Fetal Microchimeric Cells in a Fetus-Treats-Its-Mother Paradigm Do Not Contribute to Dystrophin Production in Serially Parous *mdx* Females

Elke Jane Seppanen,¹ Samantha Susan Hodgson,¹ Kiarash Khosrotehrani,¹
George Bou-Gharios,^{1,2} and Nicholas M. Fisk^{1,3}

Throughout every pregnancy, genetically distinct fetal microchimeric stem/progenitor cells (FMCs) engraft in the mother, persist long after delivery, and may home to damaged maternal tissues. Phenotypically normal fetal lymphoid progenitors have been described to develop in immunodeficient mothers in a fetus-treats-its-mother paradigm. Since stem cells contribute to muscle repair, we assessed this paradigm in the *mdx* mouse model of Duchenne muscular dystrophy. *mdx* females were bred serially to either ROSAeGFP males or *mdx* males to obtain postpartum microchimeras that received either wild-type FMCs or dystrophin-deficient FMCs through serial gestations. To enhance regeneration, notexin was injected into the tibialis anterior of postpartum mice. FMCs were detected by qPCR at a higher frequency in injected compared to noninjected side muscle ($P=0.02$). However, the number of dystrophin-positive fibers was similar in mothers delivering wild-type compared to *mdx* pups. In addition, there was no correlation between FMC detection and percentage dystrophin, and no GFP+ve FMCs were identified that expressed dystrophin. In 10/11 animals, GFP+ve FMCs were detected by immunohistochemistry, of which 60% expressed CD45 with 96% outside the basal lamina defining myofiber contours. Finally we confirmed lack of FMC contribution to satellite cells in postpartum *mdx* females mated with Myf5-LacZ males. We conclude that the FMC contribution to regenerating muscles is insufficient to have a functional impact.

Introduction

DURING PREGNANCY, FETAL cells traffic through or from the placenta to enter the maternal circulation. Small numbers of these semiallogeneic cells engraft in maternal organs and persist for decades [1,2]. Although this phenomenon seems ubiquitous in humans and mice, its clinical significance is largely unknown.

Persistent fetal microchimeric stem/progenitor cells (FMCs) have been implicated in both protective [3,4] and pathogenic [5] roles in a range of diseases, yet most evidence to date is circumstantial. FMCs selectively home to damaged tissues where they are found in a higher frequency than in uninjured control tissues [6,7]. They can either be a part of the inflammatory infiltrate or integrated into the surrounding maternal tissue, displaying niche-appropriate phenotypes [4,8]. It is these end-point phenotypes that have been used to argue that FMCs have tissue regenerative properties. Although evidence for the latter is strong, there are few data demonstrating that FMCs functionally benefit the affected maternal tissue.

A number of features indicate that FMCs are likely to be stem and/or progenitor cells: their longevity, apparent homing capacity, fetal source, and phenotypic diversity. Transfer of a more primitive pluripotent progenitor, however, cannot be excluded [9], since transplacental traffic of fetal cells begins soon after implantation in both humans and mice [8,10]. There is evidence for the transfer and persistence of diverse multi- and unipotent fetal progenitors, including hematopoietic [1,3], endothelial [4,11], mesenchymal [12], and even neuronal [14] lineages. This suggests that FMCs could act as cell therapy agents delivered naturally through pregnancy, and that FMCs from a normal fetus might potentially ameliorate maternal genetic disease in a fetus-treats-its-mother paradigm. The effect of fetus-to-mother transfer of stem/progenitor cells might arguably be expected to produce a greater effect, given the developmentally immature, but translationally advantageous, characteristics of more primitive stem/progenitor cell populations [14,15].

Muscular dystrophies are inherited myopathies characterized by cycles of skeletal muscle degeneration and regeneration. The commonest, Duchenne muscular dystrophy,

¹UQ Centre for Clinical Research, The University of Queensland, Brisbane, Australia.

²Kennedy Institute of Rheumatology, University of Oxford, Oxford, United Kingdom.

