusion. In fact, the PDE5 inhibitors decreased corporal apoptosis—specifically of smooth muscle cells in this case, as opposed to the increased apoptotic index in tunical myofibroblasts observed in the PD plaque—and oxidative stress, thus preventing or correcting CVOD. Sildenafil prevented the progression of corporal fibrosis in penile histopathology induced by cavernous neurotomy in the rat and in patients who had undergone radical prostatectomy.^{88–90} These antifibrotic effects of PDE5 inhibitors, specifically the prevention of collagen deposition and the inhibition of TGF- β 1 expression and oxidative stress, were also seen in rat models of diabetic nephropathy, experimental glomerulonephritis, myocardial infarction and hypertrophy, and pulmonary fibrosis;^{91–95} therefore, their antifibrotic effects do not seem to be restricted to

penile tissues. These effects should not be confused with the beneficial vasodilator mechanism exploited for the treatment of pulmonary hypertension.⁹⁶

Despite the two experimental papers on the effects of continuous long-term treatment with sildenafil and vardenafil on the PD-like plaque in the TGF- β 1 rat model,^{11,40} the emerging literature on this modality in other types of tissue fibrosis, and the well characterized antifibrotic effects of cGMP and guanylate cyclase stimulators, no similar experimental studies have been performed in human patients with PD. An article related to the use of PDE5 inhibitors in patients with PD in fact focused on their standard “on-demand” application for treating erectile dysfunction, and not PD itself.⁹⁷ This lack of studies in humans does not seem to be due to concerns about potential adverse effects, as several trials have shown that daily administration of sildenafil or tadalafil is well tolerated.^{98,99} Moreover, a 2006 case report described the beneficial effects of an antifibrotic regimen of drugs that upregulate nitric oxide (and, therefore, cGMP production) in two patients with refractory priapism (>48 h duration).⁴³ Based on the previous work in a rat model of PD,¹¹ the regimen included the PDE inhibitors pentoxifylline and sildenafil and the nitric oxide precursor L-arginine. At 1 year, both patients were found to have flexible corpora and no evidence of fibrosis.

Conclusions

Despite the strong preclinical evidence in animal models supporting the antifibrotic effects of continuous, long-term administration of PDE5 inhibitors in penile tissue, this approach has yet to be studied in patients with PD. The likelihood is that our wider experience of the on-demand use of PDE5 inhibitors for erectile dysfunction will eventually lead to the first clinical test of the antifibrotic hypothesis in the context of the relatively mild corporal fibrosis seen in patients after radical prostatectomy; only if successful in this application might its use be extended to PD. In any case, despite the promise of this novel approach, the progression of the human PD plaque to advanced fibrosis and calcification may restrict its application to the early stages of the disease. In addition, a combination regimen comprising PDE5 inhibitors and other agents that stimulate collagen breakdown may be needed to effectively reduce the size of an established plaque. We believe that a study in which the outcomes of men receiving a currently used treatment for PD plus a PDE5 inhibitor are compared with men receiving the same treatment plus placebo will help define the future role of PDE5 inhibitors in patients with PD.

Review criteria

We searched for original articles focusing on Peyronie's disease in PubMed published from 1980 onwards. The search terms we used were “Peyronie's disease” and “La Peyronie”. All papers identified were full-text papers (unless indicated in the reference list) and were published in English, French or Spanish.

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Acknowledgments

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BIOGRAPHICAL SKETCH

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NAME GONZALEZ-CADAVID, NESTOR F	POSITION TITLE Professor		
eRA COMMONS USER NAME (credential, e.g., agency login) NESTORGON			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
University of Buenos Aires, Argentina	M.Sc.	1961	Biochemistry
University of Buenos Aires, Argentina	Ph.D.	1964	Biochemistry
University of London, England	Ph.D.	1967	Biochemistry

A. Positions and Honors.

1961 Gold medal to the best MSc graduate, University of Buenos Aires
 1961-62 Fellowship, National Council of Scientific Research (Argentina)
 1964 Gold Medal to the best doctorate dissertation, University of Buenos Aires
 1964-66 Fellowship, Natl Council Sci Res (Argentina), work at the Courtauld Inst Biochemistry, London Univ.
 1967 Fellowship, WellcomeTrust (England), *ibid*
 1968-71 Assoc. Professor, Dept. Biochemistry., Sch. of Science, Central University, Caracas, Venezuela
 1971-92 Full Professor, Dept Cell Biology, School of Science, Central University, Caracas, Venezuela
 1978-79 Gosney Visit. Assoc. in Biology, California Institute of Technology, Biology Division, Pasadena, CA
 1980 Senior Fellowship, Guggenheim Foundation, Cal. Inst. of Technology, Biology Div., Pasadena, CA
 1982 Visiting Professor, University of Buenos Aires, School of Biochemistry, Buenos Aires, Argentina
 1984 Fellowship, Internatl Union Against Cancer, City of Hope Med Center, Div Biology, Duarte (CA).
 1987-88 Visiting Professor, UCLA School of Medicine, Div of Hematology/Oncology, Los Angeles, CA
 1987 E. Roosevelt fellowship, Internatl Union Against Cancer, UCLA Med School, Dept Medicine, Los Angeles (CA); Senior Fellowship, United Nations Univ. *ibid*
 1990-92 National Research Service Award (Senior Fellowship), Popul. Res. Center, Harbor/UCLA Med. Ctr.
 1990-96 Adj. Associate Professor, Dept of Surgery/Urology, UCLA School of Medicine, Director Urology Research Laboratory, Harbor-UCLA REI
 1996-on Adjunct Professor, Department of Urology, UCLA School of Medicine, and Director, as above
 1997-on Professor, Dept of Internal Medicine/Endocrinology, Charles R. Drew University.
 2001-07 Director, RCMi Molecular Medicine Core, Charles R. Drew University
 2009-13 Member, NIH UKGD Urology Study Section
 2010-15 Editor-in-Chief, International Journal of Impotence Research, Nature Publishing Corp.

