Sistema de ingeniería neuromuscular para el estudio in vitro de distrofias musculares

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Bilbao, 19 de noviembre del 2013

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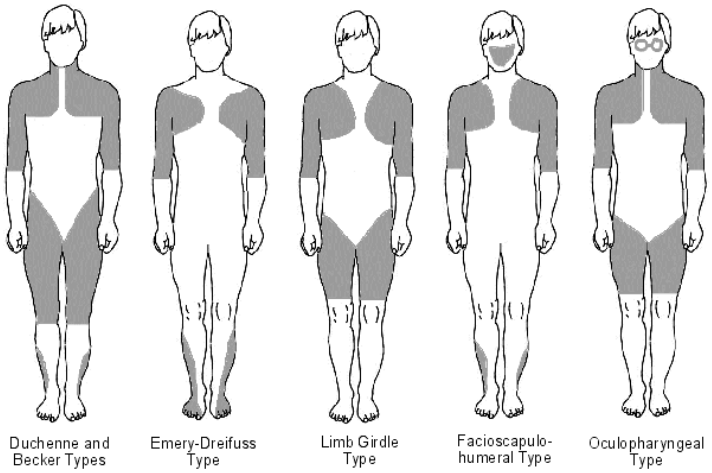
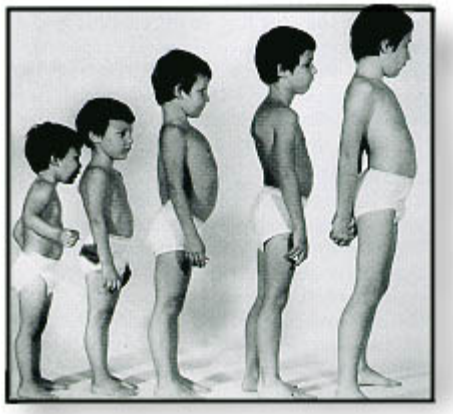
Distrofias Musculares

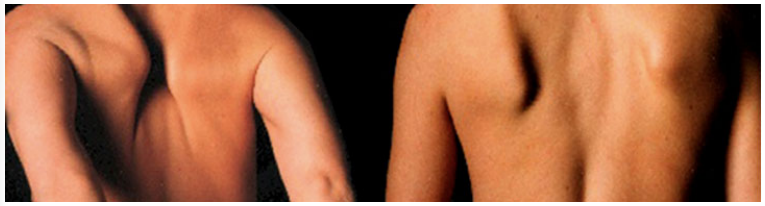
Grupo de más de 30 enfermedades hereditarias caracterizadas por debilidad y degeneración progresiva de los músculos esqueléticos.

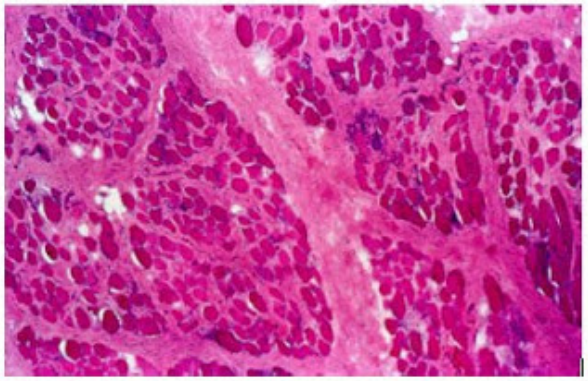
- Distrofia muscular de Duchenne
- Distrofia muscular de Becker
- Distrofias musculares congénitas
- Distrofia muscular de Emery-Dreifuss
- Distrofias musculares de cintura (LGMD)
- Distrofia muscular facioescapulohumeral
- Distrofia muscular oculofaríngea
- Distrofia miotónica….



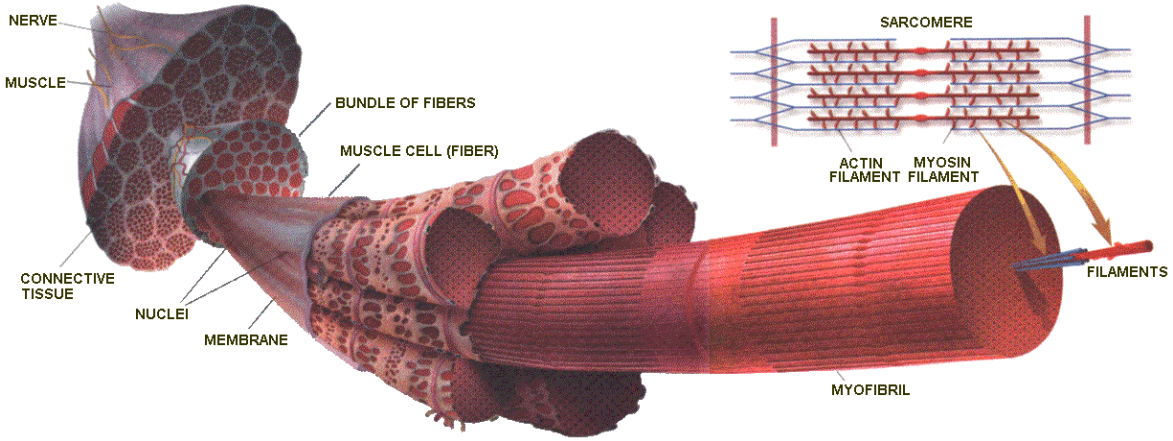
Main areas of muscle weakness in different types of dystrophy







Desarrollo del músculo esquelético



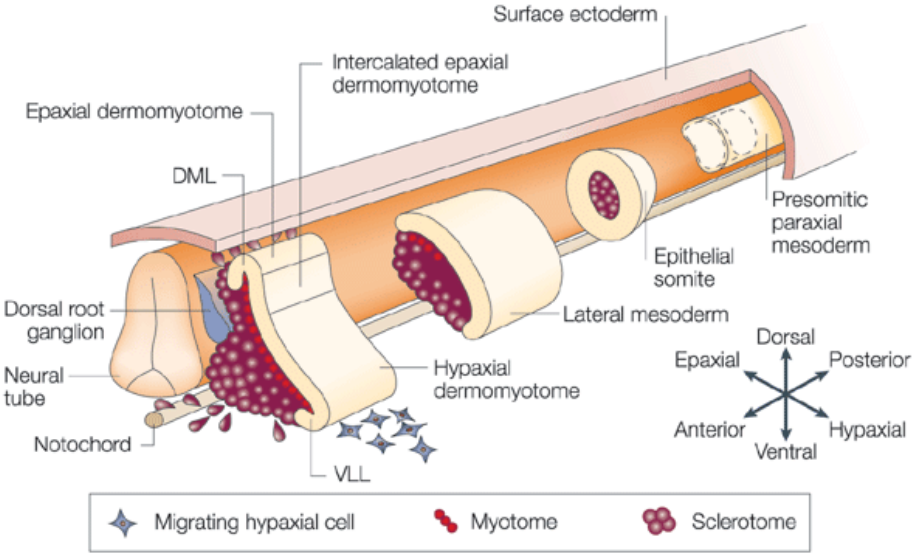
Dermomiotomo

↓

Músculo esquelético
Dermis

MRFs → determinación y fusión de los mioblastos en miotubos.

