Additional evidence supporting a potential use of microencapsulated Sertoli cells in DMD patients

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INTRODUCTION

As a consequence of the persistent muscle degeneration and poor compensatory mechanisms, muscles of DMD patients are characterized by a condition of chronic inflammation, which represents a major cause in the outcome of the pathology (*Evans et al., 2009*).

We demonstrated that a single intraperitoneal injection of **microencapsulated porcine Sertoli cells** (SeC-MC) in *mdx* mice **restrains inflammation** in muscle tissue and induces heregulin β1-dependent expression of the dystrophin paralogue, **utrophin** at the sarcolemma, resulting in improved muscle morphology and performance (*Chiappalupi et al., 2015, 2016*) **(Fig. B)**.

Once injected i.p., SeC-MC act as a "micro-biofactory" that releases in the bloodstream immunomodulatory and trophic factors that through the circulation reach every single muscle. This approach does not need pharmacological immunosuppression and is *DMD* mutation-independent.

Here, we i) performed for the first time an ultrastructural analysis of SeC inside the microcapsules; ii) investigated the direct effects of SeC on murine myoblasts/myotubes and on myotubes of higher mammals; and, iii) investigated the minimum effective dose of SeC *in vivo*.

FOCUS ON SERTOLI CELLS

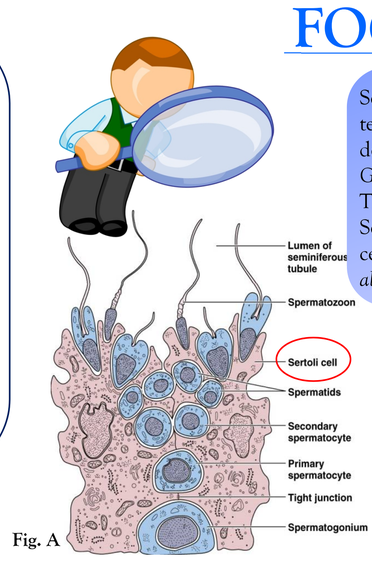


Fig. A

Sertoli cells (SeC) are the major component of the seminiferous tubules in the testis **(Fig. A)**, where they provide numerous factors required for the orderly development and immune protection of maturing germ cells (*Russell & Griswold, 1993*).

Thanks to the secretion of several trophic and immunomodulatory factors, SeC are able to create a unique **immune-privileged environment** that protects cells from the host immune system attack (*Skinner & Griswold, 2005; Mital et al., 2010*).

During the years, SeC or microencapsulated SeC (SeC-MC) have been successfully used in allogeneic and xenogeneic tissue transplantations, and in experimental models of diabetes, Parkinson's disease, amyotrophic lateral sclerosis, Laron syndrome (dwarfism) and Huntington's disease (*Kaur et al., 2015; Fallarino et al., 2009; Luca et al., 2013, 2016*).

I.p. injection of SeC-MC has been also tested in type 2 diabetes non-human primates (*Luca et al., 2014*).

1. Evaluation of the biological status of SeC inside the microcapsules

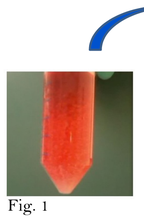


Fig. 1

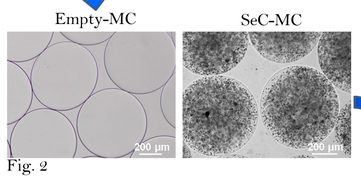


Fig. 2

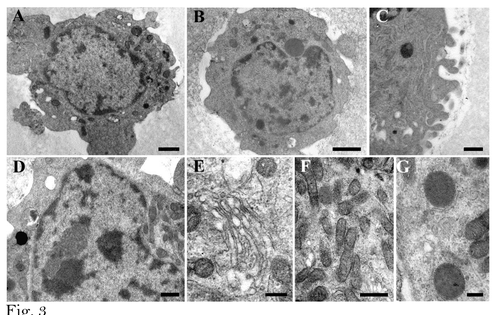


Fig. 3

The encapsulation procedure affects SeC morphology and viability to an extremely low extent.

