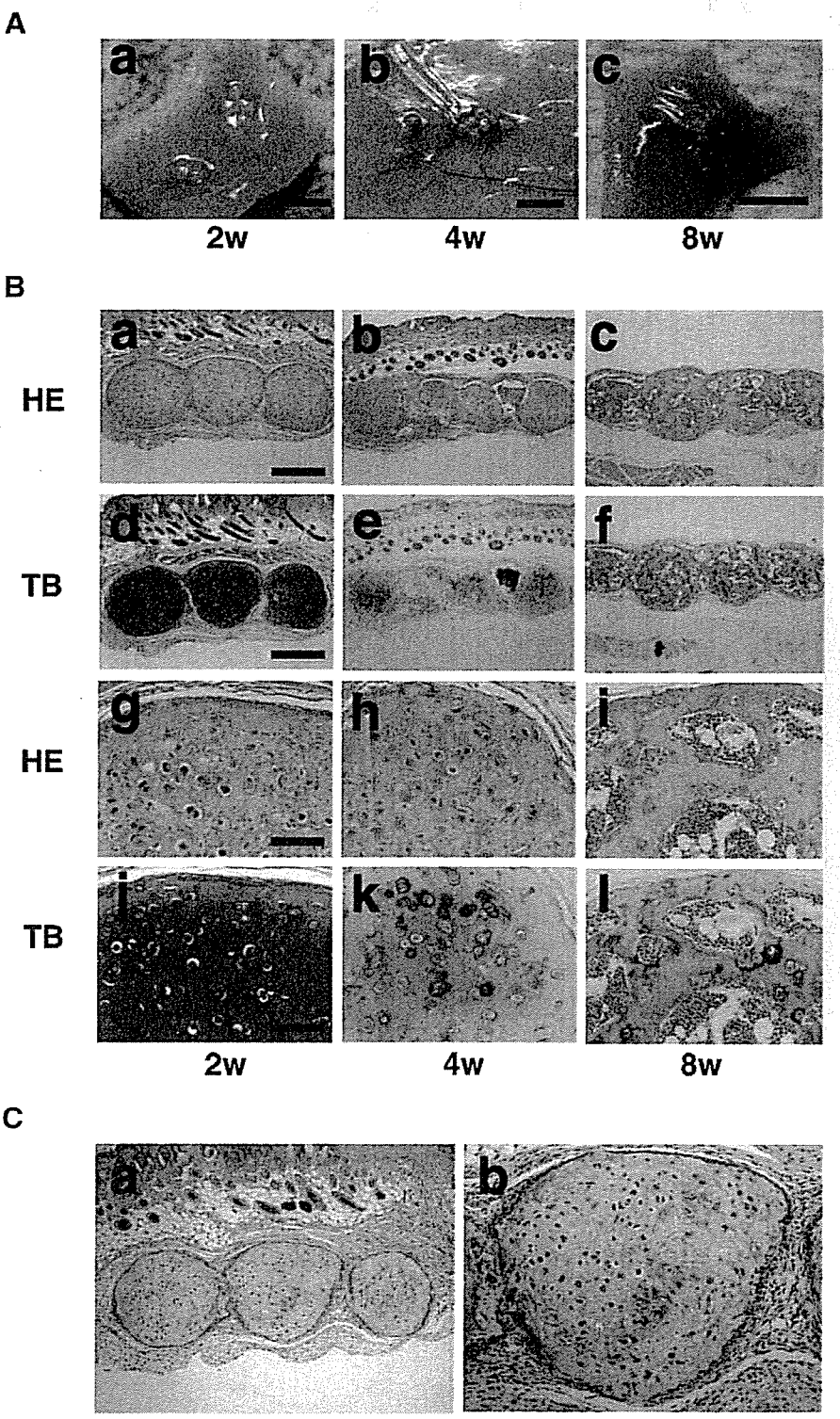

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Fig. 7. In vivo chondrogenesis of OP9 cells. In vivo chondrogenesis was examined by implantation of OP9 chondrogenic nodules. OP9 chondrogenic nodules, which were generated after pellet culture for 7 days in the CM supplemented with TGF-β3 and BMP2, were implanted just beneath the cutaneous muscle in the subcutaneous tissue and were cultivated in vivo for the number of weeks indicated. **A**: Macroscopic view of OP9 cartilage after 2 (a), 4 (b), and 8 (c)-week-in vivo cultivation. **B**: Histological analysis of OP9 cartilage after 2 (**a**,**d**,**g**,**j**), 4 (**b**,**e**,**h**,**k**), and 8 (**c**,**f**,**i**,**l**)-week-in vivo cultivation. (a,b,c,g,h,i), HE stain; (d,e,f,j,k,l), TB stain. **Panels g–l** are higher magnifications of **a–f**, respectively. **C**: Immunohistochemical analysis of the in vivo OP9 chondrogenic nodules. The OP9 chondrogenic nodules after 2-week-in vivo cultivation stained positive for collagen type II. Scale bars: 2 mm (A), 500 μm (Ba–f), 100 μm (Bg–l).

