

1 Modelling human myoblasts survival upon xenotransplantation into immunodeficient
2 mouse muscle.

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25 Abstract

26 Cell transplantation has been challenged in several clinical indications of genetic or
27 acquired muscular diseases, but therapeutic success were mitigated. To understand
28 and improve the yields of tissue regeneration, we aimed at modelizing the fate of
29 CD56-positive human myoblasts after transplantation. Using immunodeficient severe
30 combined immunodeficiency (SCID) mice as recipients, we assessed the survival,
31 integration and satellite cell niche occupancy of human myoblasts by a triple
32 immunohistochemical labelling of laminin, dystrophin and human lamin A/C. The
33 counts were integrated into a classical mathematical decline equation. After injection,
34 human cells were essentially located in the endomysium, then they disappeared
35 progressively from D0 to D28. The final number of integrated human nuclei was
36 grossly determined at D2 after injection, suggesting that no more efficient fusion
37 between donor myoblasts and host fibers occurs after the resolution of the local
38 damages created by needle insertion. Almost 1% of implanted human cells occupied
39 a satellite-like cell niche. Our mathematical model validated by histological counting
40 provided a reliable quantitative estimate of human myoblast survival and/or
41 incorporation into SCID muscle fibers. Informations brought by histological labelling
42 and this mathematical model are complementary.

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45 Introduction

46

47 Skeletal muscle is composed of syncytial myofibers produced by the extensive
48 fusions of muscle progenitors called myoblasts during embryogenesis, development
49 and growth. Lifelong, this tissue is able to regenerate consecutively to mechanical,
50 chemical or pathological injuries through the mobilisation of a population of quiescent
51 cells known as the satellite cells (1, 2). Activation of these satellite cells provides
52 myoblasts, which fuse together or with the neighbouring muscle fibers to secure
53 muscle regeneration. This natural capacity to form syncytial structures allows sharing
54 genetic material brought by exogenous myoblasts, and was considered for the
55 treatment of dystrophic muscles by myoblast transplantation. The first proof of
56 concept brought by Partridge et al. (3) was followed by clinical trials in Duchenne
57 muscular dystrophy (DMD) patients (4), which reached mitigated success and
58 suggested several issues. Immunological rejection of the donor cells, immediate or
59 delayed apoptosis, necrosis or attrition, long-term differentiation underpinned the
60 necessity to follow the early and late fate of injected cells. The use of
61 immunodeficient animals is now widely accepted in this xenogenic context of
62 transplantation (5). Severe combined immunodeficiency (SCID) mice or new
63 immunodeficient models such as Rag2⁻/Il2rb⁻/DMD mice allowed assessing the
64 survival, fate, myogenicity, transplantation efficiency and potential tumorigenicity of
65 several classes of human skeletal muscle cell populations potentially useable for
66 clinical developments, such as CD133⁺ (6), CD56⁺ (7), Aldehyde dehydrogenase +
67 (8) cells, or pluripotent stem cells committed to the myogenic lineage (9, 10). These
68 models also allowed exploring strategies to enhance the transplantation efficacies by

69 quantifying the formation of skeletal muscle fibers of human origin, such as myostatin
70 inhibition (11, 12), Wnt3a upregulation (13), or pharmacological treatments (14, 15).
71 To follow the efficiency of these approaches, researchers assessed human myoblast
72 implantation by quantifying the formation of skeletal muscle fibres of human origin (6-
73 9, 11, 16), and/or evaluated the cell survival by human Lamin A/C immunolabelling
74 (6-9, 12, 13, 16). Additionally, the assessment of satellite cell niche occupancy is of
75 particular interest to document the persistence of the regenerative capacity (6, 7, 9).
76 Standardized quantification deserves the definition of a dedicated mathematical
77 model to assess human cell survival and fate in animal experiments. In this study, we
78 used experimental data obtained in injecting human CD56-positive myoblasts in a
79 SCID recipient environment to define and validate such a mathematical model.
80 Healthy, undamaged mouse muscles were injected to avoid supplementary
81 variabilities of values linked to the setting of muscle degeneration *in vivo*. We then
82 developed histological procedures to count cells and fibers, in link with their location
83 within the injected muscle. We observed a biphasic curve of cell survival in which a
84 rapid initial loss of human cells reflecting acute cell death is followed by a progressive
85 decline over one month reflecting attrition and the lack of further integration. Human
86 cell number decline was then correlated with a low participation of donor cells in fibre
87 formation over time, without impairing the occupancy of presumptive satellite cell
88 niches. The mathematical model of cell disappearance proposed here is a good
89 predictive model based on a decline equation and also gave us information on
90 human myoblasts ability to incorporate in muscle fibers.

91

92 Material and Methods

93 **Animals**

94 Eight weeks-old CB17/SCID mice were kept in standard conditions in our own
95 facilities. All procedures were performed under anesthesia (intraperitoneal injection of
96 80 mg/kg ketamine, 16 mg/kg xylazine). The animals were sacrificed by injecting a
97 lethal dose of this anesthetic mix. All the procedures were conducted according to the
98 Guide for the care and use of laboratory animals (DHAW publication n° (NIH) 85-23
99 Office of science and health reports, DRR/NIH, Bethesda MD 20892).