Figures 1 and 2 show SeC-MC as they appear at gross and phase contrast microscopy observation, respectively. Figure 3 allows to appreciate the ultrastructure of SeC into freshly-prepared alginate microcapsules. In A, B and D it is possible to observe the nucleus with a dense and diffuse chromatin, and an extensive cytoplasm rich in organelles. The nucleolus shows fibrillar and granular components, visible in D. At high magnification, membrane extroflessions **(C)**, a well developed Golgi apparatus **(E)**, a large number of rounded or elongated mitochondria **(F)** and some lysosomes **(G)** can be observed. Bars A,B = 1 µm; C,D,F = 500 nm; E,G = 200 nm.

Background

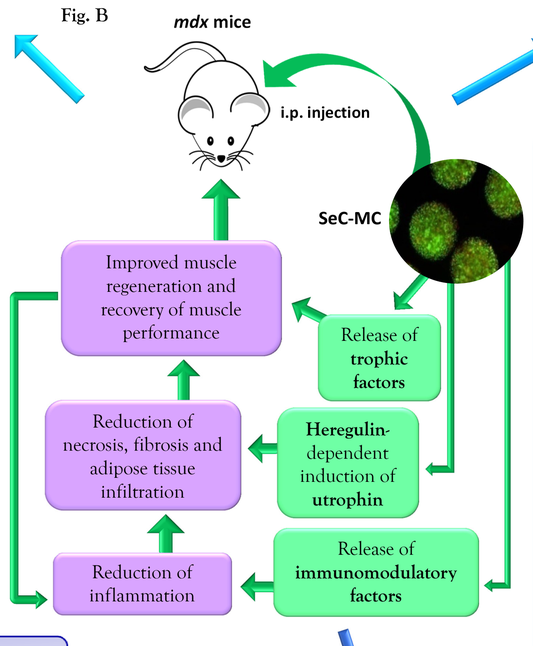
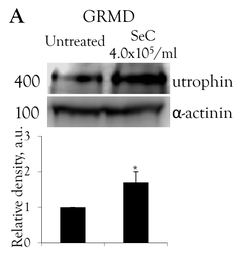


