—Brief Note—

Expression of pMGN(-4k)LacZ-neo Gene Introduced into Embryonic Stem (ES) Cells in Chimeric Mouse Fetuses

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Abstract: *We attempted to produce chimeric mouse fetuses by using an embryonic stem (ES) cell subline, OM 3, which were transfected with pMGN(-4k)LacZ-neo transgene, and to analyze expression of the transgene in chimeric mouse fetuses. In embryonic days 11-13 (E11-13), no fetuses expressing the transgene were observed. In E14, three of 11 normal fetuses recovered showed the transgene expression. In these chimeric mouse fetuses, the regions expressing the transgene were found in a wide range of fetuses, including the temporal region of the head, trunk from neck to tail, and limbs. It was shown by histochemical analysis that these patterns of transgene expression were specific to skeletal muscles. These results indicated that OM 3 cells might be useful for analyses of mouse skeletal-myogenesis and for the development of basic techniques for skeletal muscles in regenerative medicine.*
Key words: *Embryonic stem (ES) cells, Myogenin gene expression, LacZ reporter gene, Chimeric mouse fetus, Skeletal-myogenesis*

In order to examine the development or differentiation of a specific tissue, tissue-specific cell markers would be useful. Such markers could also be used for analysis of the diverse functions of a gene and the basic studies of regenerative medicine. Skeletal muscle-specific cell markers are particularly useful for not only the analysis of skeletal myogenesis but also for the study of medical therapy with myocytes or muscle transplantation for muscle diseases such as progressive muscular dystrophy.

The myogenin gene, which is one of the myogenic regulatory genes, is specifically expressed during differentiation of skeletal muscles and continues to be expressed during skeletal myogenesis, so that the regulatory region of this gene would be useful for producing skeletal muscle-specific cell markers. Fujisawa-Sehara *et al.* [1] and Kim *et al.* [2] reported that the upstream region of myogenin gene, which ranged ~4kb, directed appropriate temporal, spatial and tissue specific expression of the myogenin gene, by using a pMGN(-4k)LacZ transgene and transgenic technique with microinjection. The pMGN(-4k)LacZ gene consisted of a LacZ gene driven by the regulatory region (upstream sequence which ranged ~4kb) of myogenin gene (see Fig. 1).

We attempted to produce muscle-specific cell markers by electroporation of the pMGN(-4k)LacZ-neo gene (Fig. 1) into an embryonic stem (ES) cell line, ESD3 [3], and reported the production of transfected ESD3 cell sublines and the expression pattern of the transgene *in vitro* [4, 5]. The results suggest that the sublines could be useful as skeletal muscle specific cell markers for analysis of mouse myogenesis at least *in vitro*, but the efficacy of the sublines as skeletal muscle-specific cell markers to analyze skeletal myogenesis *in vivo* was not confirmed. In order to analyze expression of the transgene *in vivo*, chimeric mouse embryos were produced by coculture of ICR compacted morulae and OM 3 cells, which is one of the ES cell sublines transfected with the pMGN(-4k)LacZ-neo gene. Furthermore, their tissue-specific expression in chimeric mouse fetuses was analyzed.

The cells were cultured according to the method described by Robertson [6], and were used for chimera production up to 30 passages. Chimera production was carried out as described by Wood *et al.* [7, 8], excepting the use of stem cell medium [6] for coculture. After overnight culture, the embryos were transferred into uteri

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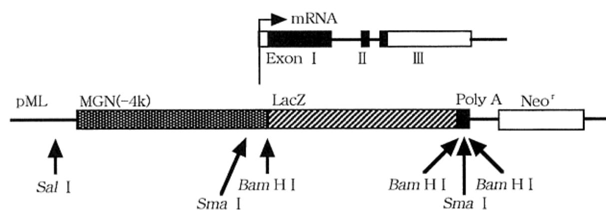

Fig. 1. Structure of the transgene, pMGN(-4k)LacZ-neo gene. The mouse myogenin gene consists of three exons (I, II, III), of which coding and non-coding sequences are represented by filled in and open boxes, respectively. The 5′ flanking and non-coding sequences of the myogenin gene are linked to the LacZ gene, and cloning into the pML vector. In order to select transfected cells *in vitro*, a neomycin resistant gene cassette from pMC1neoPolyA was fused to a pMGN(-4k)LacZ gene. pML; pML vector (about 2.6kb length), MGN(-4k); myogenin gene regulatory region (about 4kb length), LacZ; beta-galactosidase gene, Neo^r; neomycin resistant gene cassette.

of ICR recipients at 2.5 days of pseudopregnancy. The fetuses were collected on desired days of gestation, stained for LacZ expression essentially according to Bonnerot and Nicolas [9], and analyzed histochemically.

