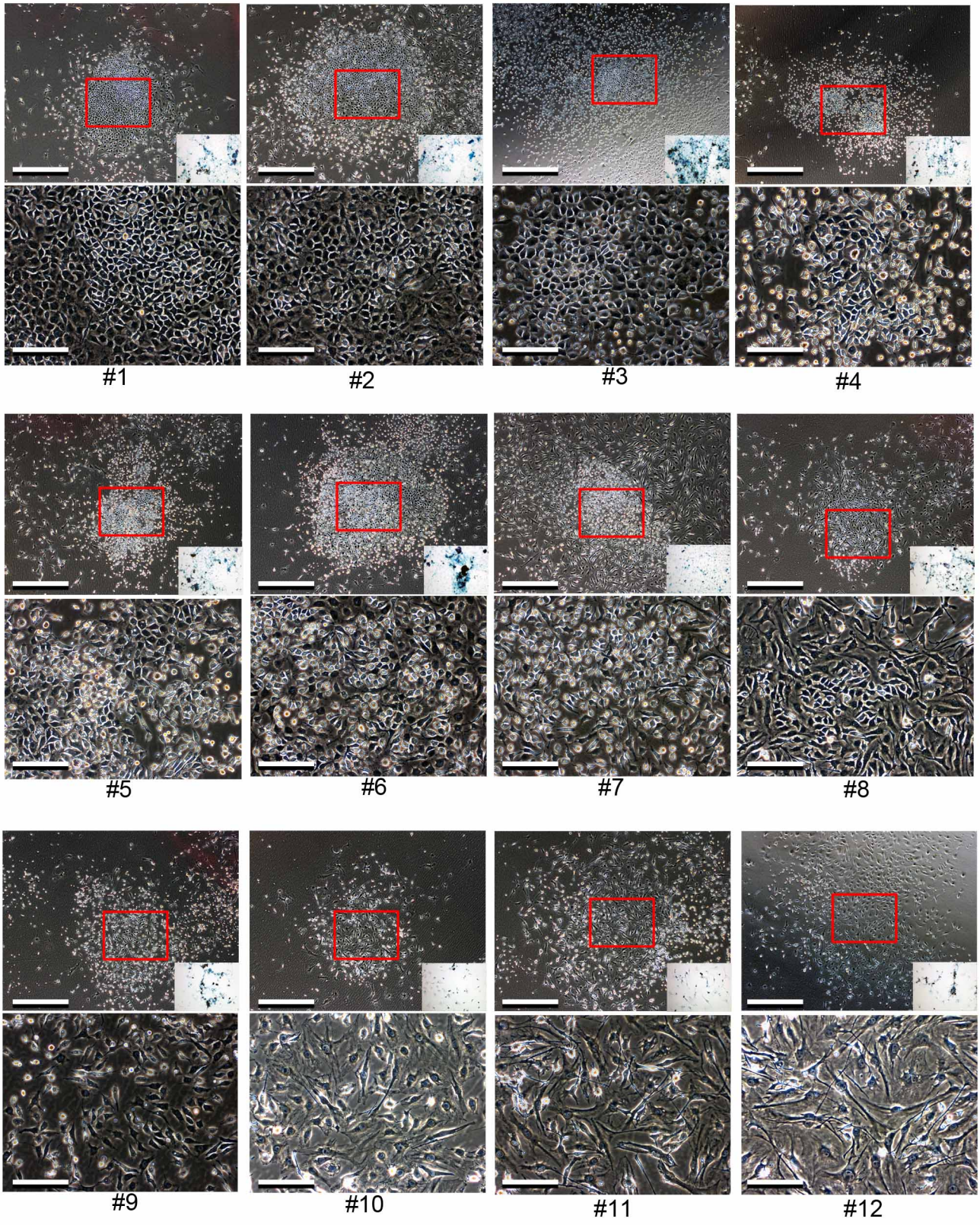
Supplemental data

Generation of hyaline cartilaginous tissue from mouse adult dermal fibroblast culture by defined factors

Kunihiko Hiramatsu, Satoru Sasagawa, Hidetatsu Outani, Kanako Nakagawa, Hideki Yoshikawa, and Noriyuki Tsumaki

