Myoblast Transplantation in the Mouse: What Cells Do We Use?

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Abstract

Implantation of myoblasts into skeletal muscle still faces a number of problems that hinder its efficient use as a cell-mediated gene therapy. In previous work, different types of cell implants, ranging from minced muscle to myoblast lines, have been used and have yielded quite different results. This raises questions about the proper type of muscle cell preparation to be used. Expanded primary cultures of pure myoblasts should provide the ideal solution in terms of cell characterization and sufficient cell numbers, and indeed, effective transplantations of such preparations have been performed recently. However, mouse myogenic cultures seem to transform readily in vitro to form permanent lines, previously shown to be tumorigenic in vivo. In this paper we report on unexpected immune responses after implantation of expanded primary myoblasts into histocompatible hosts. Furthermore, it is shown that cultured myoblasts can easily acquire immunogenic properties following a sudden change in culture medium composition. These findings suggest that culture conditions might influence cell behavior in vivo and indicate the necessity to develop adequate methods of quality control to assure predictable transplantation results.

Key words: myoblast cultures, cell transplantation, graft rejection, immune reaction.

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Several problems concerning the efficiency of myoblast transplantation as a therapeutic approach are being considered in this issue of BAM. Here we will consider the dependence of in vivo results on type of implanted cell preparation.

Transplantation of muscle tissue in the form of *whole muscle grafts* is generally quite successful [3], as is implantation *of sliced muscle* grafts into host muscles which has resulted in good incorporation and long-term survival (up to 1 year) of donor myogenic cells contained in the graft [7]. Even *minced muscle* retains high regenerative capacity in vivo and, in the mouse, can form functional muscle tissue [2]. Limitations for use of such preparations are mainly tissue availability and inapplicability of genetic cell manipulations, but also immunogenicity [22].

Muscle precursor cells in the form of *cell suspensions* have been implanted immediately after enzymatic tissue disaggregation without culturing [20], and incorporation of donor cells into the host muscle and survival for up to several months have been documented [21, 22]. Implantation of myogenic cell suspensions from normal donors into dystrophin-difficient mdx muscles has resulted in dystrophin expression in host muscles [17]. The efficiency of such transplantations can be improved if the host muscle is X-irradiated prior to cell implantation [16].

Primary cultures, i.e. myogenic cells cultured for short periods (several days) and implanted before the first subculture, survive poorly in vivo: most cells die during the first few days, and the progeny of the remaining cells disappears gradually over the next few weeks [6, 8, 9]. Prolonged survival (up to 9 months) and enhanced incorporation of donor myoblasts have been achieved after toxin damage of the host muscle preceded by X-radiation of the limb [19].

Several factors might have an impact on viability and myogenicity of implanted cells in vivo. Numerous assaults associated with cell culture (enzymatic digestions, centrifugations, artificial media, etc.) might negatively influence cell viability. Trophic factors such as basic fibroblast growth factor (bFGF) and crushed muscle mitogen [1, 4] are reduced or completely eliminated in cell suspensions and cell cultures. The influence of trophic factors on primary cell behavior is illustrated by recent findings that , addition of b FGF to the growth medium prior to implantation improves cell survival [13]. It is also likely that extracellular matrix molecules and/or non-myogenic cells (e.g. fibroblasts) present in whole, sliced or minced muscle grafts, but absent or reduced in cell cultures, support the survival, proliferation and migration of myoblasts after bulk implantation.

One obvious limitation for use of primary cell suspensions or cultures in cell-mediated gene therapy is that these methods do not allow for growth of large numbers of cells. This is not the case with immortal *myoblast lines* which are available in unlimited quantities. Cells from such permanent lines possess high myogenic potential and their progeny survives for up to 660 days in vivo [23, 24]. They give rise to large amounts of new muscle tissue, both well differentiated and innervated, and muscle contractile strength is largely increased [24]. These results clearly show that, in principle, myoblast transfer can be very effective. However, after good initial differentiation, malignant tumors (rhabdomyosarcomas) inevitably form in implanted muscles at periods of time which range, depending on the cell line clone, from 5 weeks to 4 months post-implantation [24]. This finding generally seems to preclude the use of permanent cell lines for myoblast transfer. Cells from permanent lines have also enabled the observation of "bystander" damage, i.e. damage to host tissue during rejection of donor cells, a phenomenon leading to permanent structural and functional deficits in regenerating muscles [12, 23, 25].