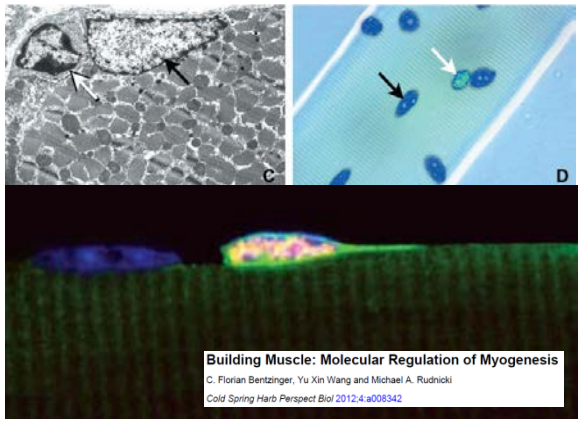
- Genes Pax: Pax3 / Pax7
- MRFs primarios : Myf5 / MyoD
- MRFs secundarios: myogenin / Myf4
- Genes específicos musculares: MyHC / MCK
- Otros: Wnts, Noggin, BMP4…

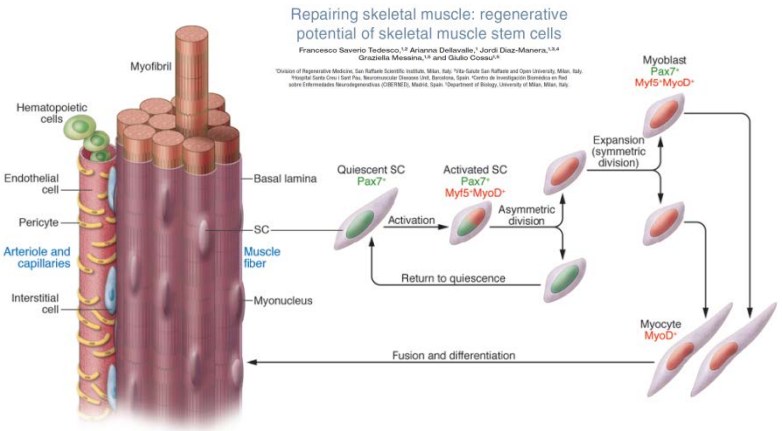


Nature Reviews | Genetics

Células relacionadas con la regeneración del músculo esquelético adulto

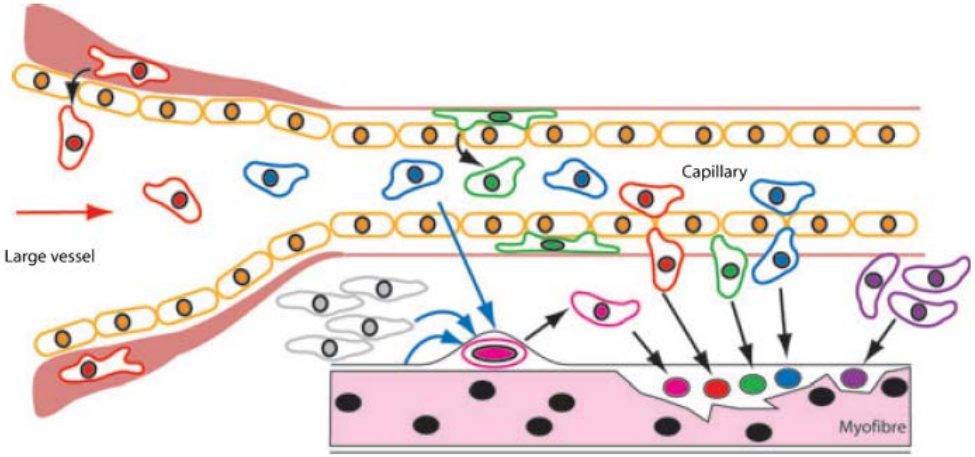
Célula satélite: primera célula *stem* miogénica (*Kartz, 1961; Mauro, 1961*)

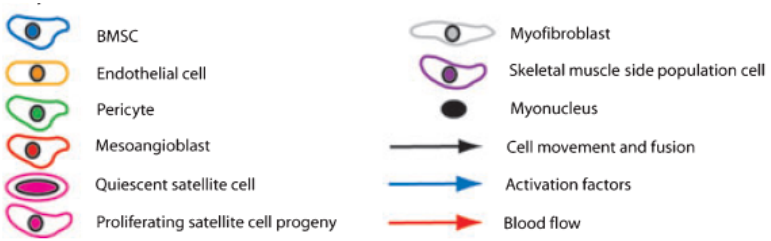




- Bajo la membrana basal.
- ↑ heterocromatina.
- Expresión Pax7 (Myf5+/-).
- Auto-renovación.
- Capacidad para formar nuevos miotubos.

Otras células con potencial miogénico





+ **SKPs (*Qiu et al. 2010*)**


J. Anat. (2009) 215, pp477–497 doi: 10.1111/j.1469-7580.2009.01138.x

REVIEW

The origin, molecular regulation and therapeutic potential of myogenic stem cell populations