100 **Cell preparation and injection**

101 Muscle biopsies were obtained as surgical wastes and kindly provided by the
102 Association Française contre les Myopathies tissue bank (Myobank, Paris, France)
103 and sampled in agreement with the French bioethics laws (Law n° 94-654 of the 29
104 July 1994, modified the 22 January 2002). Human cells were prepared using the
105 methodology previously described (17, 18). Briefly, the biopsies were minced and
106 digested using collagenase (Liberase, 0.1mg/ml, Roche Diagnostics International Ltd,
107 Germany), then trypsin-EDTA (0.025%, Hyclone, GE Healthcare Inc., France). The
108 cell suspension were filtered through 100 then 40µm cell strainers (BD Biosciences
109 Inc., Bedford, MA, USA) and seeded onto a one-layer cell factory (Nunc,
110 Thermofisher Scientific Inc., France) (17). The proliferation medium contained: 80%
111 modified MCDB medium (Hyclone, Thermofisher Scientific Inc., France), 20% defined
112 fetal bovine serum (Hyclone, GE Healthcare Inc., France), antibiotics (Sigma Inc.,
113 France), 10 ng/ml of human recombinant basic Fibroblast Growth Factor (R&D
114 Systems, United Kingdom), and 10⁻⁶M of dexamethasone (Merck Inc., France). The
115 cultures were settled for 7 days, and then the cells were harvested by trypsinization

116 and expanded before reaching 80% confluence. At this time, cells were frozen and
117 stored at -80°C. For each experiment, an aliquot was thawed and the cells were
118 proliferated in a cell factory to obtain the number of cells required using the
119 proliferation medium mentioned above. Cells were detached using trypsin-EDTA and
120 suspended in MCDB medium with 0.5% Bovine serum albumin (Sigma Inc., France)
121 for the injection. The same amount of living cells was delivered in each mouse. A
122 portion of the harvested cells underwent a specific drastic treatment to be used as a
123 negative control: they were submitted to three rapid freezing-thawing cycles, without
124 cryopreservant. This portion is then considered as a “dead cell fraction”.

125

126 **Cell phenotype and viability**

127 The CD56 phenotype and the cell viability (evaluated by propidium iodide exclusion)
128 were determined by cytofluorimetry (FACS Calibur and Cellquest software, BD
129 Biosciences Inc., San Jose, CA, USA). Human myoblasts were characterized by their
130 CD56 expression, which reached 90% of total cells within two passages as described
131 previously (17, 19, 20). After completion of the cell injection procedure, 7.8% of the
132 cells (remaining in the syringe and needle) stained positive for propidium iodide.
133 These results underlined the preservation of the cell phenotype and a low cell death
134 during the cell injection procedure.

135

136 **Experimental design**

137 Cell injections were performed in the *Tibialis anterior* muscle (TA) of anaesthetised
138 animals (n=6 for each time-point). A small incision was done to visualise the muscle
139 nearby the knee insertion. The needle (Gauge 26S) of a 10 µl Hamilton syringe filled
140 with the cell suspension (9×10^5 cells per muscle) was inserted in the proximal part

141 and up to the distal tendon (Figure 1), then pulled out while the cell suspension was
142 injected. At the end of the experiment, remaining cells not injected were used to
143 quantify the CD56 on cytometry analysis. All the injections were done with the same
144 cell suspension. Following injection, muscles were sampled at days D1, D2, D3, D4,
145 D5, D7, D14 and D28 (Figure 1). A specific *ex vivo* experimental protocol was used
146 to study the fate of cells at time 0, allowing minimally manipulating the muscles after
147 cell injection. In parallel to the classical cell transplantation procedure *in vivo*, some
148 animals were sacrificed and immediately thereafter their TA were collected, injected *ex*
149 *vivo* with the cell suspension, and frozen within 15 minutes. Muscles injected with the
150 dead cell fraction were sampled at D1.

151 **Sampling, histology and immunohistochemistry**

152 The TA muscles were snap-frozen in nitrogen-cooled isopentane. Five μm thick serial
153 cryostat cross-sections were performed and collected. They were ranked on
154 consecutive slides to obtain series spaced by 200 μm , from the knee insertion to the
155 distal tendon of the TA (Figure 1).

156 Morphology of injected muscles was analyzed on slides stained with hematoxylin-
157 eosin. Immunohistochemical analysis was performed using (i) co-immunolabelling of
158 human Cox2 and human lamin A/C to quantify the number of fibers expressing
159 human antigens and (ii) co-immunolabelling of human lamin A/C, human and murine
160 dystrophin, and human and murine laminin to follow the fate of human cells.
161 Antibodies were diluted in goat serum 1% in phosphate buffer saline (PBS, Sigma
162 Inc., France). First, 10% goat serum in PBS was applied on the sections for
163 nonspecific binding blocking. Primary antibodies were a rabbit anti-human Cox2
164 polyclonal antibody (kind gift of Dr. Anne Lombes, 1/1000 (21)), a mouse monoclonal
165 antibody directed against the C-terminus of human dystrophin (NCL-DYS2, with

166 murine dystrophin crossreactivity, Leica-Biosystems Inc., France, 1/20), a mouse
167 anti-human lamin A/C antibody (Vector Laboratories, United Kingdom, 1/100) and a
168 rabbit anti-rat laminin antibody (with human and murine laminin crossreactivity,
169 Agilent Technologies Inc., Santa Clara, CA, USA, 1/700). Labelling was visualized by
170 using Alexa 350-conjugated goat anti-mouse (Thermofisher Scientific Inc., France,
171 1/1000), FITC-conjugated goat anti-rabbit (Sigma Inc., France, 1/2000), and Cy5-
172 conjugated goat anti-mouse (IgG2b isotype, SouthernBiotech, Birmingham, AL, USA,
173 1/1000) antibodies. Slides were mounted in Mowiol or Mowiol-DAPI.