The last type of myogenic cells, which have only recently been used, are *expanded primary cultures* [10, 18]. Primary cell cultures grown under conditions which preferentially support the proliferation of myoblasts compared with that of non-myogenic cells, soon contain nearly 100% myoblasts. Rando and Blau [18] found survival of β-galactosidase expressing myogenic cells without tumor formation in vivo over a period of 6 months. Using a Y-chromosome specific probe we observed survival of male Balb/c donor cells from expanded primary cultures in female Balb/c mice for 4 months [10]. Interestingly, numbers of surviving cells in this study were dependent upon the degree of previous damage to the recipient muscles; they were lowest in intact muscles, higher in moderately cryodamaged muscles and highest in severely cryodamaged muscles. In accordance with cell survival there were corresponding increases in contractile strength as compared to control cryodamaged muscles without cell implantation: severely damaged muscles exhibited the relatively best functional improvement, moderately damaged muscles exhibited marginal improvement and intact muscles did not show improvement. In these experiments myogenic cells expanded over 2-6 passages were used and no tumor formation or specific immune response was observed within 4 months. From these results and from what is known of the behavior of other cell preparations, it would appear that expanded primary cultures are best suited for myoblast transfer. However, mouse myoblasts tend to form permanent lines in vitro, which enhances the risk for tumor formation in vivo [26], Wernig and Irintchev, unpublished results]. Also immunogenicity observed with permanent cell lines [12, 23, 25] demands stringent controls and further investigations.

We report here on a cytotoxic immune reaction in inbred mice against early passages of two expanded primary muscle cell cultures from C57Bl/10J mice but not of a third one grown under the same conditions. Such spontaneous immunogenicity was not observed with similarly grown Balb/c cultures (n = 6) [10] and unpublished results]. However, when the culture medium composition was changed (withdrawal of supplemented basic fibroblast growth factor, bFGF) non-immunogenic Balb/c cultures did acquire immunogenic properties. These results clearly indicate that culture conditions may influence cell properties and thus behavior in vivo.

Materials and Methods

Cell cultures

Primary cultures were prepared from male postnatal Balb/c (n = 3) and C57B1/10J (n = 3) mice (4-13 days old) and expanded for several passages in F-10 medium supplemented with foetal bovine serum (20%) and bFGF (2.5 ng ml^{-1}) as described elsewhere [10]. Three C57B1/10J and two Balb/c cultures expanded under these conditions (passages 2-3) were used. In addition, supplementation with bFGF in one of the Balb/c cultures was discontinued at passage 2 and at passage 3 in two independent experiments, respectively; these cells were then implanted after expansion for an additional 1-2 passages. Control implantations in Balb/c mice were performed with C2C12 cell line [23] and with Balb/c primary myoblasts grown from the onset without bFGF. Myoblast cultures were over 95% pure as estimated from desmin staining [10] and myoblasts fused to form myotubes in low-serum medium. No differences in cell growth rate or cell morphology among different primary cultures were detected in vitro.

Animals and cell implantation

Balb/c and C57B1/10J mice, purchased from Charles River Wiga (Sultzfeld, Germany) were bred in the laboratory. Primary myoblasts from male donor animals were implanted into soleus muscles of 2-3 month-old female littermates (35 Balb/c and 24 C57B1/10 mice). Cells were implanted into cryodamaged soleus muscles (single freeze-thaw cycle) as described previously [10]. The cell suspension (10^5 cells in 2 µl Hanks' balanced salt solution per muscle) was introduced as a single injection with a Hamilton syringe (10 µl, gauge 22S) along the length of the muscle. To exclude that immune reactions against immunogenic Balb/c cells are due to H-Y antigens, these cells were also implanted into male Balb/c host mice (n = 5). In two other control groups, cryodamaged muscles of female Balb/c mice were implanted with C2nlsBAG cell line cells (n = 5) or injected with 2 µl Hanks' balanced salt solution containing no cells (n = 5). Treatments of animals were in accordance with the German law for protection of experimental animals.