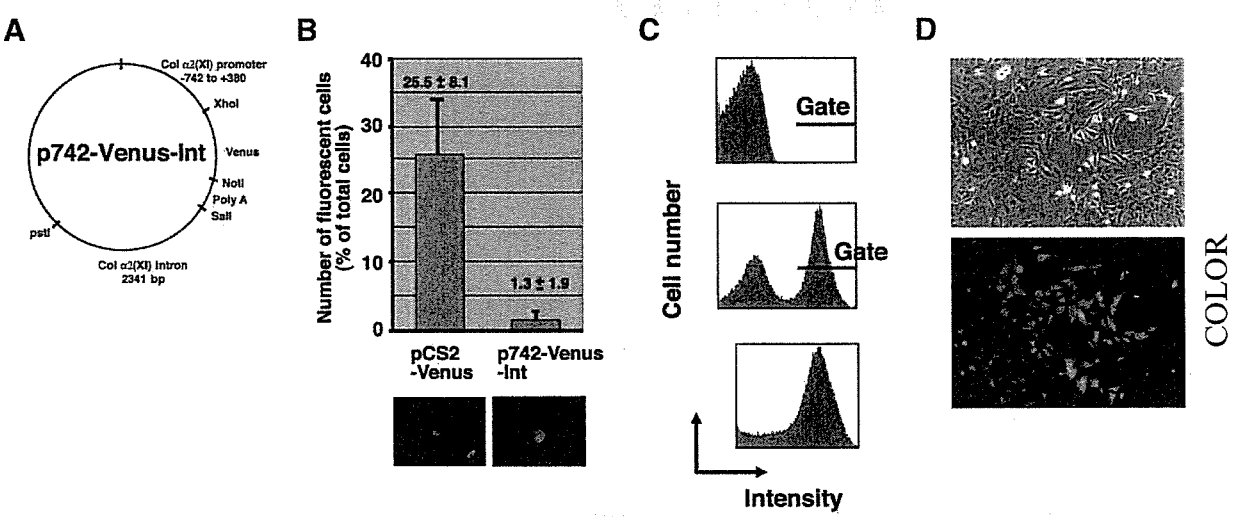

Fig. 8. Isolation of KUM5 chondroblasts using the chondroblast-specific cis-regulatory element. **A:** The p742-Venus-Int plasmid containing the fluorescent Venus gene driven by the cis-regulatory elements of the α2(XI) collagen gene. **B:** The number of fluorescent KUM5 cells (**upper**) after transfection with the p742-Venus-Int plasmid or pCS2-Venus containing the Venus gene driven by the CMV-promoter. Fluorescent photomicrograph of KUM5 cells after the first sorting (**lower**). **C:** Flowcytometric analysis of KUM5 cells after transfection with the p742-Venus-Int plasmid (**top**); The fluorescence-positive cells were sorted, propagated, and analyzed (**middle**). Again, the propagated fluorescence-positive cells were sorted, propagated, and analyzed (**bottom**). The "gate" for sorting is shown by the horizontal bar in the upper and middle panels. More than 80% of cells became positive after the final sorting. **D:** Phase contrast micrograph (upper) and fluorescent photomicrograph (lower) of the finally sorted cells (the lower panel of C).

tissue of nude mice were embedded in the hypertrophic chondrocytes and had abundant endoplasmic reticulum and a small number of mitochondria (Fig. 9H,I). The post-mitotic daughter cells in the cell nest, which are often observed in cartilage, were also detected (Fig. 9I).

DISCUSSION

In this study, we focus on the chondrogenic differentiation in vitro and in vivo using the two cell lines, KUM5 and OP9. The chondrogenic process is determined by the sequential expression of matrix component, and the differential response of differentiating cells to the growth factors may be attributed to the differentiating stages that depend on the expression patterns of the gene set as is the case for hematopoietic cells. The process of the chondrogenic differentiation is influenced by a number of growth factors including TGF-β and/or BMPs. Three isoforms of TGF-β have been known to have the ability to induce the chondrogenic differentiation. Both TGF-β2 and -β3 are more effective than TGF-β1 in promoting chondrogenesis, and TGF-β3 accelerates production of cartilaginous extracellular matrix in differentiating mesenchymal stem cells [Barry et al., 2001].