174

175 **Imaging**

176 Fluorescence imaging on tissue was done using a Zeiss Axioplan 2 Microscope and
177 a Camera Photometric CoolSnap fx (Roper Scientific Inc., Tucson, AZ, USA)
178 controlled by the Metavue 6.2r6 software.

179

180 **Cell survival analysis**

181 The first cross-section done on each TA muscles was referred as the position 0 μm .
182 As described earlier, on a given slide, consecutive cryosections were separated by
183 200 μm . Following immunohistochemical labelling, the sections were observed with
184 microscope from the proximal to the distal part of TA. The first and the last sections
185 containing nuclei expressing human lamin A/C were noted, together with their precise
186 localization within the muscles. In addition, six more sections containing human lamin
187 A/C positive nuclei were selected at regular intervals, in which lamin A/C-positive
188 nuclei were counted. Each point was drawn on a graph linking the number of human
189 nuclei to the position of the section in TA muscle in μm . A regression analysis was

190 done on this curve or in relevant portions of the curve. We hypothesized that the
191 regression curves reflected the distribution of the number of human nuclei all along
192 the muscle length. Regression curve equations were integrated over the entire
193 muscle length (Figure 1). The result obtained was divided by the human nucleus
194 mean size (9 μm , measured and averaged on more than 1000 nuclei, data not
195 shown), providing the total number of human nuclei. The yield of integration into
196 muscle fibers was studied by counting fibers containing at least one human nucleus.

197

198 **Intramuscular location of the transplanted cells**

199 Using triple labelling of dystrophin, laminin and lamin A/C, two indexes were defined
200 to assess human cell behavior in the SCID TA: the externalization index was defined
201 as the percentage of human nuclei outside the basal lamina, and the internalization
202 index was defined as the percentage of human nuclei underneath the basal lamina.
203 Human nuclei were considered at the satellite-like cell position when they were
204 positioned underneath the basal lamina detected by the laminin expression and on
205 the outside of the sarcolemma revealed by dystrophin immunodetection.

206 The time effect on the cell disappearance was evaluated by a two-way ANOVA and
207 the three populations of nuclei were compared using a Student's T test ($p < 0.05$,
208 Statview 5.0 Software, SAS Institute Inc., Cary, NC, USA).

209 $N(t)$ is the human cell nuclei number at time t . This number was modeled by the
210 following ordinary differential equation:

$$\frac{dN(t)}{dt} = A + (\lambda - \mu) \cdot N(t)$$
$$N(0) = N_0 > 0$$

211

212 Where: A is the differentiation recruitment in the cell population; λ is the proliferation
213 rate; μ is the death rate; $N(0)$ is the initial human cell number at time equal to zero.
214 These values were determined using the experimental data.

215 Results

216 **Cell survival analysis**

217 Human nuclei were counted on tissue sections after sacrifice of the animals from a
218 few minutes to 28 days after the injection. The number of human nuclei counted after
219 the *ex vivo* injection (D0) was indexed as the 100% cell survival, because it was
220 considered the maximal number of cells remaining in the tissue after injection (Figure
221 2). As a negative control, we injected the dead cell fraction obtained after 3 cycles of
222 freezing-thawing. In this case, a few cells were still expressing human Lamin A/C
223 (about 0.3% or less than 1500 cells) and still survived the drastic repeated freezing
224 and thawing sequence. When living cells were injected, rare mitotic events were
225 observed at D1 in the injection area on hematoxylin-eosin stained sections (one
226 event for 6 muscles; not shown). The number of human nuclei progressively
227 decreased with time from a percentage of 88 +/- 16.63% of the original cell
228 population still remaining in the tissue at D1 to the disappearance of 93.96 +/- 2.09%
229 of the injected cells at D28 ($p < 0.0001$) (Figure 2).

230 **Histological appraisal of cell fate**

231 Immunohistofluorescence analysis showed clusters of human nuclei into injected
232 muscles. Co-immunolabelling of human Cox2 and human lamin A/C allowed counting
233 a maximum of 40 myofibres expressing human Cox2 on a single section (Figure 3C).
234 To localise more precisely human nuclei, we performed a triple labelling of laminin,
235 dystrophin, and human-specific lamin A/C. Thus we could discriminate internalized

236 cells from externalized cells on the basis of their position according to basement
237 membranes, and the integration of some cells into satellite-like cell position.

238 At D2, 95.96 +/- 0.87% of cells were present as interstitial clusters. We observed a
239 low proportion of human nuclei within the fibers compared to those observed in the
240 interstitial space. Most fibers containing human cells were undergoing necrosis
241 (94,8% +/- 1%), as they presented a discontinuous laminin labelling and/or an
242 absence of dystrophin (Figure 3A).

243 At D7, 89.72 +/- 1.67% of human nuclei were also present in the intersitial spaces
244 while some nuclei were observed in regenerating fibers (Figure 3B, Figure 4A, Table
245 1).

246 At D14 and D28 the total number of human nuclei was greatly reduced concomitantly
247 to a relative change in proportion between the two compartments (Figure 3C-3D). We
248 first observed an increase of internalization index with time ($p=0.002$) mainly due to
249 the appearance of human nuclei in muscle fibres from D2. The percentage of
250 internalized nuclei increased mainly from D0 to D2, then decreased between D7 and
251 D28 (Figure 4A). In parallel, we observed a progressive decrease in the
252 externalization index ($p=0.0031$) (Figure 4A). This underlined a rapid loss of external
253 nuclei within the first two days, then a more progressive, slow loss of external nuclei
254 with time down to 57.33 +/- 13.98% at D28.