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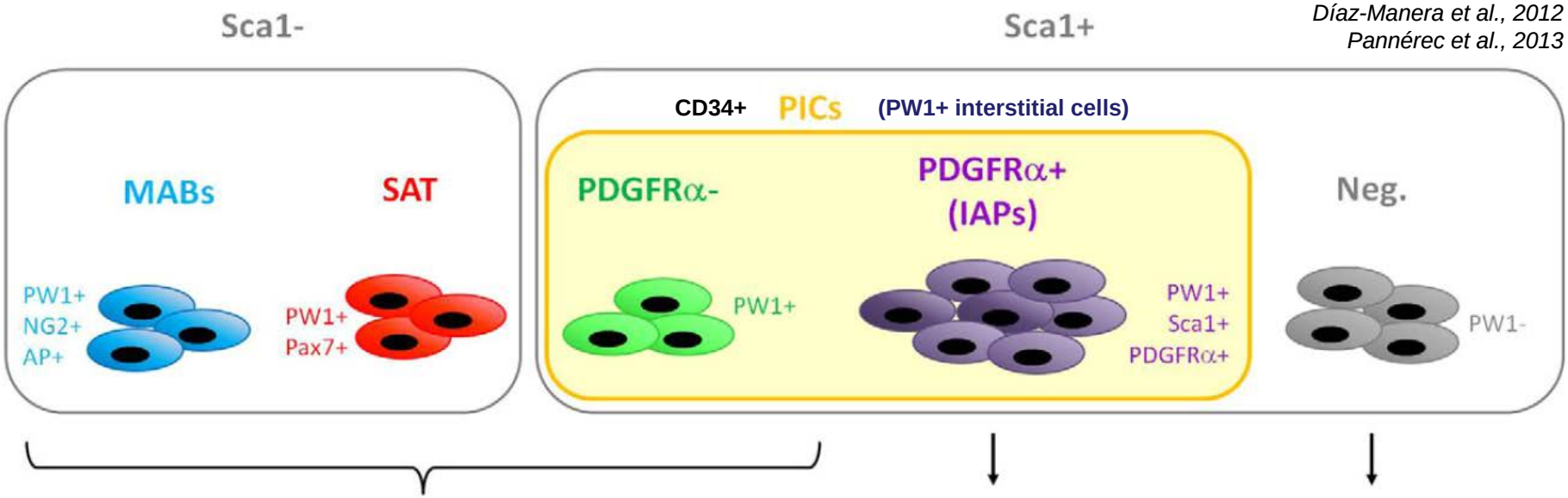
Los candidatos se multiplican…

Díaz-Manera et al., 2012
Pannérec et al., 2013



MYOGENIC

PDGFRa+
SMA+
CD146+
CD34-
CD44+
CD13+
CD63+
CD90+
NCAM-

CD34+
Sca1-
NCAM+

Sca1^{MED}
NG2+

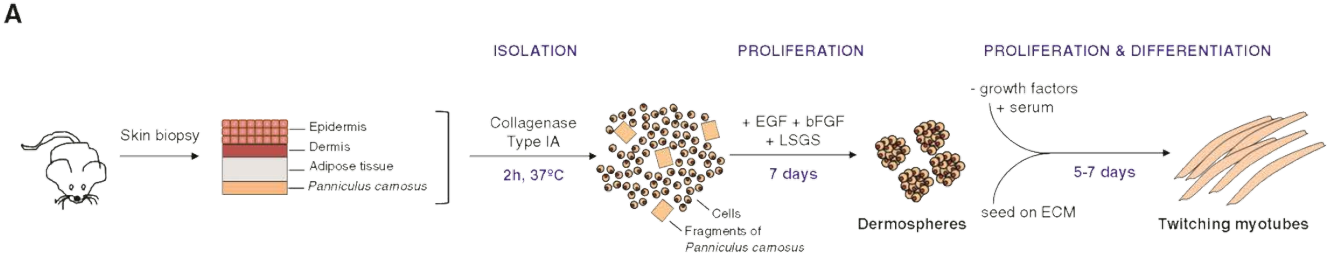
ADIPOGENIC

CD34+
Sca1^{HI}
PW1+
NG2+
Tie2+

FIBROGENIC

CD34+
Sca1^{HI}
PW1-
TCF4+

Generación de miotubos contráctiles a partir de precursores dérmicos



TISSUE ENGINEERING: Part C
Volume 20, Number 1, 2013
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DOI: 10.1089/ten.tec.2013.0146

Methods Article

Murine Muscle Engineered from Dermal Precursors: An *In Vitro* Model for Skeletal Muscle Generation, Degeneration, and Fatty Infiltration