The total of 121 embryos were cocultured with OM 3 cells, and transferred to 7 recipients (Table 1). On embryonic days 11-13 (E11-13), eighteen normal fetuses and 21 abnormal fetuses were collected. The abnormal fetuses included the absorber and the ones with hypoplasia. The normal fetuses collected on these embryonic days were analyzed by X-gal staining, but there were no fetuses with LacZ expression (Table 1). In E14, eleven normal and 4 abnormal fetuses were collected, and 3 of the normal ones showed signs of LacZ expression, indicating that they were chimeric (Table 1). LacZ-positive regions in these chimeric fetuses were observed extensively in the temporal region of the head, body trunk from neck to tail, and limbs (Fig. 2).

The chimeric fetuses with LacZ-positive cells were processed for histochemical analysis. As shown in Fig. 3, LacZ expression was restricted to skeletal muscles but not other tissues. It was thought that LacZ-positive muscle in the temporal region of the head was a part of the *Musculus masseter* (Fig. 3A), ones in the body trunk were epaxial musculature and inner, medial and outer subaxial musculatures (Fig. 3B). Some skeletal muscles in the fore and hind limbs (Fig. 3C and D) were also LacZ positive, but each muscle was unidentified. Particularly in the hind limbs, there were some skeletal muscles with no expression of the LacZ gene.

In this study chimeric embryos were produced by coculture of OM 3 cells with ICR compacted morulae, with stem cell medium as a coculture medium. Coculture of ES cells and embryos for the production of chimeras was generally performed under conditions favorable to the embryos [7, 8, 10–12], but in producing chimeras by coculture of embryos and ES cells, which had reduced ability for chimera formation, the coculture conditions favorable to ES cells might be useful. In the present study transfected ES cells which underwent numerous passages (total 20–30 passages; 15–20 before and 5–10 after transfection) were used for chimera production, but fetuses with extensive chimerisms comparable to those in the transgenic experiments with similar transgenes [1, 2, 13, 14] were obtained (Fig. 2).

As shown in Fig. 2, it was indicated that tissue-specific expression of the transgene localized skeletal muscles. These results suggested that they reflected the myogenin expression patterns *in vivo*, compared with transgenic studies with a similar transgene [1, 2, 13, 14]. But in some skeletal muscles, no transgene expression was observed. This might be due to lower migratory activities of the derivatives of transfected ES

Table 1. Production of chimeric fetuses between OM 3 cells and ICR compacted morulae by a coculture method

	No. of embryos cocultured	No. of recipients	No. of fetuses recovered Normal [Abnormal[a]]	No. of ES chimeras[b]
Total	121	7	29 [25]	3
E11[c]	34	2	7 [12]	0
E12[c]	36	2	9 [9]	0
E13[c]	18	1	2 [0]	0
E14[c]	33	2	11 [4]	3

[a]Absorber and the fetuses with hypoplasia. [b]Judgement by the presense of LacZ-positive regions. [c]Embryonic days.