B. Professional membership

1965-75 Bioch Soc (England); 1987-90 Tissue Cult Assoc (USA); 1989 Amer Assoc for Cancer Res.; 1992-Am Soc Andrology (USA); 1998-Endocrinol Society (USA); 2000 Am Urological Assoc (USA); 2000 Soc Study Impotence; 2000 Soc Study Reproduction; 2004 Sexual Medicine Soc of N Am

C. Selected peer-reviewed publications from 2003-2007 (from a list of 165 on CV) For Pubmed search, please use Gonzalez-Cadavid N, or the last name only, and not NF, to avoid missing 40 odd papers

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Ferrini MG, Moon J, Rivera S, Vernet D, Rajfer J, **Gonzalez-Cadavid NF** (2009) The genetic inactivation of inducible nitric oxide synthase (iNOS) intensifies fibrosis and oxidative stress in the penile corpora cavernosa in type 1 diabetes. J Sex Med, in press

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D. Research Support (funded, ongoing)

1. PR064756 (PI: Gonzalez-Cadavid) Department of Defense 03/01/07-02/28/10

Pharmacological prevention and reversion of erectile dysfunction after radical prostatectomy, by modulation of nitric oxide/cGMP pathways The goal is to determine whether long-term treatment with PDE5 inhibitors and nitric oxide donors can prevent CVD in a rat model of erectile dysfunction after radical prostatectomy, and whether this is due to an improvement in the underlying penile corporal fibrosis and loss of smooth muscle

2. PC061300 (PI: Gonzalez-Cadavid) Department of Defense 03/31/07-02/28/11

Modulation of stem cell differentiation and myostatin as an approach to counteract fibrosis in dystrophic muscle regeneration after injury. The goal is to determine whether skeletal muscle derived stem cells (MDSC) can ameliorate skeletal muscle atrophy and fibrosis in a mouse model of Duchenne's muscular dystrophy, and this is stimulated by ex vivo gene transfer of myostatin shRNA to stem cells, and/or treatment with agents that inhibit myostatin activity

E. Research Support (pending)

1. Takeda USA Corporation (PI Gonzalez-Cadavid) 03/01/10-09/30/11

Effects of alogliptin alone and in combination with pioglitazone on diabetic nephropathy in a rat model

F. Research Support (some, recent past)

1. NIH R21DK070003-01A1 (Gonzalez-Cadavid) NIH NIDDK 10/01/07-09/30/09

Cell-selective expression of fibrotic gene pathways (no cost extension to 09/30/10)

2. RO1 DK53069-08 (PI: Gonzalez-Cadavid) NIH/NIDDK 05/01/03-04/30/08

Erectile Dysfunction and Nitric Oxide Synthase in Aging. Renewal to be resubmitted

3. G12RR030262 NIH (PI: Francis/Baker; Core Director: Gonzalez-Cadavid) 09/01/00-08/31/07

RCMI Infrastructure Development Grant: DNA Repository and Molecular Medicine Core

4. U54 HD41748-01 NIH/NICHHD (PI: Bhasin; PI Pilot grant: Gonzalez-Cadavid) 10/01/03-09/30/07

Androgen Stimulation of Myogenic Stem Cell Differentiation"

5. Takeda North America, Inc (PI: Gonzalez-Cadavid) 04/01/08-03/31/08

Antifibrotic and Renoprotective Effects of Pioglitazone on Type 2 Diabetes Related Tubulointerstitial Fibrosis

6. Harbor/UCLA Division of Urodynamics (PI: Gonzalez-Cadavid /Bathia/Ho) 03/01/07-04/01/08

Reversion of levator ani atrophy by muscle derived stem cells in a rat model of stress urinary incontinence

7. American Diabetes Association (PI: Gonzalez-Cadavid) 08/01/05-07/31/08

Erectile dysfunction and vascular fibrosis in diabetes.

8. Eileen Norris Foundation(PI: Gonzalez-Cadavid/White) 12/01/08-11/30/09

Effect of sildenafil and muscle derived stem cells on cardiac fibrosis after myocardial infarction

9. Lilly ICOS (PI: Gonzalez-Cadavid/Rajfer) 06/01/05-05/31/06

Effect of tadalafil in preserving smooth muscle function following cavernosal nerve injury