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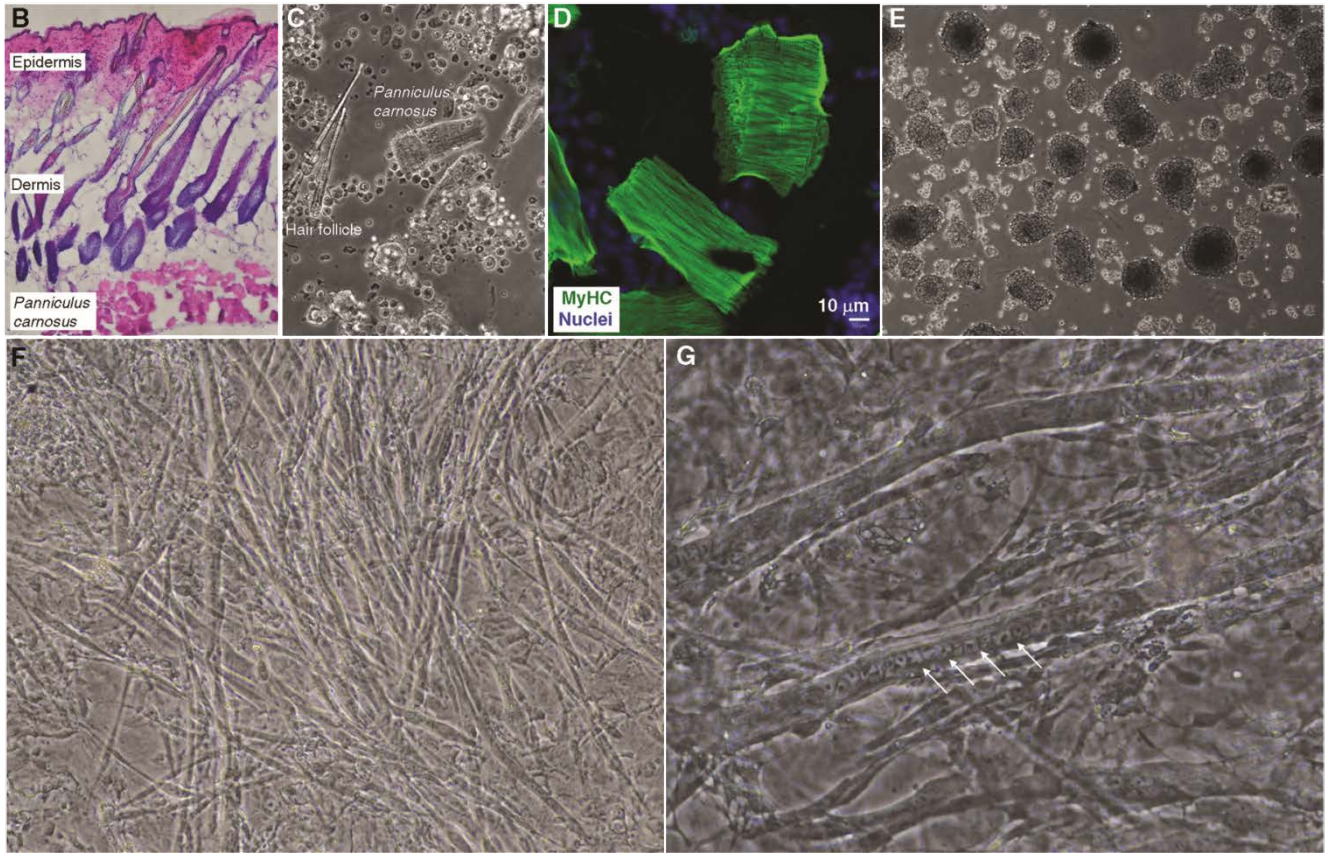
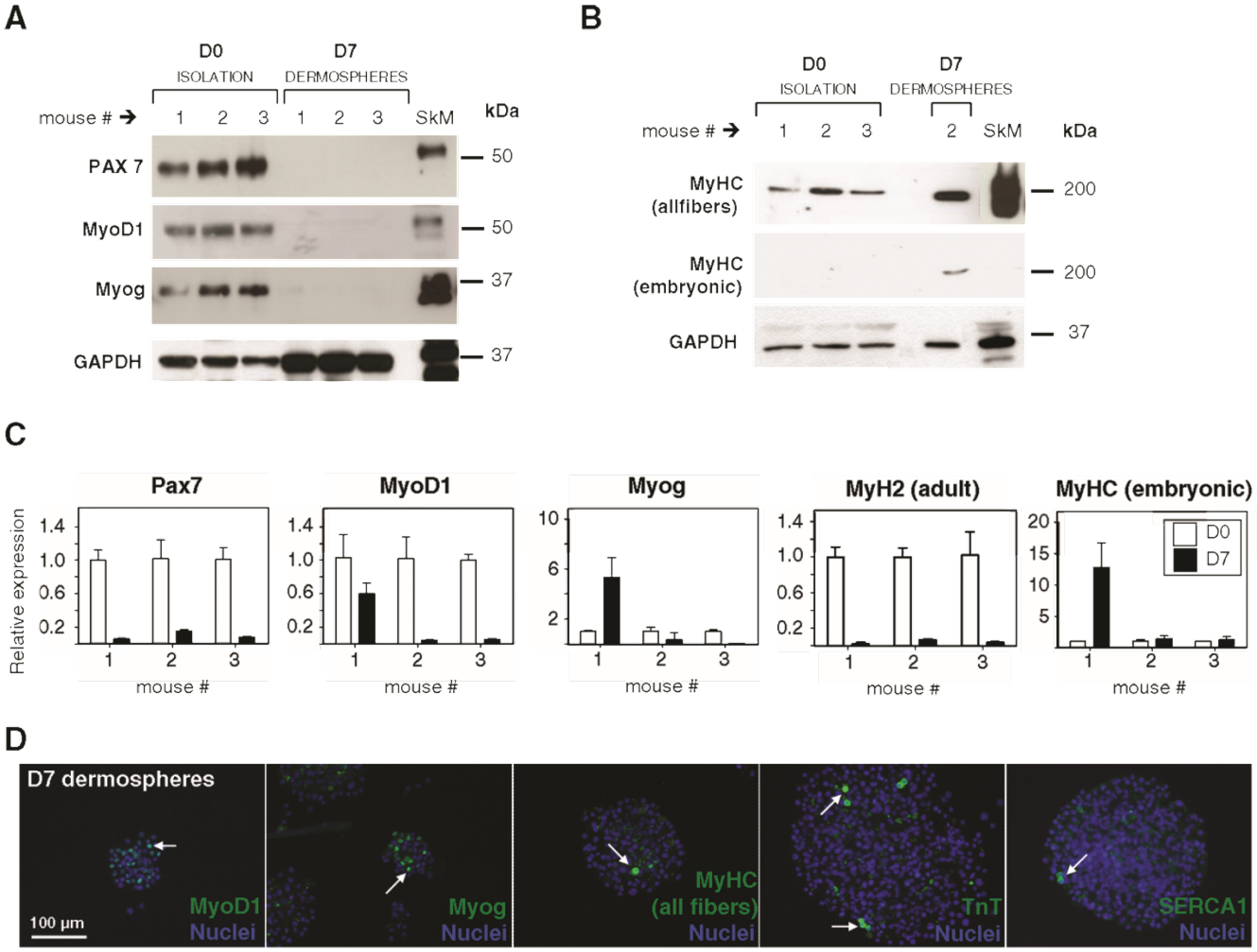