Number of Supplemental Figures: 8

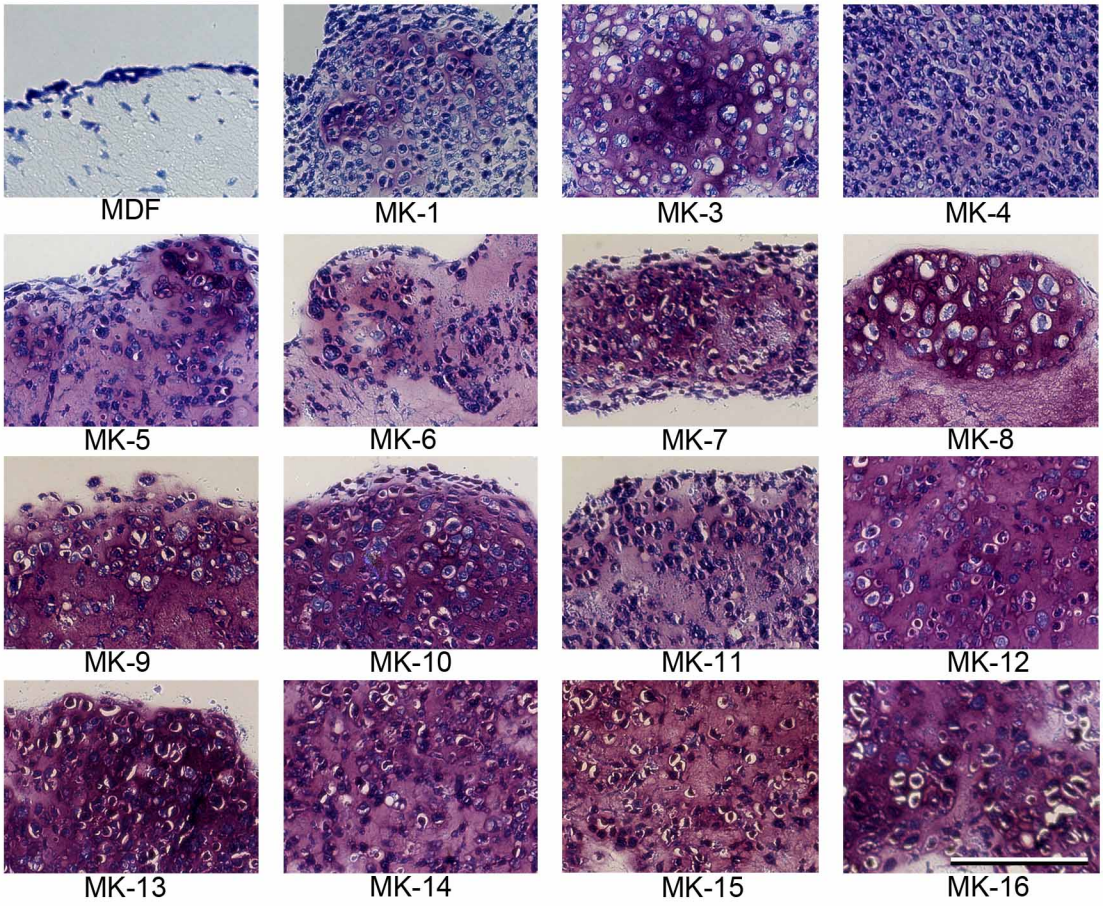
Number of Supplemental Tables: 4



Supplemental Figure 1 Images of colonies generated from *Col11a2-βgeo* MDFs by transduction of c-Myc, Klf4 and SOX9 retroviral vectors

Images of 12 randomly selected colonies are shown. For each colony, phase-contrast images with lower magnification are shown above and higher magnification images are shown below. After phase-contrast images were collected, the cells were stained with alcian blue. Insets in the top panels represent images of red boxed regions after alcian blue staining. The images of colonies are arranged according to the degree of polygonal morphology. The colony number (#1-12) is indicated below the bottom image. Colonies #1-6 consisted of polygonal-shaped cells, and colonies #7-12 consisted of non-polygonal-shaped cells. Colonies #1-6 tended to be intensely stained with alcian blue, as compared with colonies #7-12. Bars: (top panels) 500 μm; (lower panels) 100 μm.