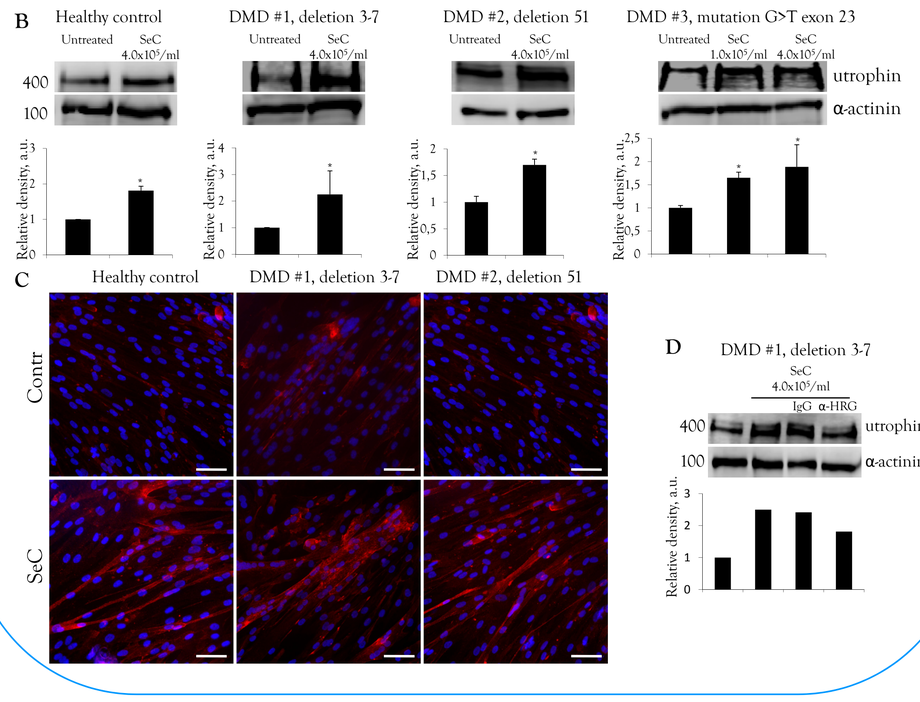
Fig. B

2. Investigation of SeC effects on dystrophic myoblasts

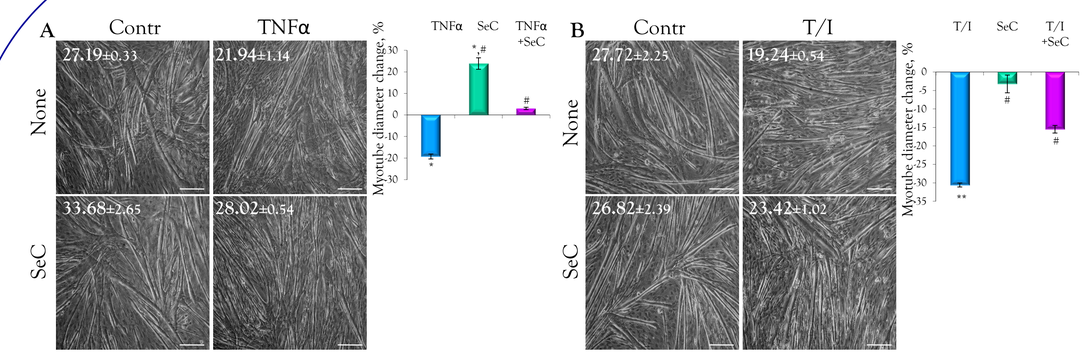
SeC induce up-regulation of utrophin expression in dystrophic myotubes from higher mammals through a heregulin-β1-dependent mechanism.

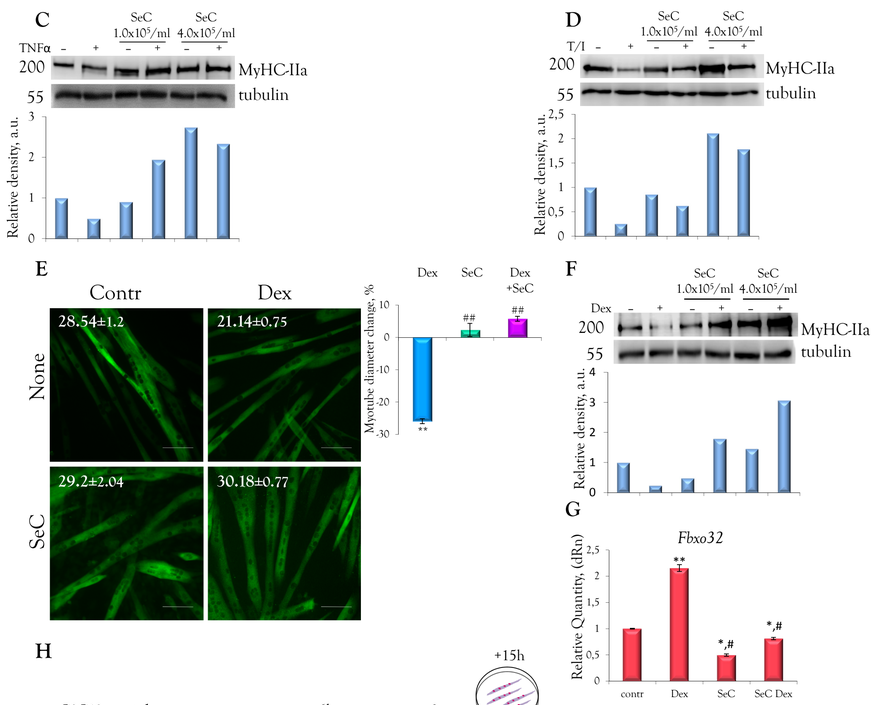
Myotubes obtained from GRMD dogs **(A)**, healthy human control or DMD patients characterized by the indicated mutations in the *DMD* gene **(B,C)** were co-cultured with or without SeC at the indicated amounts. Myotubes from a DMD patient were also cultivated in absence or presence of an anti-heregulin blocking antibody (α-HRG) **(D)**. After 48h, myotubes were analyzed by Western blotting (A,B,D) or immunofluorescence (C) for utrophin expression. Reported are the relative densities of utrophin with respect to α-actinin expression. Shown are representative blots or images of three different experiments (A-C). *, p≤0.05 *vs* untreated control.