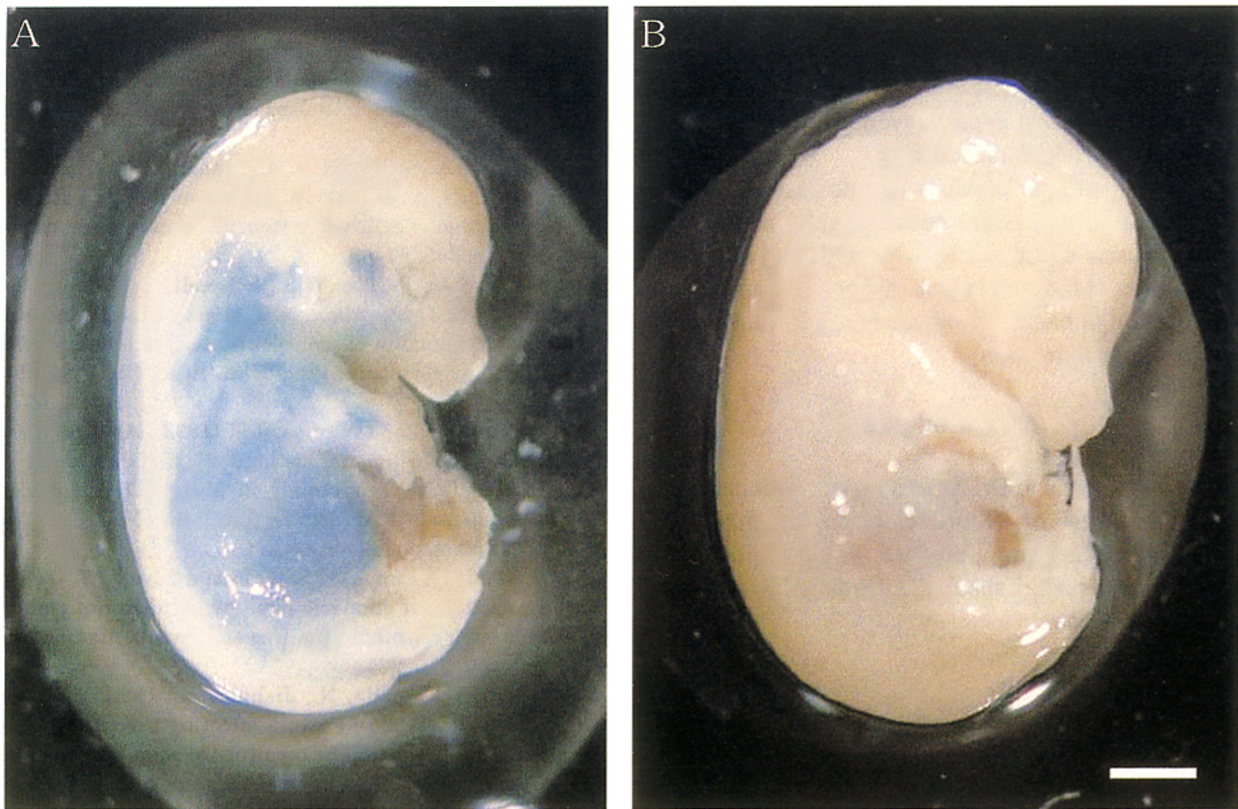

Fig. 2. Recovered fetuses on E14, which stained for LacZ expression. Panel A shows a chimeric fetus with regions expressing LacZ in the temporal region of the head, body trunk from neck to tail and limbs, and panel B shows a LacZ negative fetus. The expression pattern of the transgene shown in A, was comparable to that of transgenic fetuses with similar transgenes. The chimeric fetuses were distinguishable from non-chimeric, by eye pigmentation, before X-gal staining. Scale bar, 1 mm.

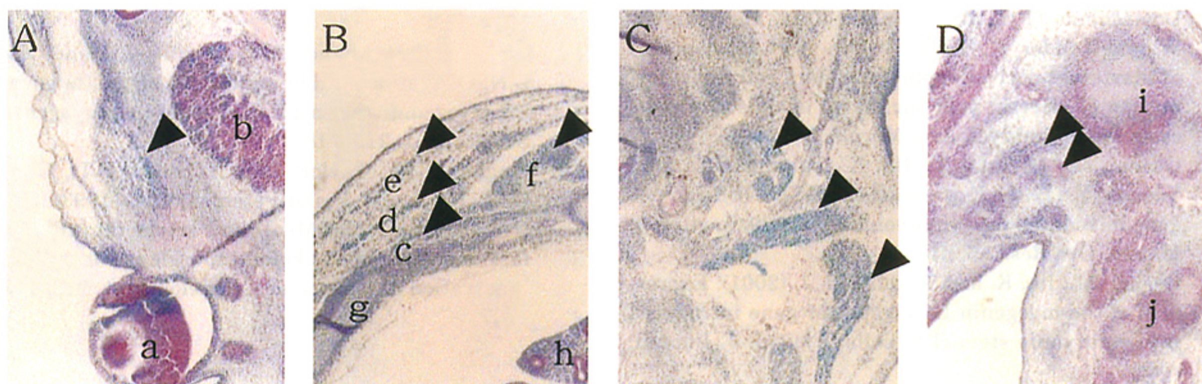

Fig. 3. Histochemical analysis of E14 chimeric fetuses. Panels A, B, C and D show the temporal region of the head, a part of the body trunk, a forelimb and a hind limb, respectively. Arrowheads indicate the skeletal muscles expressing the transgene. The LacZ-positive tissue in panel A may be a part of the *Musculus masseter*; in panel B, inner, medial and outer subaxial musculatures and epaxial musculature. In panels C and D, each muscle expressing LacZ is unidentified. Expression of the transgene was specific to skeletal muscles, but some skeletal muscles did not show a LacZ signal. a, eye; b, trigeminal ganglion; c, inner subaxial musculature; d, medial subaxial musculature; e, outer subaxial musculature; f, epaxial musculture; g, rib; h, lobe of lung; i, cartilage primordium of femur; j, cartilage primordia of talus and calcaneus.

cells, or due to a biased distribution of them. Hughes *et al.* [15] reported that the myogenin gene was expressed specific to slow muscles in rat. Our results therefore suggest that, at least in part, myogenin expression differed in each skeletal muscle type.