³Centre for Advanced Prenatal Care, Royal Brisbane and Women's Hospital, Herston, Australia.

is associated with mutations in the *dystrophin* gene, which encodes a protein integral to muscle fiber stability [16]. In the main mouse model of Duchenne muscular dystrophy (*mdx*), transplantation of various stem cell populations has shown therapeutic promise [17–19]. Muscle-resident stem cells or satellite cells have been obvious therapeutic candidates for muscular dystrophies [20], as they are activated during normal muscle repair [20,21]. Progenitors from distal sites such as bone marrow can also repair damaged muscles, although their contribution to repair is relatively small in comparison to the resident satellite cell population. We have previously shown that human fetal mesenchymal stem cells (MSCs) delivered to *mdx* fetuses *in utero* systemically engraft all muscle tissues displaying myogenic differentiation [17]. Moreover, allogeneic bone marrow transplant recipients have small numbers of muscle fibers that contain the donor nuclei [18,22].

In this study, we tested whether FMCs could act as muscle regenerative stem cells to improve dystrophin levels in maternal muscles that are dystrophin deficient. To maximize FMC recruitment to a specific muscle site and exaggerate the relatively mild phenotype of the *mdx* mouse, we generated an acute muscle insult in tibialis anterior (TA) muscles of parous mice using the myotoxin, notexin. We hypothesized that wild-type FMCs naturally acquired during gestation would preferentially target the more damaged muscles and elaborate sufficient functional dystrophin to ameliorate the muscle pathology in their mother.

Methods

Animals

C57BL/10ScSn-Dmd^{mdx} (*mdx*) females (Animal Resources Centre) were serially bred with males hemizygous for the enhanced green fluorescent protein (GFP) inserted into the ROSA26 locus Tg(Gt(ROSA)26sor-EGFP)IIAble (ROSAeGFP) (JAX®) or Myf5^{nlacZ/+} (Myf5-LacZ) males [23]. After each delivery, we ensured that at least 1 GFP mouse and 1 female mouse could be identified in the litter. For functional controls, we used *mdx* females serially bred with *mdx* males. This control group allowed us to examine exclusively the effect in *mdx* of harboring wild-type FMCs. Never-pregnant or virgin animals were used as technical controls for qPCR assays. All mice were treated in accordance with institutional guidelines and ethical approvals for the care of experimental animals.

Notexin injury

Notexin treatment was performed as described by others [24] to amplify the mild pathology in the *mdx* mouse. In brief, 4 weeks after their last delivery, *mdx* mice received an injection of 10 μ L of 10 μ g/mL notexin in 0.9% saline (Latoxan) down the length of the TA. The contralateral TA was injected with 10 μ L of saline. Seven days later when muscle regeneration peaks and new correctly localized dystrophin protein is seen in this model [24–26], mice were sacrificed and TA muscles dissected.

DNA extraction and qPCR

gDNA was extracted from each TA using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instruc-

tions. For qPCR assays, we used previously described primers and a probe to amplify a specific 75-base-pair region of the GFP transgene [27] or a region of the SRY gene located on the Y chromosome (Applied Biosystems). In our model, the GFP transgene or SRY is present in a single copy in positive FMCs. To measure total gDNA in each reaction, we amplified the murine genomic sequence of the apolipoprotein b (*apob*) gene, present in 2 copies per cell. Amplification and detection were performed using an ABI 7700 Sequence Detection System with SDS v1.9 software (Applied Biosystems). Relative amounts of fetal DNA were calculated using the comparative $\Delta\Delta$ CT method with *apob* as the reference sequence.

Tissue processing

Dissected TA muscles were fixed for 2 h in 4% PFA and subsequently infused with 30% sucrose before cryoembedding. Infused muscles were mounted in an optimal cutting medium and frozen rapidly in isopentane cooled with liquid nitrogen. Frozen 7- μ m sections were used for immunohistochemistry studies.

Immunohistochemistry

For staining of specific antigens, cryosections were permeabilized in 0.5% Triton-X-100 before blocking with 10% normal goat serum. Primary antibodies included 1 in 400 rabbit anti-dystrophin (Abcam, Cambridge, United Kingdom), 1 in 500 rabbit anti-GFP (Abcam) or 1 in 100 chicken anti-GFP (Invitrogen), 1 in 100 rat anti-mouse CD45 (Becton Dickinson), 1 in 200 chicken anti-mouse vimentin (Abcam), and 1 in 50 rabbit anti-laminin (Sigma-Aldrich). Secondary antibodies conjugated with Alexa-Fluor 568 or 488 (Invitrogen) were used for fluorescence detection. Nuclear staining was revealed in specimens mounted with a ProLong® Gold mounting medium containing DAPI (Invitrogen). Images were captured using an Axio Imager M1 (Zeiss) or an LSM 710 confocal microscope (Zeiss).