Histology

Soleus muscles (implanted and intact contralateral) were harvested between 2 and 40 days post-implantation and immediately frozen in melting isopentane. Staining was

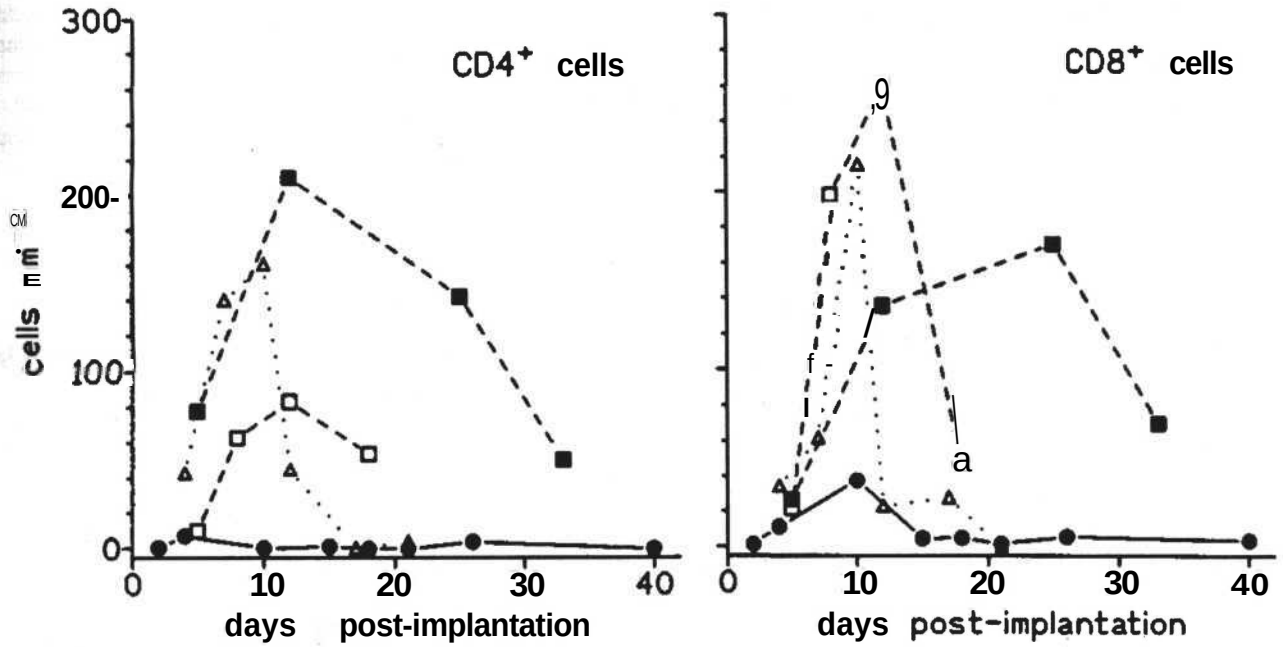

Figure 1. Numbers of CD4+ (helper T-cells, A) and CD8+ (killer T-) cells, normalized per unit cross-sectional area, in complete cross-sections from C57Bl/10 soleus muscles which had been cryodamaged and implanted 2 - 40 days earlier with cells from three different C57Bl/10 cultures (indicated by different symbols). Each symbol represents one section / muscle. One culture (squares) was implanted after 2 (open squares) and after 3 (filled squares) subcultures. The other two cultures (circles and triangles) were implanted after only 2 passages. After implantation of one culture (circles) numbers of T-cells are low and within the range observed previously in cryodamaged non-implanted muscles [23]. The other two cultures (squares and triangles) cause massive cellular infiltration.

done on serial cross-sections from the mid-belly of the muscle. Sections were stained with 1% w/v toluidine blue/ 1% w/v borax to reveal general muscle morphology. Immunofluorescent detection of major histocompatibility complex (MHC) class I molecules (monoclonal antibody, mAb, R1-9.6) [14], desmin (muscle cell marker, mAb D33, Dako, Hamburg, Germany), Lyt2 (mouse equivalent of human CD8, killer subset of T-lymphocytes, mAb 53-6.7) [15], L3T4 (CD4, helper T-cells, mAb GK 1.5) [5] and Mac1 (CD11b, macrophages/monocytes, mAb M1/70, Boehringer Mannheim, Mannheim Germany) was done as previously described [11, 12, 25]. The number of immune cells was quantified on complete cross-sections (one for each staining and muscle) and normalized per unit cross-sectional area [12].