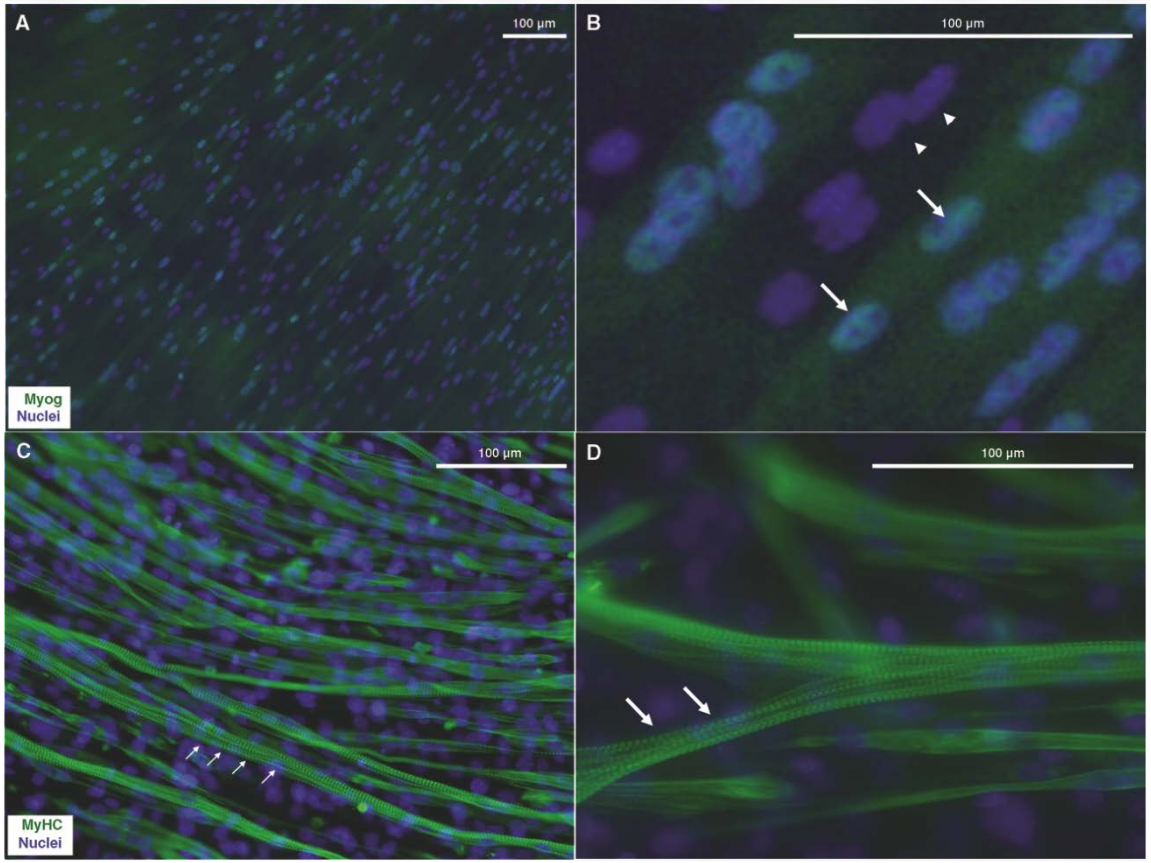
(A) Strategy to engineer twitching myotubes from dermal precursor cells in vitro. Once isolated, dermis-derived cells were expanded as dermospheres in the presence of EGF, FGF2 and LSGS. After growth factor withdrawal, dermospheres were differentiated into myotubes by seeding on ECM and supplementing with serum. (B-E) Histological analyses show the presence of Panniculus carnosus in murine skin (B, x100 magnification). After disaggregation, small muscle fragments are present in dermal cultures (C, x100), as confirmed in (D) by MyHC immunostaining (nuclei are counterstained with Hoechst; scale bar, 10 µm). No muscle fragments were detected in dermosphere cultures after 7-day proliferation (E, x100), at the time when spheres were seeded on ECM. (F-G) A large number of multinucleated (arrows in G), twitching myotubes were observed in culture after 5 to 7-day differentiation (F, x100; G, x200).

Las esferas dérmicas presentan una pequeña población de células progenitoras miogénicas

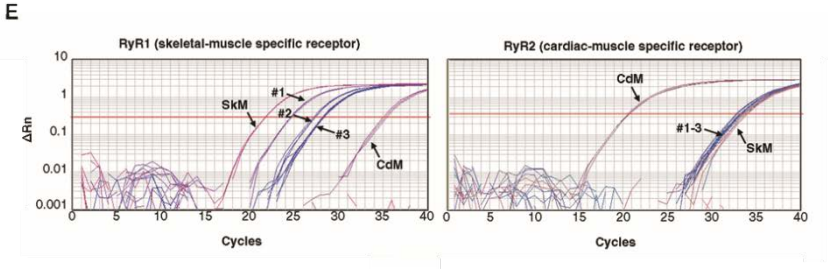


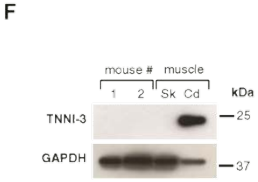
(A-B) Western blot of myogenic markers Pax7, MyoD1, Myog and MyHC (all fibers and embryonic subtypes) using GAPDH as a loading control. Proteins were detected at day 0 and day 7 of dermosphere culture, as well as in skeletal muscle (SkM) as a positive control. Position of known size markers is shown to the right of each panel, mouse replicates on the top. (C) RT-qPCR of myogenic markers Pax7, MyoD1, Myog, MyH2 and MyH3; as detected at day 0 (empty bars) and day 7 (black bars) of dermosphere culture. Expression of mRNAs is shown relative to day 0. Mouse replicates are shown on the bottom of each graph. (D) Detection of myogenic markers MyoD1, Myog, MyHC, TnT and SERCA1 by immunofluorescence at day 7 dermospheres. A discrete population of dermosphere cells expressed myogenic markers in culture (scale bar, 100 µm).