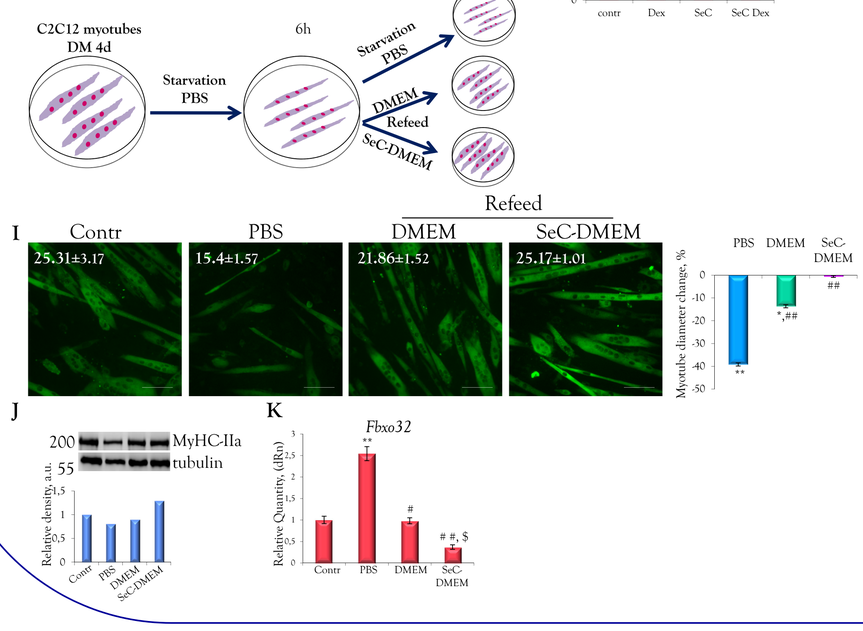




3. Effects of SeC on myotubes in *in vitro* models of muscle atrophy







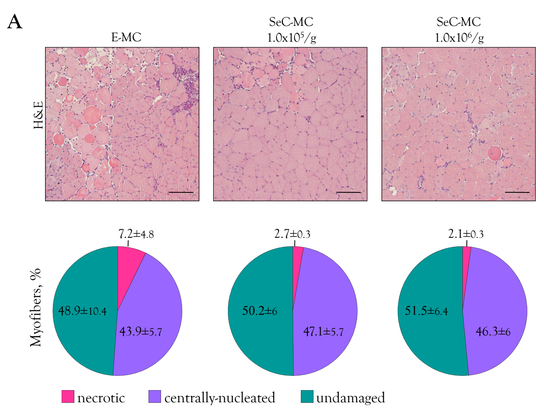
SeC protect myotubes against reduction of diameter and loss of MyHC in different *in vitro* models of muscle atrophy.

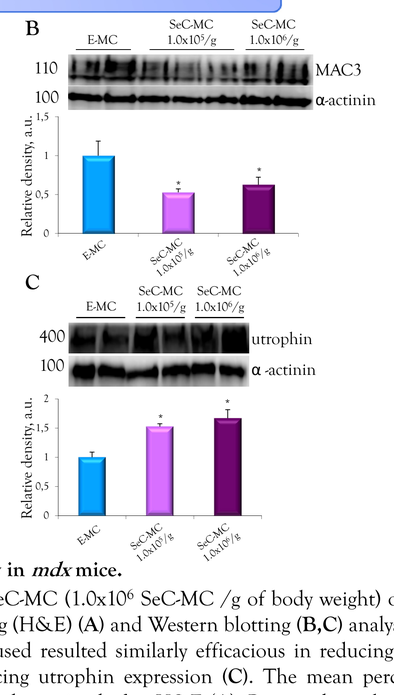
C2C12 myotubes were treated or not for 72h with TNFα alone **(A,C)** or in combination with IFNγ **(B,D)**, as pro-inflammatory stimuli, to induce atrophy *in vitro*. In the presence of TNFα or TNFα/IFNγ (T/I) a reduction in myotube diameter **(A,B)** and MyHC-IIa expression **(C,D)** was observed, which was prevented by co-culture of myotubes with SeC. Similarly, reduction of diameter **(E)** and MyHC-IIa expression **(F)** of C2C12 myotubes treated with dexamethasone (Dex, 1 µM) for 48h were counteracted by co-culturing the myotubes with SeC **(E,F)**. C2C12 myotubes treated for 24h with Dex showed an increment in *Fbxo32* (a specific E3-ubiquitine ligase involved in the degradation of MyHC-IIa) expression, which was reduced by the presence of SeC in the culture medium, as assessed by real-time PCR analysis **(G)**.