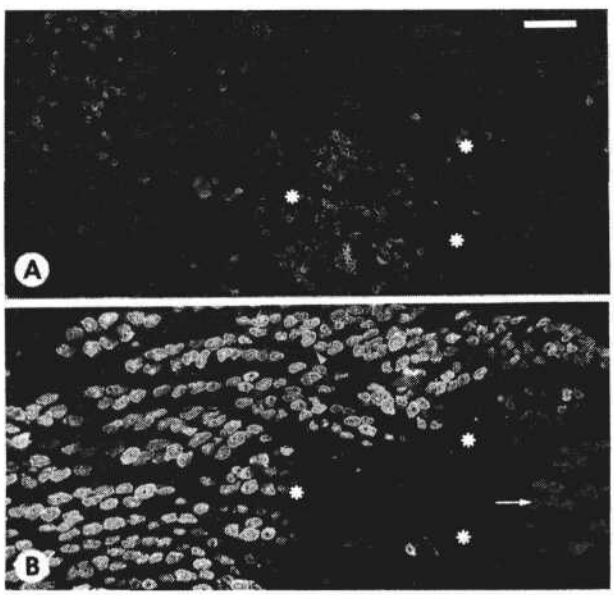

Figure 2. Immunofluorescent stainings of CD8+ T-lymphocytes (A) and desmin (B) in two neighbouring cross-sections from C57Bl/10 soleus muscle. The muscle has been implanted 8 days earlier with cultured myoblasts causing T-cell infiltration (second muscle in the group indicated with open squares in fig. 1). At this low magnification most of the muscle cross-sectional area is visible. Two regions (asterisks) contain numerous T-cells (A) but no desmin-positive muscle cells (B), indicating destruction of muscle tissue. Higher background straining (B, arrow) is seen in non-phagocytized remnants of muscle fibers or myotubes that have died either as a result of the cryodamage (an unusual finding at 8 days after damage) or, more likely, due to destruction by T-lymphocytes.