Músculo formado por miotubos esqueléticos (no cardíacos)…

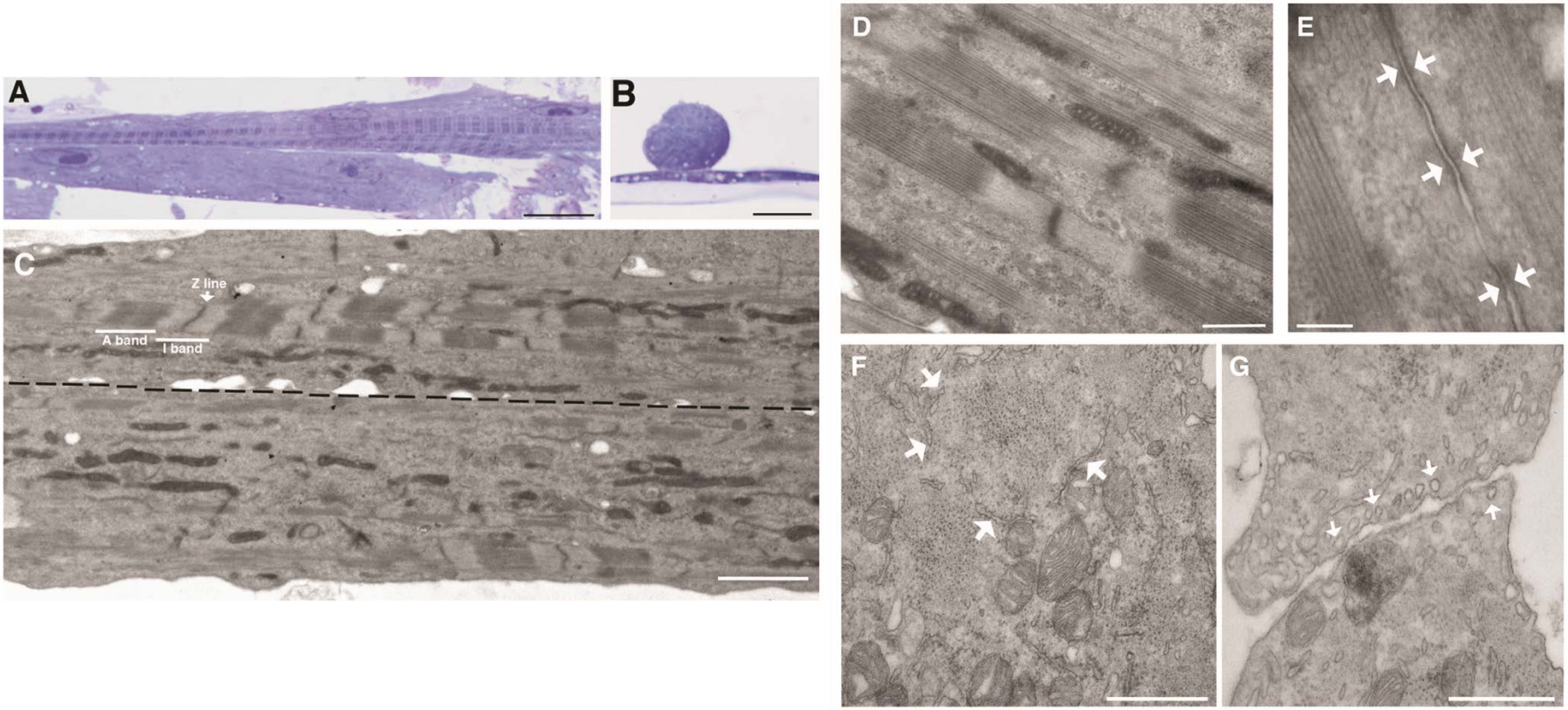


(A-D) By immunofluorescence, myotubes grown for 7 days on ECM showed high nuclear expression of myogenin (A-B, arrows; note that other non-myogenic cells are present in culture but no expression of myogenin is detected in their nuclei, arrowheads) and sarcoplasmic MyHC (C-D). Striated patterns (arrows), indicative of contractile function, are clearly visible. Nuclei were counterstained with Hoechst. Scale bars, 100 µm. (E) RT-qPCR analyses showed that skeletal-muscle specific ryanodin receptor (RyR1) mRNA is expressed by twitching myotubes, although at lower levels than skeletal muscle (SkM) positive control. (E-F) The absence of cardiac-specific RyR2 mRNA (E, as compared with positive cardiac muscle-CdM control) and cardiac specific troponin (TNNI-3) expression (F), corroborated the skeletal (not cardiac) nature of contractile myotubes.