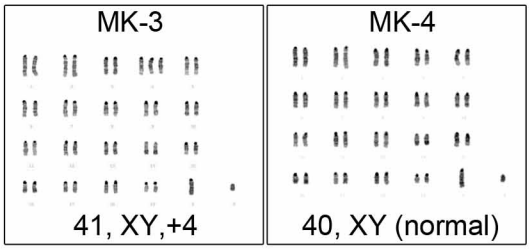
A

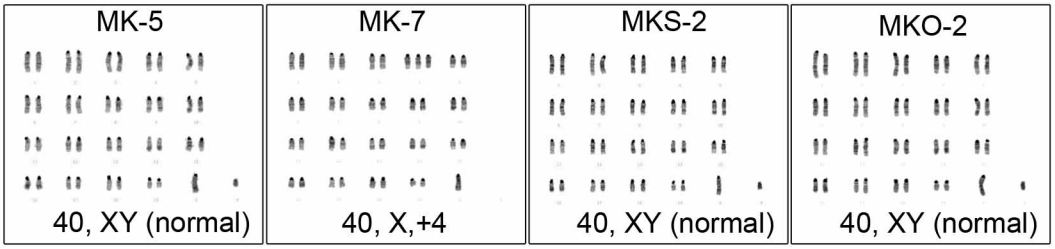


B

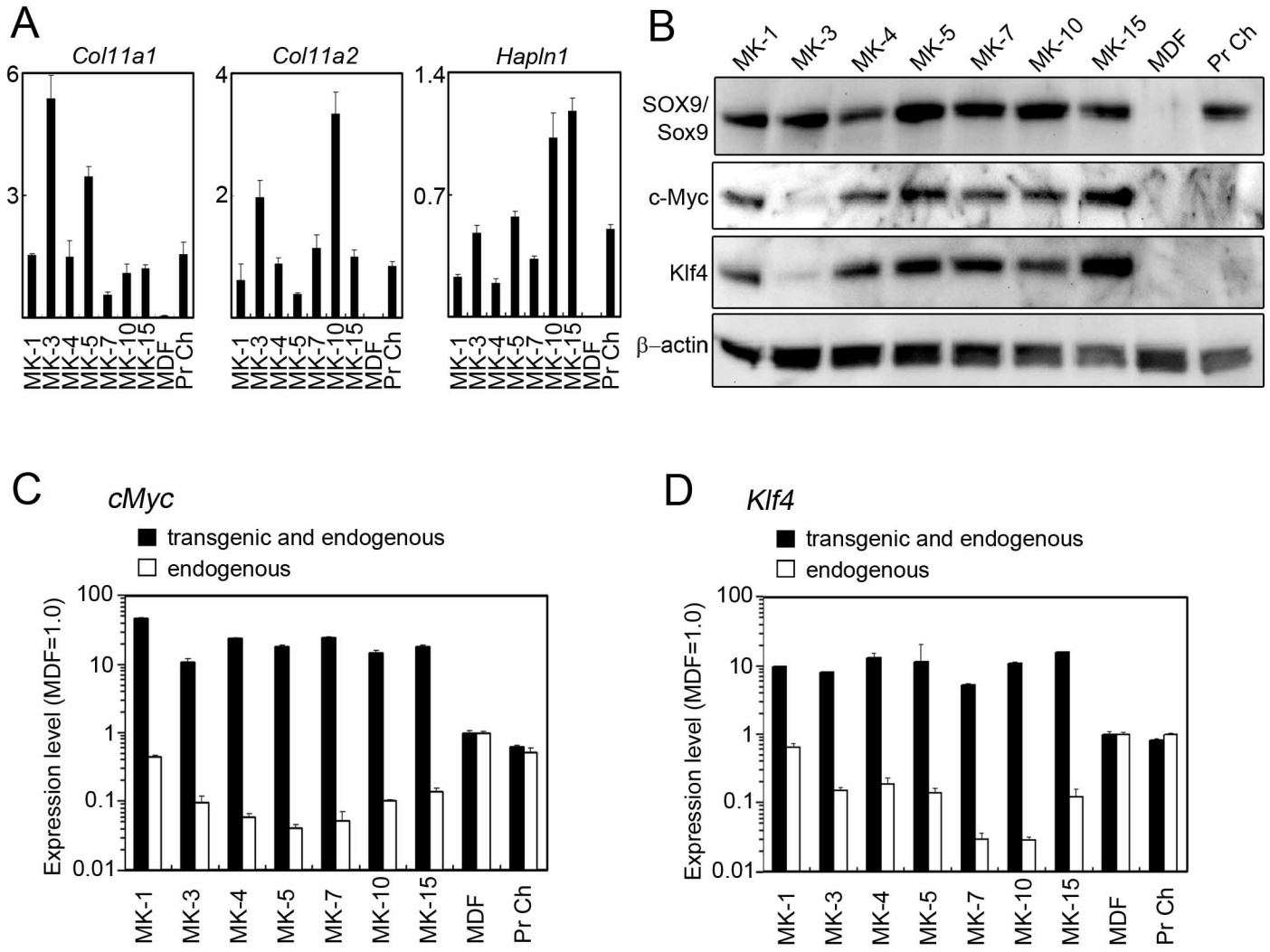
Frequencies of numbers of chromosomes in each clone