255 The triple labelling allowed detecting some human nuclei (Figure 3G) in a putative
256 satellite cell position (Figure 3H), i.e. beneath the sarcolemma detected by dystrophin
257 staining (Figure 3F) and basal lamina detected by laminin staining (Figure 3E).
258 Human nuclei in a satellite-like cell position were never observed between D0 and D7
259 and first observed at D14 (0.27 +/- 0.18%). At D28, an increase of their proportion

260 was noted up to 0.96 +/- 0.47% representing 30-50 putative satellite cells in the entire
261 muscle (data not shown).

262 **Modelization of cell behavior**

263 Our mouse model did not undergo muscle fiber regeneration, except for the muscle
264 fibers damaged by the injection. We observed only extremely rare figures of mitosis.
265 The ordinary differential equation describing a decline was then simplified by
266 approximating the human cell proliferation to zero. The new resulting equation
267 becomes:

$$268 \quad \frac{dN(t)}{dt} = A - \mu \cdot N(t)$$
$$N(o) = N_o > 0$$

269 By solving the ordinary differential equation, we obtained explicitly:

$$270 \quad N(t) = \frac{A}{\mu} \cdot (1 - e^{-\mu t}) + N_o \cdot e^{-\mu t}$$

271 Taking into account the experimental results, we obtained μ at 0.18 and A at 7000.
272 N(t) tends to 38889 cells.

273 A modelization curve is presented in the figure 4B. Maximal predicted cell loss (at 1
274 month) was of 93.37% of injected cells and was very close to that observed
275 experimentally (93.96%).

276

277 Discussion

278 Muscle cell therapy has been developed to improve skeletal muscle function in
279 several human diseases. Major targets of this approach are chronic genetic diseases
280 such as muscular dystrophies (Duchenne, facio-scapulo-humeral or oculo-
281 pharyngeal muscular dystrophies (22)) or acquired diseases such as urethral
282 incontinence (23, 24). Causes of these disorders are usually a progressive or acute
283 defect of the regeneration process. Although some clinical results have been
284 encouraging, most of these approaches did not become therapeutic solutions and a
285 better knowledge of muscle cell biology and integration is mandated to improve
286 efficacy.

287 Strengths and limitations of the mouse model

288 Our present goal was to modelize both the cell survival and the cell integration of
289 human CD56-positive myoblasts into skeletal muscle. To validate our mathematical
290 model, we acquired experimental data by injecting human cells at a single
291 longitudinal site into the healthy (non-injured) TA muscles of SCID mice. This
292 protocol avoids both an important damaging of the muscle tissue, and the dispersal
293 of cells at too many sites. Therefore, it limits variability and allows standardization.
294 On the other hand, the regenerative potential in these conditions is presumably very
295 low due to the absence of a stimulating, regenerative environment, which is an
296 extrinsic limitation of the model.

297 An intrinsic limitation of this xenotransplantation model is represented by interspecies
298 molecular differences between human grafted cells and the murine recipient tissue,
299 which may limit the efficacy of cell recognition and fusion. Indeed, several proteins
300 are involved in cellular interactions and close adhesions, such as ADAM12, CD36,
301 integrin alpha3, integrin alpha9, integrin beta1, Jamb, Jamc, Kirrel, M-Cadherin,

302 NCAM, N-Cadherin, neogenin, nephrin (25-28). While these structures are extremely
303 conserved from Homo Sapiens to Pan Troglodytes (mean, 99%) or Macacca Mulatta
304 (mean, 97%), they may show important evolutions in Mus Musculus (mean identity
305 with Homo sapiens is 89%). Of note the murine Jamb and Jamc proteins involved in
306 cell recognition and close adhesion through heterophilic interactions show only 80%
307 and 87% identity with their human homolog, which may hamper a perfect fitting
308 between human and murine actors of cell fusion. These observations would deserve
309 further studies of efficacy of direct protein-protein interactions. Therefore, both
310 extrinsic and intrinsic factors may limit the regenerative processes that usually
311 culminate in cell fusion and fiber accretion.

312 Robustness of the analysis

313 In spite of these limitations, to characterize our experimental model, we developed a
314 cell count methodology based on the co-immunolocalisation of human lamin A/C
315 nuclei and murine markers of the sarcolemma (dystrophin) and of the basal lamina
316 (laminin). This direct detection method allowed us to quantify both the human cell
317 survival and cell fate. To reflect more closely the reality, we quantified human nuclei
318 throughout length of the muscles analysed. As a cell count in the negative control, we
319 used a preparation of cells that was presumably killed by repeated freezing-thawing
320 cycles without cryoprotectant (29). Surprisingly, approximately 0.3% of the cells
321 (1500) apparently survived these drastic treatments and could be detected 24 hours
322 after injection. Although unexpected, this low percentage of survival validated the
323 methodology of measuring the presence and viability of the cells. Such a
324 phenomenon has been described previously using a methodology based on
325 radionucleotide counting (29), and it was hypothesized that the inserted
326 radionucleotide was not immediately cleared by macrophages after killing the cells by

327 repeated freezing and thawing. In our study, the nuclear lamin protein integrity was
328 assessed as it labelled the perinuclear membrane, however it is still possible that all
329 cellular debris were not removed by inflammatory cells at the time of histological
330 assessment. Whether the cells that we counted were still viable, or should be
331 considered as false positive, their number was very low when compared to the
332 number of injected cells and we considered that this count validated indeed the
333 sensitivity of our counting method.