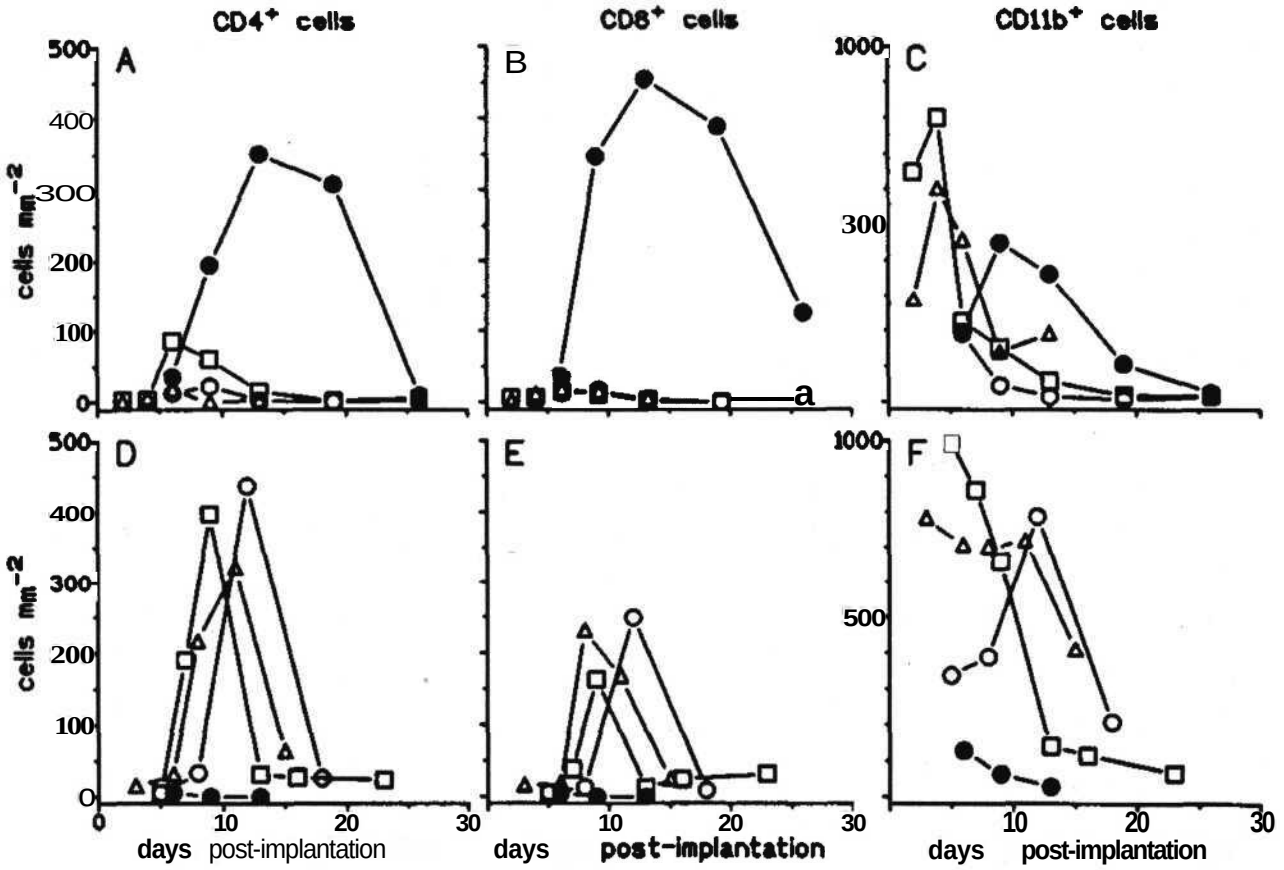

Figure 3. Numbers of CD4⁺ (T-helper; A, D), CD8⁺ (T-killer; B, E) and CD11b (Mad, macrophages/monocytes; C, F) cells normalized per unit muscle cross-sectional area in Balb/c soleus muscles 2-26 days after cryodamage and implantation of different syngenic primary cultures, and in different control muscles. Each point represents one animal. Controls (A-C): cryodamaged non-implanted muscles (O), and muscles implanted with non-histocompatible C2nlsBAG cell line (●) or cells from two different primary cell cultures grown with bFGF (O, Δ). Numerous T-cells associated with rejection of C2nlsBAG myoblasts in the second and third week after implantation (A, B); delayed disappearance of macrophages as compared to muscles with inconspicuous T-cell infiltrates is evident (C). Muscles implanted with cells from one of the primary cultures (□) contain low numbers of CD4 cells (A), but no T-killer cell infiltrates (B); reduction of macrophages with time procedes rapidly (C). Fig. 4D-E show muscles implantated with cells deprived of bFGF (○, □, A). Deprivation was undertaken independently two times (O and □ + Δ) starting with the same passage of cells grown with bFGF. Deprived cells induce massive infiltration of T-helper cells (D) and to a lesser extent T-killer cells (E), and macrophages persist for longer periods in the muscles (F). Immune infiltrates were not obviously caused by H-Y antigens since identical cells caused similar reactions in female (O) and male (Δ) animals. In contrast, in muscles implanted with cells from a culture initially established without bFGF (9) T-cell infiltration was not observed (D, E) and macrophage numbers declined more rapidly (F).

Results

Immune reactions against C5 7Bl/10 cells but not Balb/c cells

Myoblasts from early passages of expanded primary cultures from Balb/c mice formed muscle fibers after implantation into syngenic animals and contributed to muscle contractile force [10]. Survival of donor-derived male cells in female hosts for periods of up to 4 months, shown previously with in situ hybridisation of a Y-chromosome specific DNA probe [10], and the lack of significant infiltration of muscles by immune cells at early post-implantation periods in the present study (see below) indicate that these Balb/c myoblast preparations were not immunogenic. Similarly, numbers of lymphocytes present 2-40 days after implantation of C57B1/10 cells from one culture (passage 3) into C57Bl/10 host muscles (circles in fig. 1) were within the range previously observed for muscles cryodamaged only, which indicates unspecific infiltration of inflammatory cells. However, in muscles implanted with cells from two other cultures from different C57Bl/10 donors (both cultures at passage 2), there was significant infiltration with a maximum in the second week post-implantation (fig. 1, open triangles and open squares). Im-

plantation of one of these cultures (open squares) after an additional passage (passage 3) caused an even more massive infiltration (filled squares in fig. 1).