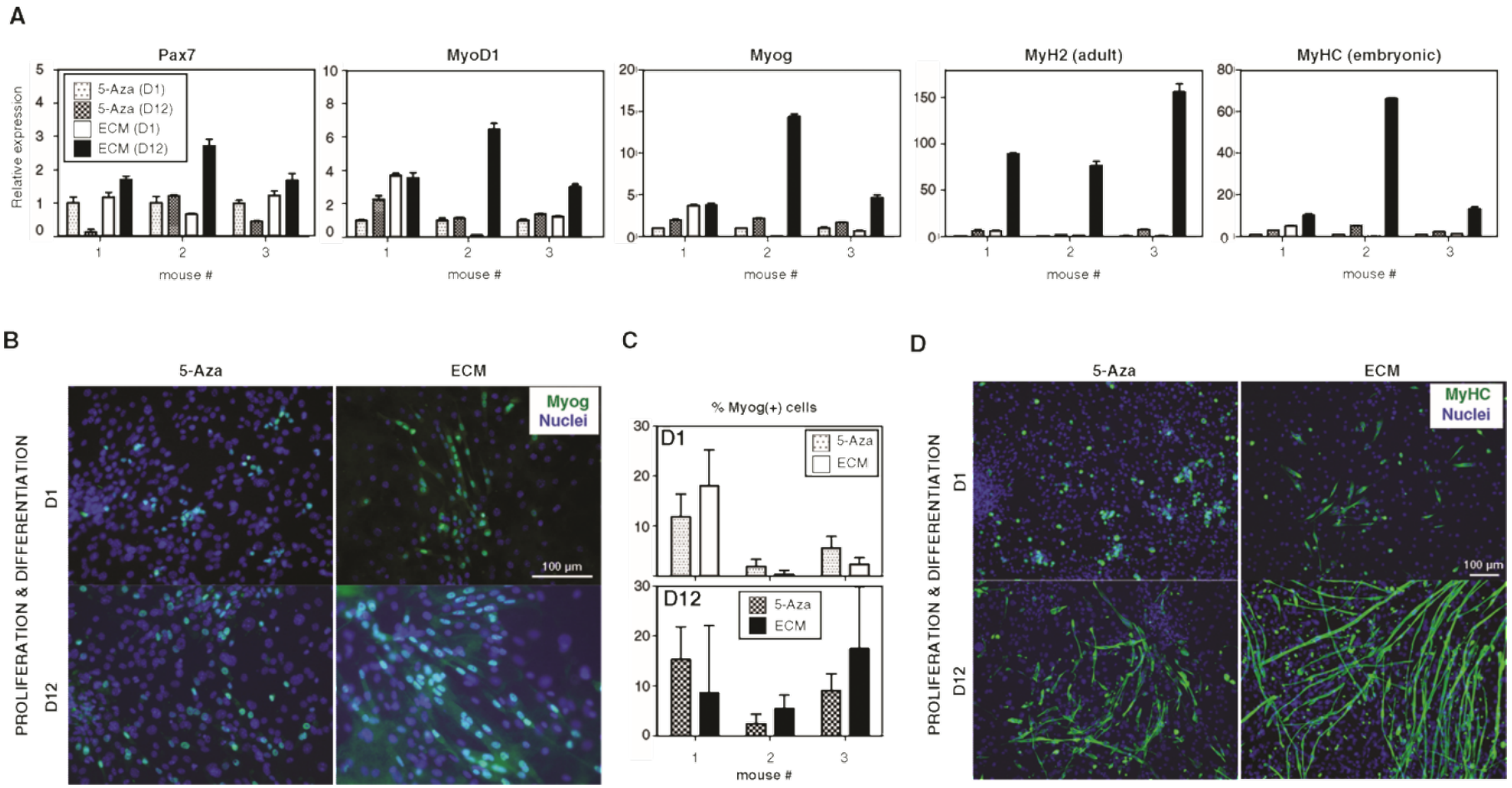


...también confirmados mediante caracterización ultraestructural



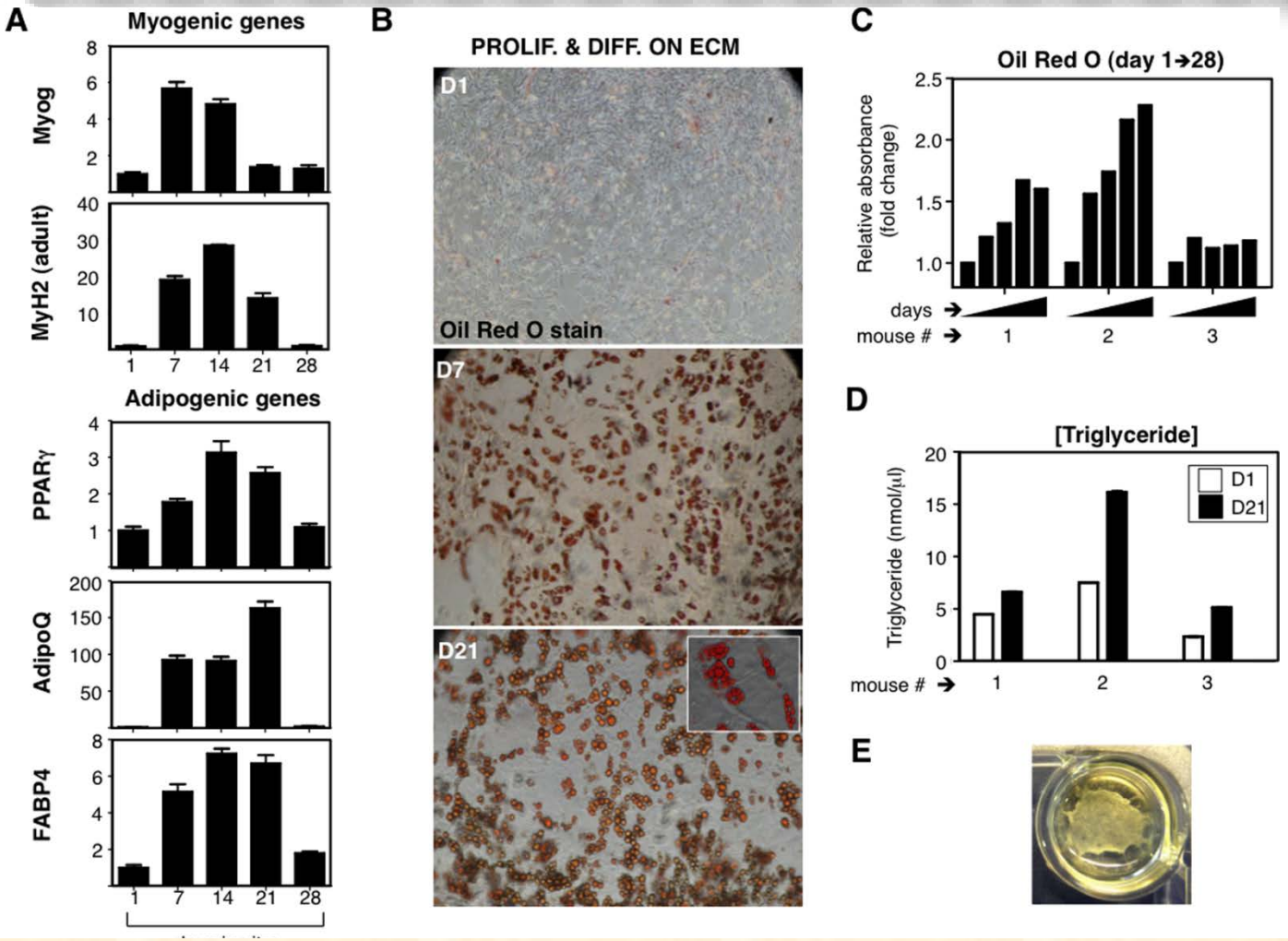
(A-C). Aspect of semithin (1.5 µm) sections stained with toluidine blue. (A) Semithin section showing tubular and spherical cells (scale bar, 100 µm). (B) Longitudinally oriented myofibers show clear striations (scale bar, 40 µm). (C) A transversally sectioned fiber sits on top of a longitudinally sectioned fiber (scale bar, 30 µm). (D-H) Ultrathin (70nm) sections of muscle fibers, as seen by electron microscopy. (D) Panoramic view of two adjacent cells (separated by a discontinuous line) where typical striations of skeletal muscle may be seen. Position of A band, I band and Z line are indicated (scale bar, 2 µm). Enlarged mitochondria running parallel to fibers are also vivible. (E) Enlarged image of myofiber organisation. Smooth endoplasmic reticulum (SER) cisternae and mitochondria are alternatively detected between myofiber groups (scale bar, 1 µm). (F) Transversal section where myofiber organisation is observed: myofibrils are surrounded by SER (arrows) and mitochondria (scale bar, 500nm). (G) Dense muscular (adherens) junctions (arrows) in between adjacent muscular fibers (scale bar, 200nm). (H) Two transversally sectioned myofibers show abundant caveoles (arrows) in the proximity of the cell membrane and myofibrils (scale bar, 500nm).

La diferenciación de los cultivos sobre la matriz extracelular mejora la diferenciación miogénica.



Cultivos de diferenciación de 12 días, estudio comparativo entre matriz extracelular y azacitidina. (A) Análisis de RT-qPCR para mRNAs de Pax7, MyoD1, Myog, MyH2 and MyHC (B-D) Análisis de inmunofluorescencia para miogenina (B,C) y MyHC (D).

Cultivos de larga duración muestran infiltración de adipocitos

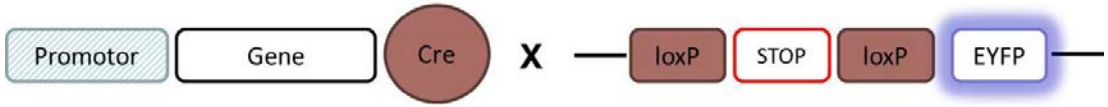


(A) Gene expression analyses by RT-qPCR revealed an early expression of myogenic mRNAs (Myog and MyH2; peaking at 7-14 days), followed by a clear decrease after 20 days differentiation. Concomitantly, an increased expression of adipogenic genes PPARγ, AdipoQ and FABP4 was detected peaking at day 14-21. (B-C) A progressive differentiation of adipocytes was visible by Oil Red O staining (B, x100), peaking after 21 days in culture (C). (D) Triglyceride content confirmed these data. (E) The aged appearance of engineered skeletal muscle after 1 month in culture is shown. Note that the ECM is progressively peeling off the edges of the culture plate due to the force generated during the contractions of myotubes. The abundance of adipocytes makes the aged culture turn yellow.