Colony	Chromosome number					
	≦38	39	40	41	42≦	Total
MK-3	0	1	14	26	9	50
MK-4	3	1	23	9	14	50
MK-5	2	6	23	15	4	50
MK-7	0	2	40	6	2	50
MKS-2	2	1	28	5	14	50
MKO-2	0	1	44	0	5	50

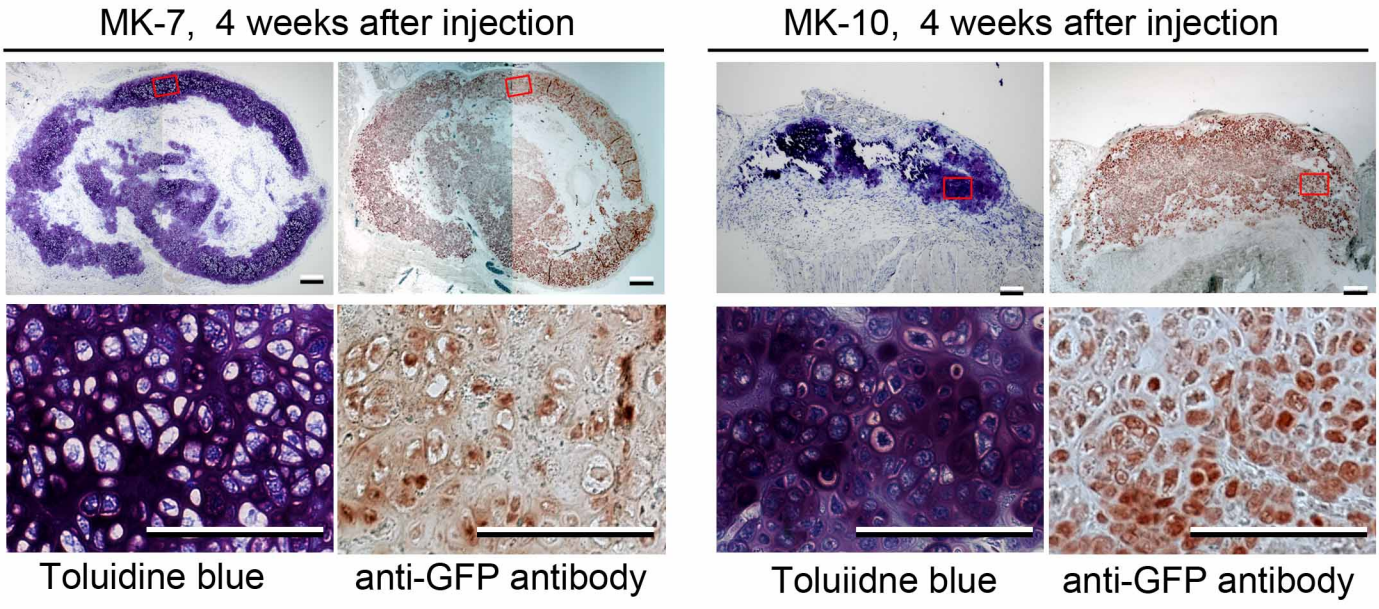


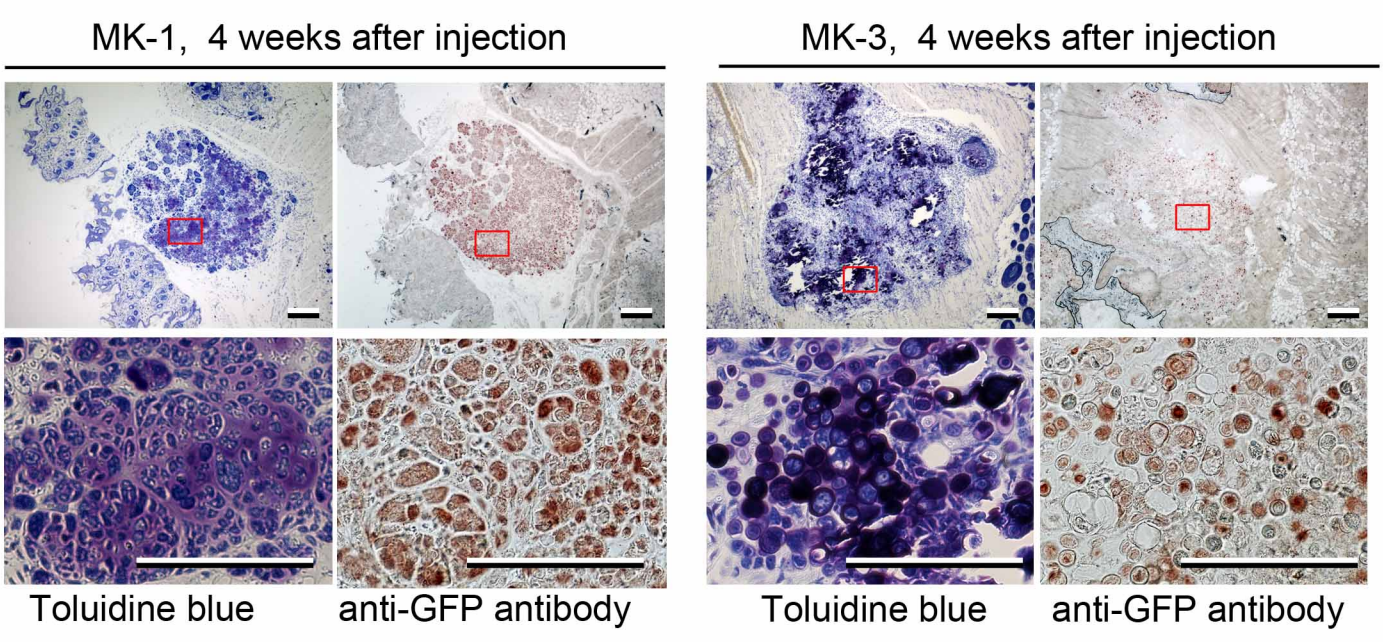


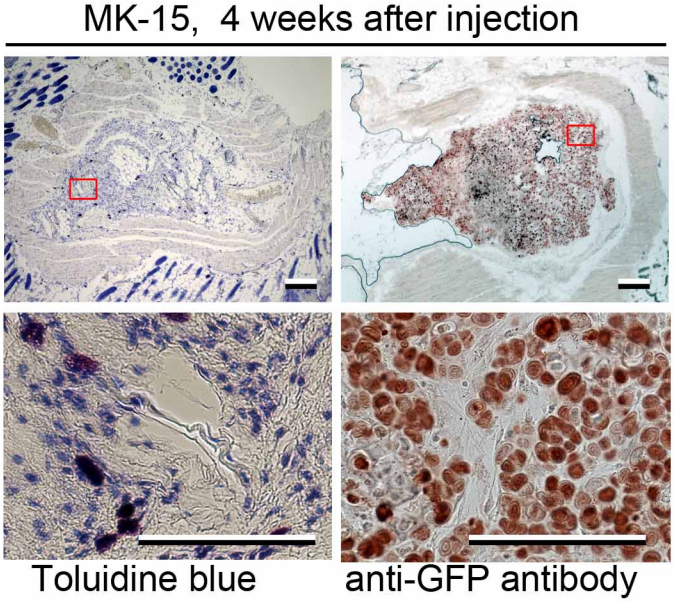
Supplemental Figure 2 Three-dimensional culture and karyotypes of induced MK cell lines
(A) Three-dimensional culture in type I collagen gels. After a 3-week culture, gels were recovered and processed for histological sections. Sections were stained with toluidine blue. Results from MDFs and MK-1 and MK-3 to -16 cell lines are shown. All MK cell lines generated cartilage-like matrix with glycosaminoglycan, as indicated by the metachromatic toluidine blue staining. Parental MDFs did not produce cartilage-like matrix. Bar: 100 μm.
(B) Karyotypes of induced cell lines. (Table) Frequencies of numbers of chromosomes. Fifty cells were examined for each induced cell line. (Panels) Most frequent karyotype of each cell line is shown. MK-3, -4, -5 and -7 are cell lines isolated from individual colonies in cultures transduced with c-Myc, Klf4, and SOX9. MKS-2 is a cell line isolated from a colony in cultures transduced with c-Myc, Klf4, Sox2, and SOX9. MKO-2 is a cell line isolated from a colony in cultures transduced with c-Myc, Klf4, Oct3/4, and SOX9.