Scoring and quantification

To eliminate bias, all slides for dystrophin quantification and FMC engraftment were scored blind to the gestational history of the animals. Background green-specific autofluorescence, emitted by oxidative skeletal muscle fibers [28], was factored into the stringent criteria for analyzing engraftment of FMCs to prevent identification of false-positive cells. Slides were first scanned using a dual GFP/dsRED filter and selected cells emitted only green-specific fluorescence. To include these as FMCs, the selected cells also had to contain a nucleus and manifest a regular cell membrane. Cells considered as FMCs also had to emit GFP fluorescence at comparable levels to the mononuclear cells in muscle from the control ROSAeGFP animals. Finally, to be counted as true FMCs, cells had to have homogenous GFP staining under a high magnification (40 $\times$ objective).

Statistical analysis

Analyses were performed using GraphPad Prism v5c software. Normally distributed data were evaluated by standard *t*-tests, while nonparametric tests, such as

Mann–Whitney, were used on skewed data, and Fisher exact testing was used for categorical analyses. A P value <0.05 was considered significant.

Results

FMCs are specifically recruited to muscles after notexin injury

To investigate the role of FMCs in muscle repair, we generated a cohort of *mdx* female mice that had been serially pregnant with GFP+ve fetuses. The breeding strategy and pregnancy history are outlined in Fig. 1. To allow engraftment of pregnancy-acquired FMCs, mothers were housed for further 4 weeks after their last delivery. A local muscle insult using the myotoxin, notexin, was then employed [24] to amplify the continuous muscle regeneration that occurs in this mouse mutant. In addition to satellite cell-mediated regeneration, notexin injection also induces the rapid recruitment and differentiation of bone marrow-derived stem cells to the muscle, substantially increasing the proportion of newly formed fibers [18]. Notexin-induced damage was confirmed by hematoxylin and eosin staining (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd).

To examine whether FMCs are recruited specifically to notexin-damaged muscles, we amplified the paternally inherited *GFP* transgene [29]. *GFP* gDNA was detected in all postpartum *mdx* females that had produced GFP transgenic

fetuses. *GFP* gDNA was detected more often in TA muscles exposed to notexin compared to saline injection (9/11 vs. 4/11, $P=0.04$ Fisher exact test). In addition, more *GFP* gDNA was detected in TA muscles of *mdx* females regenerating after notexin insult compared to their contralateral saline-injected limbs ($P=0.02$). Of note, although *GFP* gDNA could be detected in some saline-injected contralateral limbs, this did not reach statistical significance compared to never-pregnant notexin-treated control animals (Fisher exact=0.1) (Fig. 2A). The number of GFP+ve pups delivered by *mdx* females did, however, correlate linearly with the amount of the *GFP* sequence detected in notexin-treated limbs (Pearson's r $P=0.01$, $R^2=0.6$) (Fig. 2B). The presence of fetal gDNA in *mdx* muscles was also confirmed using a qPCR strategy directed specifically against the Y-chromosome (Supplementary Fig. S2).

FMCs do not contribute to the percentage of dystrophin+ve fibers

We next sought to evaluate whether naturally acquired FMCs could actively contribute to maternal tissue repair. Dystrophin is central for maintaining muscle fiber integrity during contraction, and without it, mechanical stress associated with contraction leads to muscle fiber necrosis, and eventually fibrosis. Idiosyncratic exon skipping produces multiple in-frame *dystrophin* transcripts that are translated into a functional protein [30]. These so-called revertant fibers are, however, too few in proportion (ranging from 1% to 6%

FIG. 1. *mdx* breeding strategy and pregnancy history. (A) Experimental group: *mdx* females mated serially with ROSAeGFP males that harbor a single copy of *GFP* in the ROSA26 locus. In this breeding strategy, 50% of the progeny and thus trafficked FMCs are labeled by the paternally inherited transgene. Female FMCs that traffic into their *mdx* mothers are also unaffected by the dystrophin mutation, and thus essentially normal. (B) Control group: *mdx* females mated serially, but only with *mdx* males. As a consequence, all the trafficked FMCs are affected by the dystrophin mutation (no normal cells). (C) Pregnancy history: *mdx* females mated with ROSAeGFP males had at least 2 pregnancies. If litters were not cannibalized, the sex and genotype of the progeny were recorded. M, male; F, female; ?, sex, and genotype of pups were unknown due to their cannibalization; FMC, fetal microchimeric stem/progenitor cells; GFP, green fluorescent protein.