In C57Bl/10 muscles implanted with the non-immunogenic cultured cells (filled circles in Fig. 1), neither MHC class I expression in muscle fibers nor areas of muscle fiber destruction (as seen in Fig. 2 B) were detected at 2-40 days (not shown). In contrast, cytotoxic reaction was evident in muscles into which immunogenic cells were implanted (squares or triangles in fig. 1). These muscles exhibited areas filled with numerous lymphocytes but devoid of muscle cells (fig. 2A, B); in areas containing muscle cells MHC class I positive muscle fibers were present (not shown). These features were previously observed in the course of rejection of non-histocompatible cells [12, 23].

Induction of immunogenicity in Balb/c myoblasts

While none of the Balb/c muscles implanted with regularly grown cell preparations from inbred Balb/c mice used in a previous study (n = 6) [10] displayed unusual T-cell reactions between 5 and 30 days (open symbols in Fig. 3 A-C), muscles implanted with two samples, derived from the same cultures and independently expanded in a medium deprived of bFGF, contained large numbers of CD4 and CD8 lymphocytes (open symbols in fig. 3D-F). By the second week after implantation these muscles contained MHC class I positive muscle fibers as well as necrotic myofibres (not shown). It is unlikely that infection with microorganisms caused this T-cell response, since transfer of conditioned medium did not enhance immunogenicity of cells grown continually in the presence of bFGF (data not shown). Thus, due to different culture conditions, the cells may be altered to cause T-cell reactions.

Discussion

We found T-cell reactions after implantation of bFGF-deprived Balb/c cultures and this effect was reproducible. Immunogenicity was not due to H-Y antigens since implantation of these cells (male) into male recipients caused similar infiltrates as in female hosts (fig. 3D-F). Nor can immunogenicity be attributed to infection with microorganisms (e.g., viruses or mycoplasma), since immunogenic properties could not be transfered to other culture preparations by transfer of conditioned medium. Acquisition of immunogenicity in myogenic cells of Balb/c mice is attributable to the culture conditions, specifically, the change in medium composition, and not the absence of bFGF supplementation per se, since myoblast cultures established and expanded without bFGF (fig. 3D-F) were notimmunogenic.

How cultured cells develop immunogenic properties is not clear. One may assume that initial culturing in the presences of bFGF results in selection of cells dependent on this substance for proliferation. Subsequent deprivation may now cause selection of cells which are both immunogenic and less mitogen-dependent. It is also possible that changes in growth conditions only modify the expression of (a) cell surface or secreted molecule(s) such as adhesion, chemotactic or mitogenic molecules causing accumulation and activation of T-cells in implanted muscles.

Why immune reaction after implantation of C57B1/10 cultures occurred apparently "spontaneously" in two out of the three preparations is not clear. The reason might be a trivial one such as viral infection of the cultures, which we have not excluded in these particular preparations. Another possibility is that myoblasts react to culture conditions in a strain-specific manner. Thus, C57B1/10 cells, unlike Balb/c cells, might tend to become immunogenic even without dramatic manipulation of culture conditions. Vulnerability of myogenic cells to culture conditions may even be species-specific: formation of permanent cell lines which are tumorigenic, for example, readily occurs with mouse cultures but have not been reported for human myogenic cells.

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References

[1] Bischoff R: A satellite cell mitogen from crushed adult muscle. *Dev Biol* 1986; 115: 140-147.

[2] Carlson BM: The regeneration of minced muscles. *Monogr Dev Biol* 1972; 4: 1-128.

[3] Carlson BM, Faulkner JA: The regeneration of skeletal muscle fibers following injury: a review. *Med Sci Sports Exerc* 1983; 15: 187-198.

[4] Clarke MS, Khakee R, McNeil PL: Loss of cytoplasmic basic fibroblast growth factor from physiologically wounded myofibers of normal and dystrophic muscle. *J Cell Sci* 1993; 106: 121-133.