Together with our previous reports [4, 5], these results indicated that OM 3 cells might be useful as a skeletal muscle-specific cell marker and were applicable for analyses of mouse skeletal-myogenesis and for the development of basic techniques in regenerative medicine for skeletal muscles.

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References

1) Fujisawa-Sehara, A., Hanaoka, K., Hayasaka, M., Hiromasa-Yagami, T. and Nebeshima, Y. (1993): Upstream region of the myogenin gene confers transcriptional activation in muscle cell lineage during mouse embryogenesis. Biochem. Biophys. Res. Com., 191, 351–356.
2) Kim, S.Y., Takahashi, J., Tsutsumi, K., Kang, M.S. and Yasuda, Y. (1995): Expression of the myogenin-LacZ reporter gene during early postimplantation development of the mouse. J. Reprod. Dev., 41, 241–247.
3) Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985): The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J. Embryol. Exp. Morph., 87, 27–45.
4) Osonoi, M., Mimaki, Y., Ito, K., Takahashi, J. and Yasuda, Y. (1999): Expression of pMGN(-4k)LacZ-neo gene introduced into embryonic stem cells in vitro. J. Mamm. Ova Res., 16, 31–36.
5) Osonoi, M., Ito, K. and Takahashi, J. (2001): Expression of the myogenin-LacZ reporter gene introduced into embryonic stem (ES) cells *in vitro*. J. Reprod. Dev., 47, 45–52.
6) Robertson, E.J. (1987): Embryo-derived stem cell lines. In: Teratocarcinomas and Embryonic Stem Cells; A Practical Approach (Robertson, E.J. ed.), pp. 71–112, IRL Press.
7) Wood, S.A., Allen, N.D., Rossant, J., Auerbach, A. and Nagy, A. (1993): Noninjection methods for the production of embryonic stem cell-embryo chimaeras. Nature, 365, 87–89.
8) Wood, S.A., Pascoe, W.S., Schmidt, C., Kemler, R., Evans, M.J. and Allen, N.D. (1993): Simple and efficient production of embryonic stem cell-embryo chimeras by coculture. Proc. Natl. Acad. Sci. USA., 90, 4582–4585.
9) Bonnerot, C. and Nicolas, J.F. (1993): Application of LacZ gene fusions to postimplantation development. In: Guide to Techniques in Mouse Development. (Wassarman, P.M. and Depamphilis, M.L. eds.), pp 451–469, Academic Press.
10) Sugino, T., Azuma, S., Sato, E. and Toyoda, Y. (1993): Production of chimeric mice by coculture of embryonic stem cells and zona-free embryos. J. Mamm. Ova Res., 10, 216–221.
11) Ueda, O.,Kamada, N., Jishage, K., Uchida, S. and Suzuki, H. (1995): Factors affecting the efficiency of chimera production by coculture of zona-free embryos with frozen-thawed embryonic stem cells in mice. J. Reprod. Dev., 41, 181–186.
12) Khillan, J.S. and Bao, Y. (1997): Preparation of animals with a high degree of chimerism by one-step coculture of embryonic stem cells and preimplantation embryos. BioTechniques, 22, 544–549.
13) Cheng, T.C., Hanley, T.A., Mudd, J., Merlie, J.P. and Olson, E.N. (1992): Mapping of myogenin transcription during embryogenesis using transgenes linked to the myogenin control region. J. Cell Biol., 119, 1649–1656.
14) Cheng, T.C., Wallance, M.C., Merlie, J.P. and Olson, E.N. (1993): Separable regulatory elements governing myogenin transcription in mouse embryogenesis. Science, 261, 215–218.
15) Hughes, S.M., Taylor, J.M., Tapscott, S.T., Gurler, C.M., Carter, W.J. and Peterson, C.A. (1993): Selective accumlation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. Development, 118, 1137–1147.