FIG. 2. More fetal gDNA is specifically detected in *mdx* TA muscles after treatment with notexin. **(A)** GFP gDNA is present only in *mdx* females mated with GFP males. GFP gDNA is selectively found in *mdx* TA muscles treated with notexin (paired *t*-test $P=0.02$). GFP gDNA was also detected in saline-injected TA muscles of some dystrophic mice. **(B)** The amount of GFP sequence detected in *mdx* muscles correlates with the number of GFP+ve pups delivered by *mdx* females (Pearson's $r P=0.01$, $R^2=0.6$). TA, tibialis anterior.

of total myofibers) [31] to overcome the phenotype in either the human or murine disease (Fig. 3A). We therefore evaluated the efficacy of wild-type FMCs as opposed to dystrophin-deficient FMCs to contribute to dystrophin production in *mdx* females. Notexin-treated TA muscles were chosen for this investigation, as the day-7 timepoint reflects the expected peak for the production of new appropriately localized dystrophin protein [26,32], and *mdx* muscles previously transplanted with young satellite cells have been shown to have donor-derived dystrophin+ve fibers 7 days after notexin treatment [33]. Individual dystrophin+ve fibers were counted and normalized to the total number of fibers present in each field of view. The average percentage of dystrophin+ve fibers was calculated for entire sections at 2-sectional depths so that $\sim 3,000$ muscle fibers were counted in each mouse. Since FMCs could potentially contribute to the satellite cell pool, we also compared the number of revertant fiber clusters between the 2 groups as a possible influence of FMC engraftment.

The percentage of dystrophin+ve fibers (2.6% vs. 2.9% $P=0.5$ Mann-Whitney) (Fig. 3B) or the number of dystrophin+ve clusters (5.8 vs. 7.5 $P=0.12$) (Fig. 3C) did not differ significantly between the 2 cohorts.

Dystrophin+ve fibers cannot be attributed to delivery of female offspring

The single-base mutation in *mdx* is X-linked, and as a consequence, loss of functional dystrophin occurs in homozygous females and hemizygous males. Under our breeding strategy, only FMCs from female fetuses had the possibility of producing wild-type dystrophin. Because our qPCR (Fig. 2B) and other recent data have linked the proportion of FMCs with the litter size [34], we undertook a subanalysis to explore any link between dystrophin+ve fibers and delivery of female fetuses. Linear regression analysis of dystrophin percentage to either the number of female fetuses (Fig. 4A) or the percentage of female fetuses (Fig. 4B) showed no

FIG. 3. FMCs do not contribute substantially to dystrophin levels in regenerating TA muscles. **(A)** Examples of dystrophin+ve fibers (red) in *mdx* TA 7 days after local administration; (a) saline, (b) notexin, (c) or in wild-type TA and (d) rabbit IgG isotype control. White arrows point to revertant clusters. Scale bars represent 50 μ m. All images were taken with the Zeiss LSM and Zen 2008 software. **(B)** Percentage of individual dystrophin+ve fibers relative to the total number of fibers. **(C)** Total number of dystrophin+ve clusters. The graphs represent pooled data from 2-sectional depths. Color images available online at www.liebertpub.com/scd

FIG. 4. Levels of dystrophin+ve fibers in regenerating TA muscles cannot be attributed to female progeny. Linear regression analyses comparing dystrophin percentage to the number of female fetuses delivered among those of known gender (A) or percentage of female fetuses delivered (B) showed no correlation. (C) *GFP* gDNA detection in postpartum *mdx* females did not correlate with dystrophin+ve fiber detection by IHC.

correlation with dystrophin levels. Animals that cannibalized their pups were excluded for this analysis. There was also no correlation between the dystrophin percentage and qPCR detection of *GFP* gDNA (Fig. 4C), suggesting that these dystrophin+ve fibers identified were likely revertant and not the product of even small numbers of wild-type FMCs that engrafted.