[5] Dialynas DP, Quan ZS, Wall KA, et al: Charactarization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK 1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J Immunol* 1983; 13: 2445-2451.

[6] DiMario JX, Stockdale FE: Differences in the developmental fate of cultured and noncultured myoblasts when transplanted into embryonic limbs. *Exp Cell Res* 1995; 216: 431-442.

[7] Fan Y, Beilharz MW, Grounds MD: A potential alternative strategy for myoblast transfer therapy: The use of sliced muscle grafts. *Cell Transplant* 1996; 5: 421-429.

[8] Fan Y, Maley M, Beilharz M, et al: Rapid death of injected myoblasts in myoblast transfer therapy. *Muscle Nerve* 1996; 19: 853-860.

[9] Huard J, Acsadi G, Jani A, et al: Gene transfer into skeletal muscles by isogenic myoblasts. *Hum Gene Ther* 1994; 5: 949-958.

[10] Irintchev A, Langer M, Zweyer M, et al: Functional improvement of damaged adult mouse muscle by implantation of primary myoblasts. *J Physiol* 1997; 500:775-785.

[11] Irintchev A, Zeschnigk M, Starzinski-Powitz A, et al: Expression pattern of M-cadherin in normal, denervated and regenerating mouse muscles. *Dev Dynam* 1994; 199: 326-337.

[12] Irintchev A, Zweyer M, Wernig A: Cellular and molecular reactions in mouse muscles after myoblast implantation. *J Neurocytol* 1995; 24: 319-331.

[13] Kinoshita I, Vilquin JT, Roy R, et al: Successive injections in mdx mice of myoblasts grown with bFGF. *Neuromuscul Disord* 1996; 6: 187-193.

[14] Koch S, Koch N, Robinson P, et al: Comparison of allogeneic and xenogeneic determinants on the H-2Kk molecule. *Transplantation* 1983; 36: 177-180.

[15] Ledbetter JA, Herzenberg LA: Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol Rev* 1979; 47: 63-90.

[16] Morgan JE, Hoffman EP, Partridge TA: Normal myogenic cells from newborn mice restore normal histology to degenerating muscles of the mdx mouse. *J Cell Biol* 1990; 111: 2437-2450.

[17] Partridge TA, Morgan JE, Coulton GR, et al: Conversion of mdx myofibers from dystrophin-negative to positive by injection of normal myoblasts. *Nature* 1989; 337: 176-179.

[18] Rando TA, Blau HM: Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J Cell Biol* 1994; 125: 1275-1287.

[19] Vilquin JT, Wagner E, Kinoshita I, et al: Successful histocompatible myoblast transplantation in dystrophin-deficient mdx mouse despite the production of antibodies against dystrophin. *J Cell Biol* 1995; 131: 975-988.

[20] Watt DJ, Lambert K, Morgan JE, et al: Incorporation of donor muscle precursor cells into an area of muscle regeneration in the host mouse. *J Neurol Sci* 1982; 57: 319-331.

[21] Watt DJ, Morgan JE, Partridge TA: Use of mononuclear precursor cells to insert allogeneic genes into growing mouse muscles. *Muscle Nerve* 1984; 7: 741-750.

[22] Watt DJ, Morgan JE, Partridge TA: Allografts of muscle precursor cells persist in the non-tolerized host. *Neuromuscul Disord* 1991; 1: 345-355.

[23] Wernig A, Irintchev A: "Bystander" damage of host muscle caused by implantation of MHC-compatible myogenic cells. *J Neurol Sci* 1995; 130: 190-196.

[24] Wernig A, Irintchev A, Haertling A, et al: Formation of new muscle fibres and tumours after injection of cultured myogenic cells. *J Neurocytol* 1991; 20: 982-997.

[25] Wernig A, Irintchev A, Lange G: Functional effects of myoblast implantation into histoincompatible mice with or without immunosuppression. *J Physiol* 1995; 484: 493-503.

[26] Wernig A, Salvini TF, Irintchev A: Axonal sprouting and changes in fibre types after running-induced muscle damage. *J Neurocytol* 1991; 20: 903-913.