334 In this study, we used the experimental data described above to propose a
335 mathematical model assessing the cell disappearance. The several and consecutive
336 experimental data harvested allowed validating the proposed model. We discarded
337 the potential immediate proliferation of the injected cells as this parameter in our
338 model is negligible compared to the death rate. Indeed we only observed rare mitotic
339 events at D1 in the injection area. This parameter, when known or calculated, may
340 still be introduced in the equation to improve the predictivity. As described in the
341 results section, maximal predicted cell loss was very close to that observed
342 experimentally that makes this mathematical model a good predictive one. Finally this
343 model measured also the ability of human myoblasts to incorporate muscle fibers as
344 $N(t)$ tends to the number of human nuclei incorporated in SCID mice skeletal muscle
345 fibers. But it cannot modelize the number of skeletal muscle fibers repopulated by
346 human nuclei.

347 Kinetics of cell survival

348 The cell survival study showed a progressive disappearance of CD56-positive
349 myoblasts, which was slower than the acute cell death obtained by injecting human
350 cells into normal immunocompetent mice in a xenogenic context (21). This is due to
351 the SCID immunodeficient environment, which avoids the short- and long-term

352 rejection of the grafted cells. However, we observed a 94% disappearance of injected
353 cells at 1 month. This cell disappearance was associated with a great limitation in cell
354 integration into myofibers as demonstrated by the low number of human Cox2-
355 expressing fibers observed (less than 1% of total fibers).

356 The disappearance of externalized human nuclei paralleled that of total human
357 nuclei. It suggests the loss of differentiation or fusion capacity of injected cells as
358 observed in previous studies (30). Human cells remain during a few weeks in the
359 endomysium in which time window they could be solicited to improve regeneration.
360 However, cells that did not participate to fibre formation and regeneration were
361 progressively committed to disappearance. The number of human nuclei integrated
362 within the first two days was indeed maintained at D7, D14 and D28. Then once
363 integrated in regenerative fibers, these nuclei were maintained. The unavailability to
364 fuse with uninjured myofibres could be related to the factors presented above (1) the
365 molecular differences between human and murine species and lack of appropriate
366 recognition or interaction of molecules involved in cell-cell fusion, (2) the low
367 regenerative context and the absence of expression of adhesion molecules at the
368 surface of recipient myofibers or (3) a decrease of the regenerative potential of
369 myoblasts due to the high density of cells at the injection site. This last hypothesis
370 may be conformed by the observation by Skuk et al. (31) that cell death was
371 proportionally linked to the number of cells injected due to the local lack of oxygen
372 and nutrients.

373 Do human myoblasts contribute to satellite cell niche repletion in mouse?

374 Although the integration of injected cells was very low, we observed some human
375 cells in satellite-like position in the recipient SCID muscles at D14 and D28. The
376 proportion of cells expressing human lamin A/C at this specific location reached

377 0.96% of the total human nuclei counted at D28, suggesting the capacity of the
378 injected myoblasts to integrate or constitute *de novo* a satellite cell niche. On one
379 hand, some injected myoblasts may have kept some stemness attributes, at least the
380 capacity to form a stock of repopulating cells, and maybe the capacity to self-renew,
381 despite the species barrier encountered in our model. The long-term persistence of
382 myoblasts within non-muscle tissue (myocardium) has been documented previously
383 (32). On the other hand, the true capacities of these satellite-like cells are unknown.
384 The topography may not reflect the biology. The mechanism by which these human
385 nuclei occupy a satellite-like cell niche in the murine tissue is unclear. The
386 regeneration observed in our model was consecutive to a local, segmental necrosis
387 and focal disruption of basal lamina due to needle penetration, allowing some cells to
388 be injected underneath basal lamina. After necrosis, basal lamina was restored
389 around the original structure (as observed in figure 3 at D7). It is conceivable that, at
390 this moment, some internalized human nuclei may be trapped between the myofiber
391 sarcolemma and basal lamina deposition before any cell-cell fusion. By consequence
392 the trapped cells seem to occupy a satellite-like cell position. Nevertheless, the
393 formation of Pax7-positive cells occupying a murine satellite cell niche after
394 intramuscular transplantation in SCID mice has been also previously described (7).
395 The number of human nuclei incorporated in fibers seemed to be determined at D2
396 after the injection and represent less than 3% of the cell number injected. And, the
397 injected cells occupying a satellite-like position did not appear before D14 and
398 represent less than 1% of the internalized nuclei. Such a paucity makes difficult the
399 modelization of the human nuclei internalization and of satellite cell positioning.

400

401

402 Conclusion

403 Our experimental model underlines the low integration rate of the grafted myoblasts
404 in an healthy muscle in the context of xenogenic transplantation (human into mouse)
405 and in the absence of extensive muscle fibre regeneration. The use of
406 xenotransplantation of human myoblasts into immunodeficient mice is routinely used
407 for different transplantation studies, and this method is the easiest to study their
408 behavior after grafting, because it documents both survival and distribution of cells.
409 The experimental model used in this study allowed us to validate a predictive
410 mathematical model of the cell survival that could be used in further studies to
411 estimate and compare the cell survival of different types of myogenic progenitors in a
412 xenogenic transplantation context and their ability to fuse with SCID mice muscle
413 fibers.

414

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517 Table 1: Quantification of the CD56-positive human myoblast behavior in SCID TA

518 muscle from D0 to D28 after their injection.