FMCs recruited to notexin-treated TA muscles are part of the inflammatory infiltrate and do not express dystrophin

Although there were no significant outcomes to dystrophin levels from wild-type FMC in *mdx* muscles, we sought to examine FMC properties by analyzing their engraftment and immunophenotype. First, multiple sections of notexin-treated TA muscles were analyzed for engraftment. GFP+ve muscle fibers were never found, indicating a lack of myogenic capacity by FMCs (minimum of 12 sections per animal were scanned). However, single, mononuclear GFP+ve FMCs were detected in the inflammatory exudate using two different anti-GFP antibodies (Fig. 5A). Although few in number, GFP+ve FMCs were found in 10 of 11 animals (Fig. 5B), and in 36%, they were present in at least 2 consecutive slides (Fig. 5C). Approximately 60% (17/28) of GFP+ve FMCs identified expressed the pan-hematopoietic marker, CD45 (Fig. 5D, F), whereas costaining with GFP and vimentin revealed that 25% of GFP+ve cells (3/12) identified expressed vimentin (Fig. 5E G). Additional costaining with GFP and laminin revealed that GFP+ve FMCs were nearly always found outside the basal lamina (23/24) (Fig. 5F-a, F-b, G). However, an example of a single GFP+ve FMC contained within the basal lamina was identified and confirmed by confocal microscopy (Supplementary Fig. S3), suggesting their capacity to integrate in the appropriate stem cell niche. However, GFP+ve FMCs coexpressing dystrophin were never found (0/16) (Fig. 5F-c, F-d, G). To explore further whether FMC could definitively contribute to satellite cells, we repeated the experiments but instead crossed *mdx* females with Myf5-LacZ males that express the LacZ reporter in satellite cells. Using this approach, only Myf5+ FMC and therefore pregnancy-acquired satellite cells would be labeled. Myf5+ FMC could not be found in either saline or notexin-treated TA muscles from postpartum *mdx* females (Supple-

mentary Fig. S4). These data suggest that FMCs in regenerating muscle do not have significant myogenic propensity.

Discussion

The contribution of FMCs to injured maternal tissue has been investigated in maternal heart muscle [35] and skin [4], but not as yet in skeletal muscle. Indeed, very few studies have explored the active contribution of persistent FMCs to tissue repair in postpartum mothers. Our data demonstrate that long-lived FMCs are selectively recruited to damaged muscle, in particular, the muscle undergoing acute inflammation in response to a myotoxin. The recruited FMCs found in damaged, but regenerating, muscles were associated with an inflammatory exudate, and 60% expressed the pan-hematopoietic marker, CD45. Moreover, ~96% of recruited FMCs were found outside the basal lamina. FMCs expressing dystrophin were also never detected. Contrary to our original hypothesis, the acquisition of wild-type FMCs through multiple pregnancies did not significantly impact dystrophin expression in deficient mothers.

The lack of significant impact by FMCs was surprising, given that the *mdx* mouse undergoes continuous muscle regeneration, as indicated by the high proportion of centrally nucleated myotubes [36]. It is thought that these constant cycles of muscle degeneration-regeneration eventually lead to premature satellite cell exhaustion and consequent demise in this animal [37]. Since a local notexin insult is known to recruit bone marrow populations to aid in this repair process [38], we postulated that persistent FMCs (likely from the bone marrow compartment [8,29]) would be similarly recruited and participate in this repair process. Our results however cannot exclude the possibility that FMCs below detectable levels reside locally, proliferate, and become detectable in response to injury. Since most FMCs found *in situ* were not part of the muscle syncytia and were predominantly either CD45+ or vimentin+ (Fig. 5), we conclude that FMCs do not contribute to the fibrosis observed in the *mdx* mouse [39].