This study was undertaken to obtain mesenchymal stem cells with chondrogenic potential that retain critical in vivo cell functions, as do mammary gland epithelial cells, skin keratinocytes, and pigmented epithelial cells. To achieve this, we attempted to identify marrow-derived cells with chondrogenic nature and immortality without transformation among the cells obtained by the limiting-dilution method [Umezawa et al., 1992], defining "immortality" simply as indefinite cell division.

OP9 cells are known to serve as a niche or a specific microenvironment for the regulation of self-renewal and differentiation of stem cells [Nakano, 1996], and the question is raised of whether marrow stromal cells or marrow-derived mesenchymal cells with chondrogenic potential are capable of constituting a microenvironment for stem cells. It is inconceivable that cartilage can form a niche for cells in the living body based on structural and morphological considerations; however, a cell with chondrogenic or adipo-chondrogenic potential may serve as a niche not only in the case of OP9 cells but also as a general concept, at least in vitro.

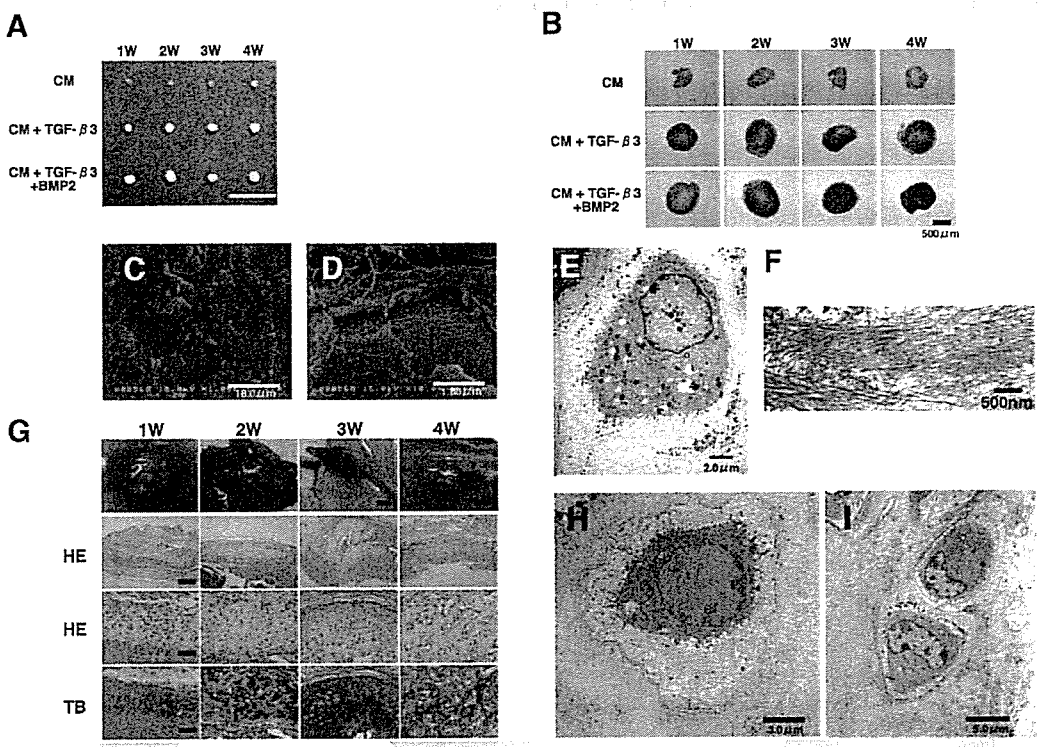

Fig. 9. In vitro and in vivo chondrogenesis of KUM5 cells sorted according to the activity of the chondrocyte-specific cis-regulatory element. **A,B:** Macroscopic view of the chondrogenic nodules which were generated after pellet culture of the finally sorted KUM5 cells for 1–4 weeks in the CM supplemented with growth factors as indicated (A) and toluidine blue stained section (B). **C–F:** Ultrastructural analysis of the micromasses of KUM5 cells sorted according to the activity of the Col α2(XI) cis-regulatory element (KUM5-Venus) after culturing in the CM supplemented with TGF-β3 for 3 weeks. (C,D), SEM; (E,F), TEM. **G:** In vivo chondrogenesis was examined 1–4 weeks after direct injection of the finally sorted KUM5 cells. From top to bottom: Macroscopic view, scale bars: 2 mm; histological analysis, scale bar: 600 μm, HE stain; histological analysis, scale bar: 120 μm, HE stain; histological analysis, scale bar: 120 μm, TB stain. **H,I:** Ultrastructural analysis (TEM) of the sorted KUM5 cartilage. The sorted KUM5 cells were implanted into the subcutaneous tissue of Balb/c nu/nu mice, and the generated cartilage was resected 2 weeks after implantation. Scale bars: 5 mm (A), 500 μm (B), 2 mm (G, **top row**), 500 μm (G, **2nd row**), 100 μm (G, **3rd** and **bottom row**).

The sequence of enchondral or perichondral ossification by KUM5 and OP9 cells was as follows: deposition of homogeneous matrix surrounding the small nests of the injected cells that subsequently became positive for type II collagen and exhibited metachromasia with toluidine blue staining, trapping them in the secreted homogeneous matrix, and the appearance of small nests of isogenous chondrocytes that probably resulted from repeated cell division. At a later stage, that is, 4–8 weeks after injection, the peripheral region of the generated cartilage became ossified. Importantly, the chondrogenesis by KUM5 and OP9 cells was irreversible and reproducible, and the implanted cells never transformed into malignant cells, formed any abnormal extracellular matrices, or induced any significant inflammatory reactions. It is again noteworthy that the osteogenesis by these two different lines of cells was mediated by chondrogenesis, and it was therefore considered to be chondral ossification. Thus, the unique characteristics of these two cell lines provide an opportunity to analyze the process of enchondral or perichondral ossification in an experimental system in detail.