Time	D1 DC	D0	D2	D7	D14	D28
Externalization index	100 (0)	100 (0)	95.96 (0.87)	89.72 (1.67)	78.42 (8.01)	57.33 (13.98)
Internalization index	0	0	4.04 (0.87)	10.34 (1.67)	21.58 (8.01)	42.67 (13.98)
% cell survival	0.27 (0.13)	100 (10.65)	50.14 (13.75)	34.49 (6.89)	17.48 (6.28)	6.04 (2.09)
% nuclei at satellite cell position	0	0	0	0	0.27 (0.18)	0.96 (0.47)

519

520 D1DC describes the control muscles injected with dead cell fraction. D0 corresponds

521 to the muscles grafted *ex vivo* and immediately frozen, which serve to observe the

522 location of the cells immediately after the injection and to establish the 100% of the

523 grafted cells. Externalization and internalization indexes were provided as mean

524 percentages (S.E.M.) compared to the total human lamin A/C positive nuclei in the

525 respective muscles. The mean percentage of human nuclei located in a satellite-like

526 cell position is also presented.

527

528 Figure legends:

529 Figure 1: Experimental design and procedures.

530 This figure summarizes all the experimental procedures performed in this study. The
531 experimental design was represented by the timeline from injection time (INJ) at time
532 0 to all the sampling days (S. Day axis) from D0 to D28. The injection and histological
533 procedures were schematized to illustrate and support the textual descriptions. The
534 total human lamin A/C positive nuclei calculation was illustrated by the graph linking
535 the number of human nuclei to the position of the section in TA muscle in μm done
536 for each muscle and the mathematical formula. Number of human nuclei in each
537 muscle was calculated by integration of linear functions (obtained by linear
538 regression on relevant sections in the curve) and divided by the mean nucleus size.

539

540 Figure 2: Survival of human cells grafted in SCID mouse muscles.

541 Follow-up of human nuclei from D0 to D28. The human lamin A/C-positive nuclei
542 were counted on eight serial sections, using six muscles per time point. Bar graph
543 shows the mean and standard deviation. The number of human nuclei statistically
544 decreased with time ($p < 0.0001$).

545

546 Figure 3: Human myoblast fate in SCID mouse TA muscle (dystrophin in blue, human
547 lamin A/C in red and laminin in green, co-immunolabellings).

548 **A-** At D2, grafted cells were present in clusters, some nuclei being internalized in
549 necrotic myofibres demonstrating a dystrophin negative pattern and/or a
550 discontinuous laminin labelling (white arrow). **B-** At D7, the injected cells were still
551 gathered in clusters and internalized nuclei were observed in regenerating myofibres
552 (white arrow). **C-** At D14, human mitochondria transferred upon fusion of human cells

553 exhibit the Cox-positive staining (green) in some muscle fibers, which were also
554 frequently containing human nuclei. The human-derived muscle fibers were not
555 numerous and their absolute number did not dramatically evolve from D2 to D28. **D-**
556 At D28, human internalized nuclei were observed in myofibres (asterisk). **E to H-**
557 Human lamin A/C-positive nuclei (**G**) were observed at several positions in the
558 injected muscle, some being in interstitial place (thick arrow), some being trapped
559 between the laminin sheath and the dystrophin edging (therefore, in a satellite-like
560 cell position, asterisk), some being integrated within muscle fibers below their
561 dystrophin edging (fine arrow). **E** is the laminin staining, **F** the dystrophin staining, **G**
562 the lamin A/C staining and **H** the merge image.

563 Scale bar at the bottom left: A to C- 100 μm ; D- 65 μm ; E to H-20 μm .

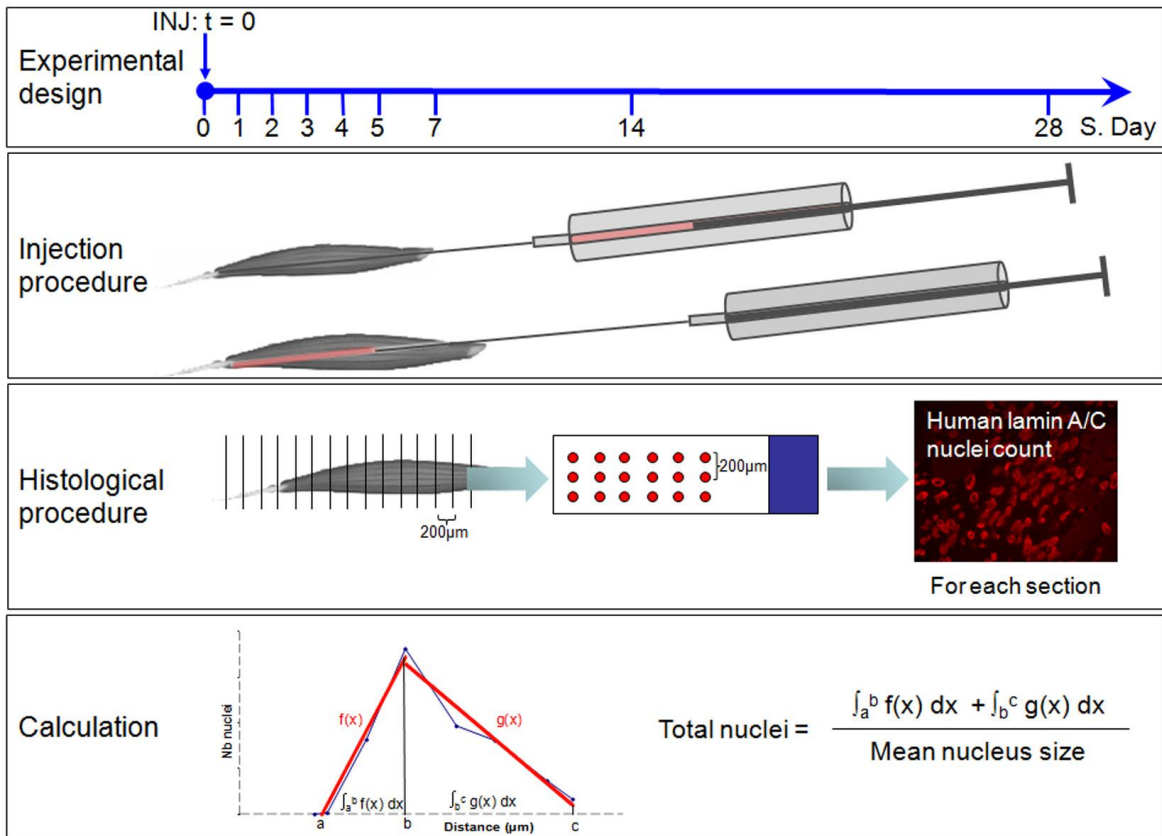
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565 Figure 4: Quantification of human cell survival and modelization of cell survival.