(H) C2C12 myotubes were starved by removing the growth medium and incubating them with PBS for 6h. Then, myotubes were refed for 15h with DMEM or DMEM conditioned for 48h with SeC (SeC-DMEM). We found a lower extent of the reduction of myotube diameter **(I)** and MyHC-IIa expression **(J)** induced by starvation in the presence of SeC-DMEM **(I,J)**. In accordance, the expression of *Fbxo32*, which was induced by starvation, was maximally reduced in the presence of SeC-DMEM, as assessed by real-time PCR analysis **(K)**.

Reported are the percentage changes in myotube diameters (A,B,E,I) or the relative densities of MyHC-IIa with respect to tubulin expression (C,D,F,J) compared to untreated controls (Contr). *, p≤0.05 and **, p≤0.01 *vs* Contr. #, p≤0.05 and ##, p≤0.01 *vs* internal control (TNFα, T/I, Dex or PBS). $, p≤0.05 *vs* unconditioned DMEM.

4. Investigating the minimum effective dose of SeC-MC





Low doses of SeC-MC are efficacious in recovering muscle morphology in *mdx* mice.

Four-weeks-old *mdx* mice were i.p. injected with the standard dose of SeC-MC (1.0x10⁶ SeC-MC /g of body weight) or a ten-fold lower dose. Three weeks after injection, haematoxylin/eosin staining (H&E) **(A)** and Western blotting **(B,C)** analyses were performed on *Quadriceps femoris* muscles. The two doses of SeC-MC used resulted similarly efficacious in reducing muscle necrosis **(A)** and inflammation (MAC3 expression) **(B)**, and in inducing utrophin expression **(C)**. The mean percentages (±SEM) of undamaged, centrally-nucleated and necrotic myofibers were determined after H&E (A). Reported are the relative densities of MAC3 (B) or utrophin (C) with respect to α-actinin expression. Shown are representative blots and images of three independent experiments. *, significantly different from control (E-MC treated mice).

CONCLUSIONS

1. Ultrastructural investigation revealed that the procedure used to prepare the microcapsules affects SeC morphology and viability to an extremely low extent since the nucleus, cell wall and cytoplasmic organelles appear in a viable state in ready-to-inject SeC-MC.
2. In line with data obtained in *mdx* mice (*Chiappalupi et al., 2015, 2016*), SeC were found able to induce up-regulation of utrophin expression also in myotubes from higher mammals, i.e. GRMD dogs and DMD patients with different mutations in the *DMD* gene, with a heregulin β1-dependent mechanism.
3. SeC protect myotubes against reduction of diameter and loss of MyHC-IIa in *in vitro* models of muscle atrophy induced by pro-inflammatory cytokines, starvation or dexamethasone.
4. Significantly lower doses of SeC-MC than those previously used (*Chiappalupi et al., 2015, 2016*), are still efficacious in reducing inflammation, inducing utrophin expression, and recovering morphology in muscles of dystrophic mice.

Altogether, our data further support the use of i.p. injection of SeC-MC as a potential treatment of DMD patients.

References: Chiappalupi S. *et al.*, 2015, *Data in Brief*, 5:1015-21; Chiappalupi S. *et al.*, 2016, *Biomaterials*, 75:313-26; Evans N. P. *et al.*, 2009, *Am. J. Phys. Med. Rehabil.*, 1:755-68; Fallarino F. *et al.*, 2009, *J. Exp. Med.*, 206: 2511-26; Kaur G. *et al.*, 2015, *Anim. Reprod.*,12:105-17; Luca G. *et al.*, 2013, *J. Control Release*, 165:75-81; Luca G. *et al.*, 2014, *Transplant Proc.*, 46:1999-2001; Luca G. *et al.*, 2016, *CNS Neurosci. Ther.*, 22:686-90; Mital P. *et al.*, 2010, *Reproduction*,139:495−504; Russell L.D. & Griswold M. D., 1993, *Cache River Press*; Skinner M. K. & Griswold M. D., 2005, *Elsevier Academic Press*; 8:107-120.

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