Although we observed a selective recruitment of what were likely bone marrow-derived FMCs (based on their expression of CD45), they had little functional impact. Despite chronic FMC therapy, the lack of functional contribution can

An alternative, and more likely, explanation for the limited impact we observed could be the relative infrequency of persistent FMCs among maternal cells [29]. Despite maximizing the dose of FMCs acquired by mothers through serial breeding [29], their proportion was small (0.001%) compared to maternal cells. Moreover, the dystrophin mutation in *mdx* is X-linked [43], limiting potential benefits to FMCs acquired from female fetuses, and thus 50% of the progeny (regardless of their GFP expression). Notwithstanding this, we found no correlation between the number of female progeny and ei-

Bone marrow from postpartum women contains a small population of fetal mesenchymal stem cells (MSCs) with a known myogenic differentiation capacity [12]. Although we successfully found FMCs expressing the mesenchymal marker, vimentin, they were probably too few in proportion (3/12 FMCs) to have a significant influence on muscle pathology. Even 0.7% engraftment of fetal MSCs after intra-uterine transplantation did not ameliorate disease severity in *mdx* [17]. Moreover, we did not find FMCs robustly expressing proteins associated with muscle differentiation or

integration. Despite using notexin to amplify the muscle regeneration and stimulate production of dystrophin [32], FMCs expressing dystrophin were never found (0/16). Furthermore, only one FMC (1/24) was detected within the basal lamina. The basal lamina is important for coordinating satellite cell and fibroblast positioning and differentiation during muscle regeneration [45]. Although we found an example of a GFP+ve FMC in this area, which is a known satellite cell niche [20], this was undeniably rare. Additionally, we also demonstrated the absence of Myf5+ FMC in post-partum *mdx*, suggesting a lack of contribution by FMC to satellite cells. Together these data suggest that FMCs do not include a functional myogenic precursor.

Conclusion

The functional contribution of FMCs to women's health has long-generated debate. In this study, the transfer, persistence, and preferential engraftment of FMCs did not ameliorate disease manifestations in murine mothers with muscular dystrophy. This may reflect model specific limitations of muscular dystrophy or that FMC numbers at least in these experiments were insufficient to contribute actively to tissue repair. Notwithstanding this, we report the use of repeated mobilization of FMCs into the maternal circulation during pregnancy as a proxy method to test the effect of chronic stem/progenitor cell administration to treat maternal disease.

Acknowledgments

This work was funded by the National Health & Medical Research Council in Australia, project grant no. 569822. The authors are also grateful for the Myf5-LacZ transgenic mice, kindly provided by professor Peter Zammit.

Author Disclosure Statement

No competing financial interests exist.