566 **A-** Follow-up of human nuclei from D0 to D28 according to their localization: in
567 (internalized) and out (externalized) of the muscle fibre. Different fonts were used to
568 distinguish each nucleus localization. **B-** After modelization of cell survival using the
569 experimental points, the mathematical model shows a progressive cell
570 disappearance of human nuclei.

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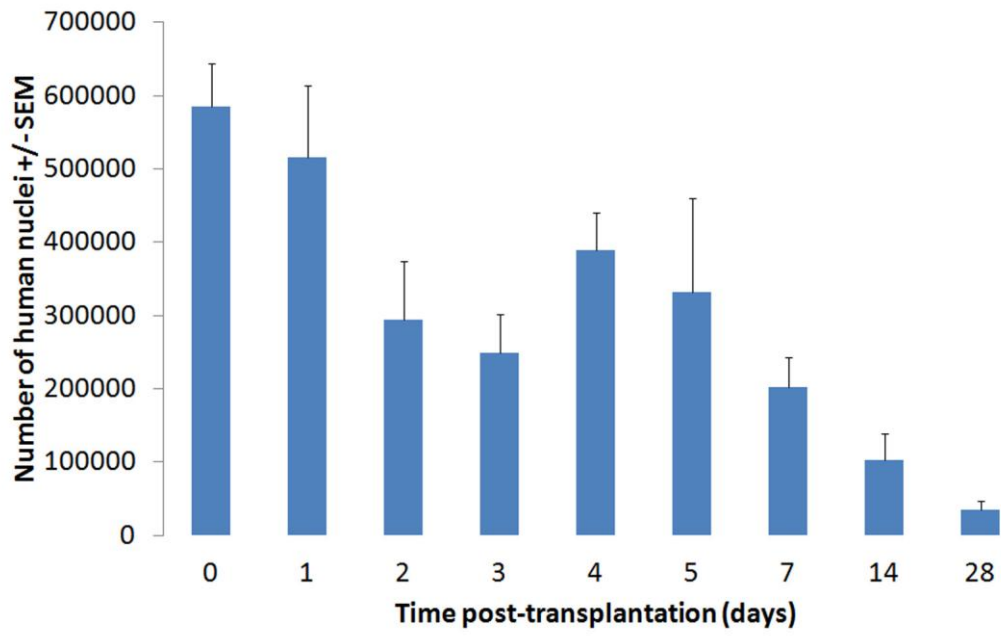
572 Figure 1