In fetal life, primary ossification centers form by one of two processes: enchondral ossification or membranous ossification. Enchondral ossification refers to bony replacement of cartilage and is the mode of formation of the long bones. During membranous ossification mesenchymal cells form membranes within which ossification occurs and this is the mode of formation of the scapula and skull and, in part, of the clavicle and pelvis. After birth, bone growth continues by both enchondral and membranous ossification. Further enchondral ossification occurs in

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the physes and results in continuous longitudinal growth of the long bones until skeletal maturity. KUM5 and OP9 cells were obtained from long bone and calvaria, respectively, and showed enchondral ossification. We have also reported that KUSA-A1 cells form bone by membranous ossification in vivo, and thus we have three different types of cells showing distinctive in vivo characteristics. The process of chondrogenesis or enchondral ossification may also serve as a model for chondromatosis and osteochondromatosis in a joint cavity.

The expression pattern of chondrocyte-specific genes in OP9 and KUM5 cells is different from that in ATDC5 cells, which are a mouse embryonal carcinoma-derived chondrogenic cell line. ATDC5 cells exhibit a multistep differentiation process encompassing the stages from chondrogenesis to enchondral ossification [Shukunami et al., 1996]. Early-phase differentiation is characterized by the expression of type II collagen, followed by induction of the aggrecan gene. Late stage differentiation is characterized by the start of expression of short-chain collagen type X genes. By contrast, marrow-derived mesenchymal stem cells express the aggrecan genes at an early stage and then type II collagen during chondrogenic differentiation [Pittenger et al., 1999]. Surprisingly, gene expression pattern determined by the gene chip analysis was consistent with protein levels of cell surface molecules; this consistency indicates that the expression profiling is valid. Expression of "structural proteins" on Gene Ontology, including the extracellular matrix, was much higher by OP9 and KUM5 cells than by non-chondrogenic cells such as KUSA-A1 osteoblasts, H-1/A preadipocytes, and 9-15c mesenchymal stem cells, implying that the OP9 and KUM5 cells are mainly engaged in synthesizing extracellular matrix.

Can we inhibit enchondral or perichondral ossification after the completion of chondrogenesis? This is a challenge for the future, probably the not-too-distant future. We could not prevent the generated hyaline cartilage from ossifying at present even after selection based on the chondrocyte-specific cis-regulatory element of the collagen α2(XI) gene, probably due to the inability to inhibit vasculogenesis from the neighboring connective tissue. However, these established murine marrow-derived mesenchymal cells with in vivo chondrogenic activity and expression profiles provide a powerful model for studies of chondrogenic differentiation and our further understanding of cartilage regeneration. Bone marrow-derived chondroblasts with chondrogenic potential are useful candidate cell sources in addition to dedifferentiated chondrocytes obtained from cartilage for transplantation in osteoarthritis and rheumatoid arthritis.

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