Las miofibras maduras puede diferenciarse sobre la matriz extracelular a partir de células precursoras miogénicas presentes en las dermoesferas murinas, en un sistema in vitro que presenta características semejantes a las encontradas en procesos de envejecimiento y degeneración muscular.

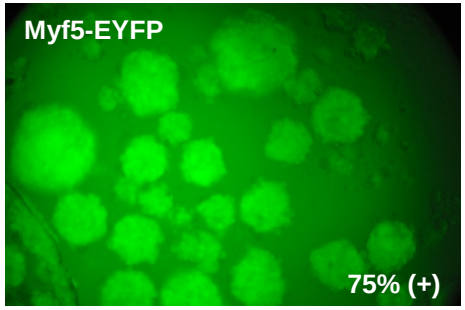
¿En qué estamos? A) Identificación de los precursores dérmicos miogénicas

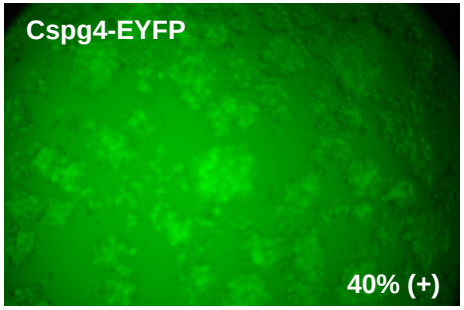
Líneas de trazado de linaje: sistemas **Cre-loxP-EYFP…**

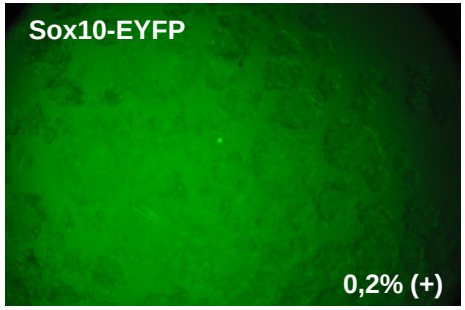


Gen de interés	Ratón	*Para tracear el linaje de…*
Pax3/Pax7	Pax3-GFP / inducible Pax7-Cre	Células satélite
Myf5	B6.129S4-Myf5<tm3(cre)Sor>/J	Células del músculo esquelético y células de la dermis
Cspg4	B6;FVB-Tg(Cspg4-cre)1Akik/J	Pericitos
Sox10	B6;SJL-Tg(Sox10-cre)507Mcln/J	Células derivadas de la cresta neural
ROSA26EYFP	B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J	*Reportero (expresión EYFP bajo control de la recombinasa Cre)*

Dermoesferas de Myf5-EYFP, Cspg4-EYFP ySox10-EYFP…



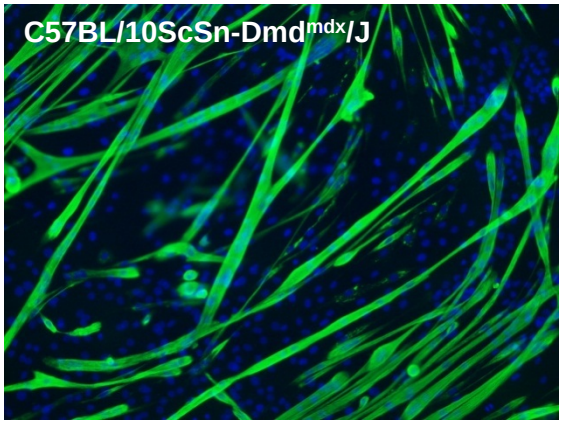


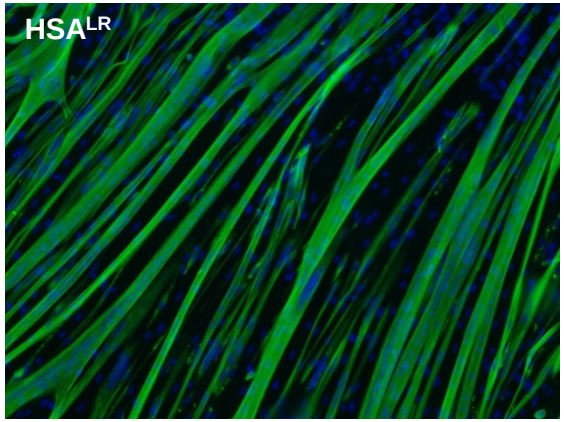


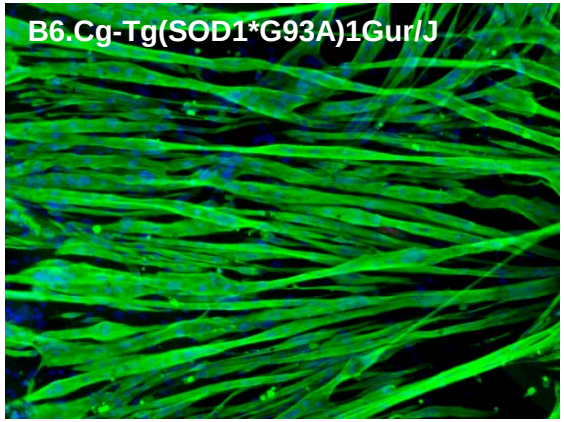
B) Extrapolación a modelos de enfermedades neuromusculares

Modelos murinos de…	Ratón	*Información*
Distrofia Muscular de Duchenne (DMD)	C57BL/10ScSn-Dmd^{mdx}/J	Mutación en el gen de la distrofina, su degeneración muscular comienza hacia las 3 semanas de edad. Modelo de DMD murino más utilizado (*Grounds et al., 2008*)
Distrofia Miotónica1 (MD1)	HSA^{LR}	Modelo generado por *Mankodi et al. 2000*, posee *250 repeticiones de (CTG)* insertados en la mitad del úlltimo exon del gen de la actina.
Esclerosis Lateral Amiotrófica (ALS)	B6.Cg-Tg(SOD1*G93A)1Gur/J	Presenta todas las características histopatológicas observadas clínicamente en ALS esporádica y familiar.

Estudio de miotubos obtenidos *in vitro* a partir de células precursoras dérmicas:







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