573

574

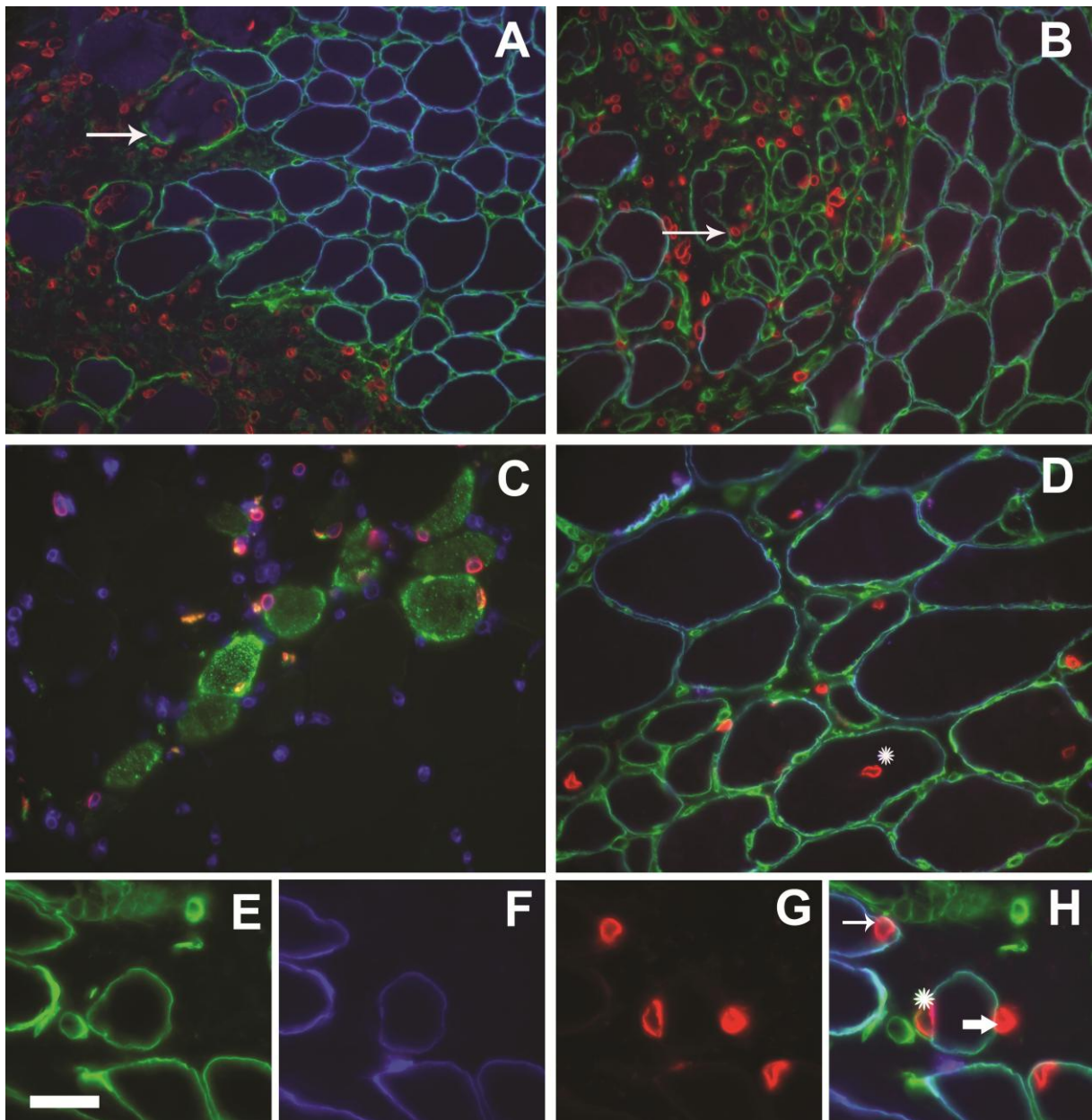
575 Figure 2



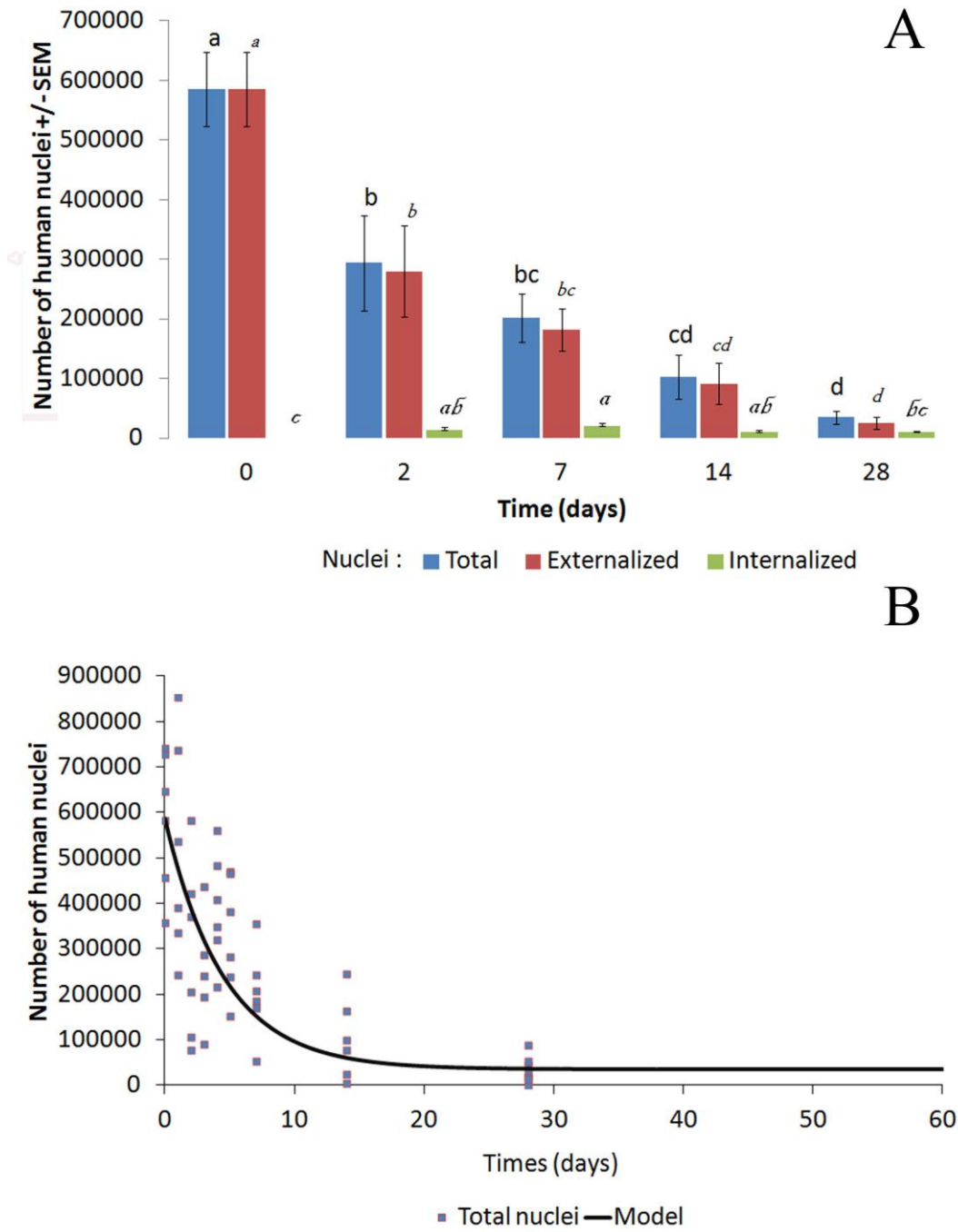
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578 Figure 3



581 Figure 4



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