References

- Bianchi DW, GK Zickwolf, GJ Weil, S Sylvester and MA DeMaria. (1996). Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci U S A* 93:705–708.
- Khosrotehrani K, KL Johnson, DH Cha, RN Salomon and DW Bianchi. (2004). Transfer of fetal cells with multilineage potential to maternal tissue. *JAMA* 292:75–80.
- Khosrotehrani K, M Leduc, V Bachy, S Nguyen Huu, M Oster, A Abbas, S Uzan and S Aractingi. (2008). Pregnancy allows the transfer and differentiation of fetal lymphoid progenitors into functional T and B cells in mothers. *J Immunol* 180:889–897.
- Nassar D, C Droitcourt, E Mathieu-d'Argent, MJ Kim, K Khosrotehrani and S Aractingi. (2012). Fetal progenitor cells naturally transferred through pregnancy participate in inflammation and angiogenesis during wound healing. *FASEB J* 26:149–157.
- Nelson JL. (1998). Microchimerism and the causation of scleroderma. *Scand J Rheumatol Suppl* 107:10–13.
- Nguyen Huu S, M Oster, MF Avril, F Boitier, L Mortier, MA Richard, D Kerob, E Maubec, P Souteyrand, et al. (2009). Fetal microchimeric cells participate in tumour angiogenesis in melanomas occurring during pregnancy. *Am J Pathol* 174:630–637.
- O'Donoghue K, HA Sultan, FA Ai-Allaf, JR Anderson, J Wyatt-Ashmead and NM Fisk. (2008). Microchimeric fetal cells cluster at sites of tissue injury in lung decades after pregnancy. *Reprod Biomed Online* 16:382–390.
- Sunami R, M Komuro, T Yuminamochi, K Hoshi and S Hirata. (2010). Fetal cell microchimerism develops through the migration of fetus-derived cells to the maternal organs early after implantation. *J Reprod Immunol* 84:117–123.
- Mikhail MA, H M'Hamdi, J Welsh, N Levicar, SB Marley, JP Nicholls, NA Habib, LS Louis, NM Fisk and MY Gordon. (2008). High frequency of fetal cells within a primitive stem cell population in maternal blood. *Hum Reprod* 23: 928–933.
- Thomas MR, R Williamson, I Craft and CH Rodeck. (1994). The time of appearance, and quantitation, of fetal DNA in the maternal circulation. *Ann N Y Acad Sci* 731:217–225.
- Nguyen Huu S, M Oster, S Uzan, F Chareyre, S Aractingi and K Khosrotehrani. (2007). Maternal neoangiogenesis during pregnancy partly derives from fetal endothelial progenitor cells. *Proc Natl Acad Sci U S A* 104:1871–1876.
- O'Donoghue K, J Chan, J de la Fuente, N Kennea, A Sandison, JR Anderson, IA Roberts and NM Fisk. (2004). Microchimerism in female bone marrow and bone decades after fetal mesenchymal stem-cell trafficking in pregnancy. *Lancet* 364:179–182.
- Zeng XX, KH Tan, A Yeo, P Sasajala, X Tan, ZC Xiao, G Dawe and G Udolph. (2010). Pregnancy-associated progenitor cells differentiate and mature into neurons in the maternal brain. *Stem Cells Dev* 19:1819–1830.
- Guillot PV, C Gotherstrom, J Chan, H Kurata and NM Fisk. (2007). Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. *Stem Cells* 25:646–654.
- Zhang ZY, SH Teoh, MS Chong, JT Schantz, NM Fisk, MA Choolani and J Chan. (2009). Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells. *Stem Cells* 27:126–137.
- Winder SJ. (1997). The membrane-cytoskeleton interface: the role of dystrophin and utrophin. *J Muscle Res Cell Motil* 18:617–629.
- Chan J, SN Waddington, K O'Donoghue, H Kurata, PV Guillot, C Gotherstrom, M Themis, JE Morgan and NM Fisk. (2007). Widespread distribution and muscle differentiation of human fetal mesenchymal stem cells after intrauterine transplantation in dystrophic *mdx* mouse. *Stem Cells* 25:875–884.
- Ferrari G, G Cusella-De Angelis, M Coletta, E Paolucci, A Stornaiuolo, G Cossu and F Mavilio. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 279:1528–1530.
- Sohn RL and E Gussoni. (2004). Stem cell therapy for muscular dystrophy. *Expert Opin Biol Ther* 4:1–9.
- Tedesco FS, A Dellavalle, J Diaz-Manera, G Messina and G Cossu. (2010). Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *J Clin Invest* 120:11–19.
- Zammit PS, TA Partridge and Z Yablonka-Reuveni. (2006). The skeletal muscle satellite cell: the stem cell that came in from the cold. *J Histochem Cytochem* 54:1177–1191.
- Gussoni E, RR Bennett, KR Muskiewicz, T Meyerrose, JA Nolte, I Gilgoff, J Stein, YM Chan, HG Lidov, et al. (2002). Long-term persistence of donor nuclei in a Duchenne muscular dystrophy patient receiving bone marrow transplantation. *J Clin Invest* 110:807–814.

23. Zammit PS, JJ Carvajal, JP Golding, JE Morgan, D Summerbell, J Zolnerchiks, TA Partridge, PW Rigby and JR Beauchamp. (2004). Myf5 expression in satellite cells and spindles in adult muscle is controlled by separate genetic elements. *Developmental biology* 273:454–465.
24. Plant DR, FE Colarossi and GS Lynch. (2006). Notexin causes greater myotoxic damage and slower functional repair in mouse skeletal muscles than bupivacaine. *Muscle Nerve* 34:577–585.
25. Lefaucheur JP and A Sebille. (1995). The cellular events of injured muscle regeneration depend on the nature of the injury. *Neuromusc Disord* 5:501–509.
26. Vater R, MJ Cullen, LV Nicholson and JB Harris. (1992). The fate of dystrophin during the degeneration and regeneration of the soleus muscle of the rat. *Acta Neuropathol* 83:140–148.
27. Pan D, R Gunther, W Duan, S Wendell, W Kaemmerer, T Kafri, IM Verma and CB Whitley. (2002). Biodistribution and toxicity studies of VSVG-pseudotyped lentiviral vector after intravenous administration in mice with the observation of *in vivo* transduction of bone marrow. *Mol Ther* 6:19–29.
28. Jackson KA, DS Snyder and MA Goodell. (2004). Skeletal muscle fiber-specific green autofluorescence: potential for stem cell engraftment artifacts. *Stem Cells* 22:180–187.
29. Khosrotehrani K, KL Johnson, S Guegan, H Stroh and DW Bianchi. (2005). Natural history of fetal cell microchimerism during and following murine pregnancy. *J Reprod Immunol* 66:1–12.
30. Lu QL, GE Morris, SD Wilton, T Ly, OV Artem'yeva, P Strong and TA Partridge. (2000). Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. *J Cell Biol* 148:985–996.
31. Wernig G, V Janzen, R Schafer, M Zweyer, U Knauf, O Hoegemeier, RR Mundegar, S Garbe, S Stier, et al. (2005). The vast majority of bone-marrow-derived cells integrated into mdx muscle fibers are silent despite long-term engraftment. *Proc Natl Acad Sci U S A* 102:11852–11857.
32. Yokota T, QL Lu, JE Morgan, KE Davies, R Fisher, S Takeda and TA Partridge. (2006). 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* 119:2679–2687.
33. Boldrin L, PS Zammit, F Muntoni and JE Morgan. (2009). Mature adult dystrophic mouse muscle environment does not impede efficient engrafted satellite cell regeneration and self-renewal. *Stem Cells* 27:2478–2487.
34. Fujiki Y, KL Johnson, H Tighiouart, I Peter and DW Bianchi. (2008). Fetomaternal trafficking in the mouse increases as delivery approaches and is highest in the maternal lung. *Biol Reprod* 79:841–848.
35. Kara RJ, P Bolli, I Karakikes, I Matsunaga, J Tripodi, O Tanweer, P Altman, NS Shachter, A Nakano, V Najfeld and HW Chaudhry. (2012). Fetal cells traffic to injured maternal myocardium and undergo cardiac differentiation. *Circ Res* 110:82–93.
36. Coulton GR, JE Morgan, TA Partridge and JC Sloper. (1988). The mdx mouse skeletal muscle myopathy: I. A histological, morphometric and biochemical investigation. *Neuropathol Appl Neurobiol* 14:53–70.
37. Heslop L, JE Morgan and TA Partridge. (2000). Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. *J Cell Sci* 113 (Pt 12):2299–2308.
38. Corbel SY, A Lee, L Yi, J Duenas, TR Brazelton, HM Blau and FM Rossi. (2003). Contribution of hematopoietic stem cells to skeletal muscle. *Nat Med* 9:1528–1532.
39. Morrison J, DB Palmer, S Cobbold, T Partridge and G Bou-Gharios. (2005). Effects of T-lymphocyte depletion on muscle fibrosis in the mdx mouse. *Am J Pathol* 166:1701–1710.
40. Tinsley J, N Deconinck, R Fisher, D Kahn, S Phelps, JM Gillis and K Davies. (1998). Expression of full-length utrophin prevents muscular dystrophy in mdx mice. *Nat Med* 4:1441–1444.
41. Wakefield PM, JM Tinsley, MJ Wood, R Gilbert, G Karpati and KE Davies. (2000). Prevention of the dystrophic phenotype in dystrophin/utrophin-deficient muscle following adenovirus-mediated transfer of a utrophin minigene. *Gene Ther* 7:201–204.
42. Chandrasekharan K, JH Yoon, Y Xu, S deVries, M Camboni, PM Janssen, A Varki and PT Martin. (2010). A human-specific deletion in mouse Cmah increases disease severity in the mdx model of Duchenne muscular dystrophy. *Sci Transl Med* 2:42ra54.
43. Sicinski P, Y Geng, AS Ryder-Cook, EA Barnard, MG Darlison and PJ Barnard. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* 244:1578–1580.
44. Fujiki Y, KL Johnson, I Peter, H Tighiouart and DW Bianchi. (2009). Fetal cells in the pregnant mouse are diverse and express a variety of progenitor and differentiated cell markers. *Biol Reprod* 81:26–32.
45. Sanes JR. (2003). The basement membrane/basal lamina of skeletal muscle. *The J Biol Chem* 278:12601–12604.

Address correspondence to:
 Elke Jane Seppanen
 UQ Centre for Clinical Research
 The University of Queensland
 Building 71/918, RBWH campus
 Brisbane 4029
 Australia

E-mail: e.seppanen@uq.edu.au

Received for publication January 29, 2012

Accepted after revision June 25, 2012

Prepublished on Liebert Instant Online June 25, 2012