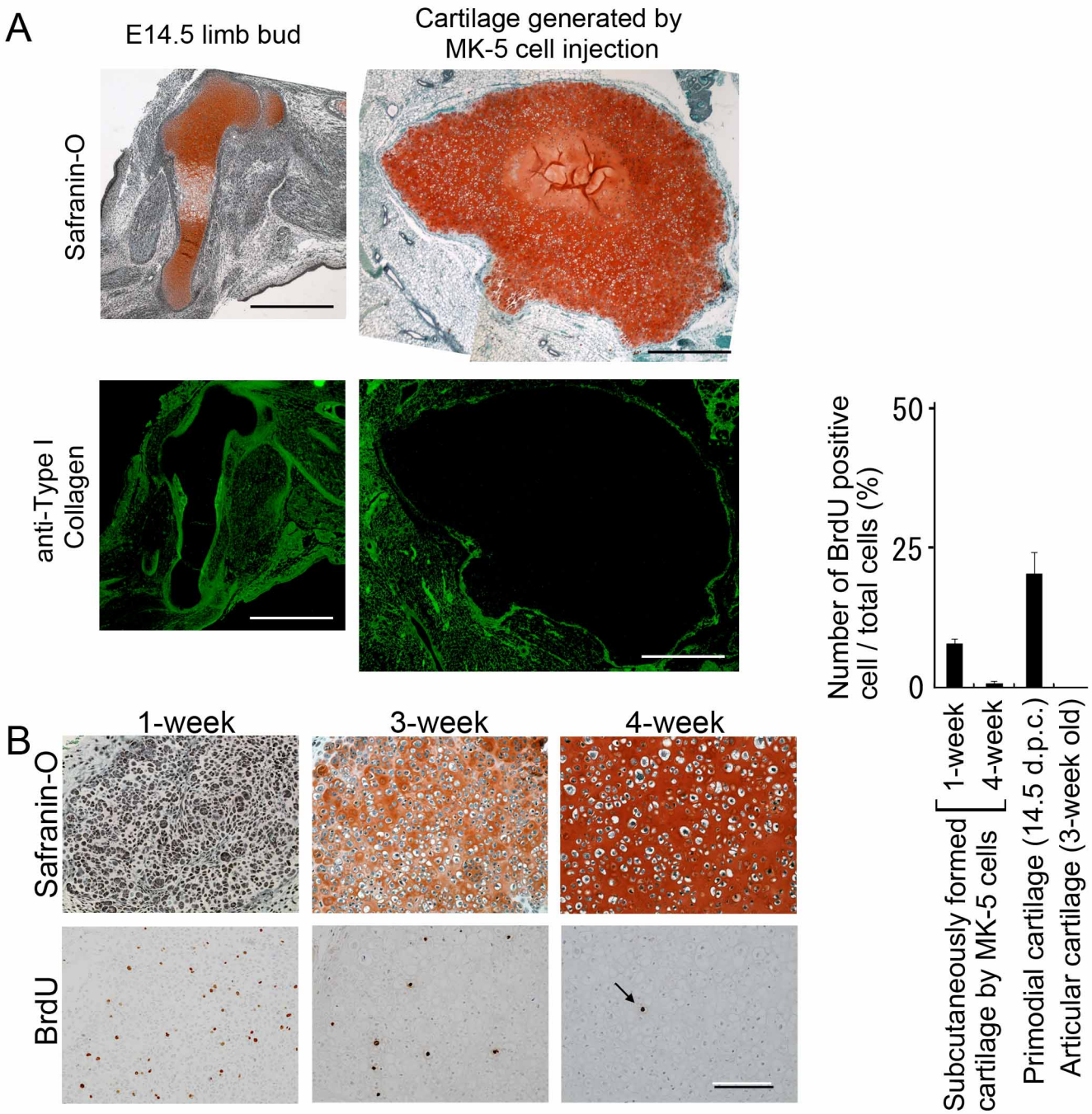
Supplemental Figure 3 Expression of marker genes and transgenes in induced cells
(A) Real-time RT-PCR analysis of chondrocyte-marker gene expression in MK-induced cells, MDFs, and primary chondrocytes. *Col11a1*, type XI collagen α1 chain gene; *Col11a2*, type XI collagen α2 chain gene; *Hapln1*, link protein 1 gene. Error bars indicate means ± SD (n = 3).
(B) Western blot analysis of SOX9 and reprogramming factors in induced cells, MDFs and primary chondrocytes.
(C and D) mRNA levels of *cMyc* (C) and *Klf4* (D) in MK induced cells, MDFs, and primary chondrocytes. The relative expression levels compared to MDFs are shown. RNA levels were determined with real-time RT-PCR analysis by using primers specific for endogenous transcripts (white columns) and those common for both endogenous and transgenic transcripts (black columns).



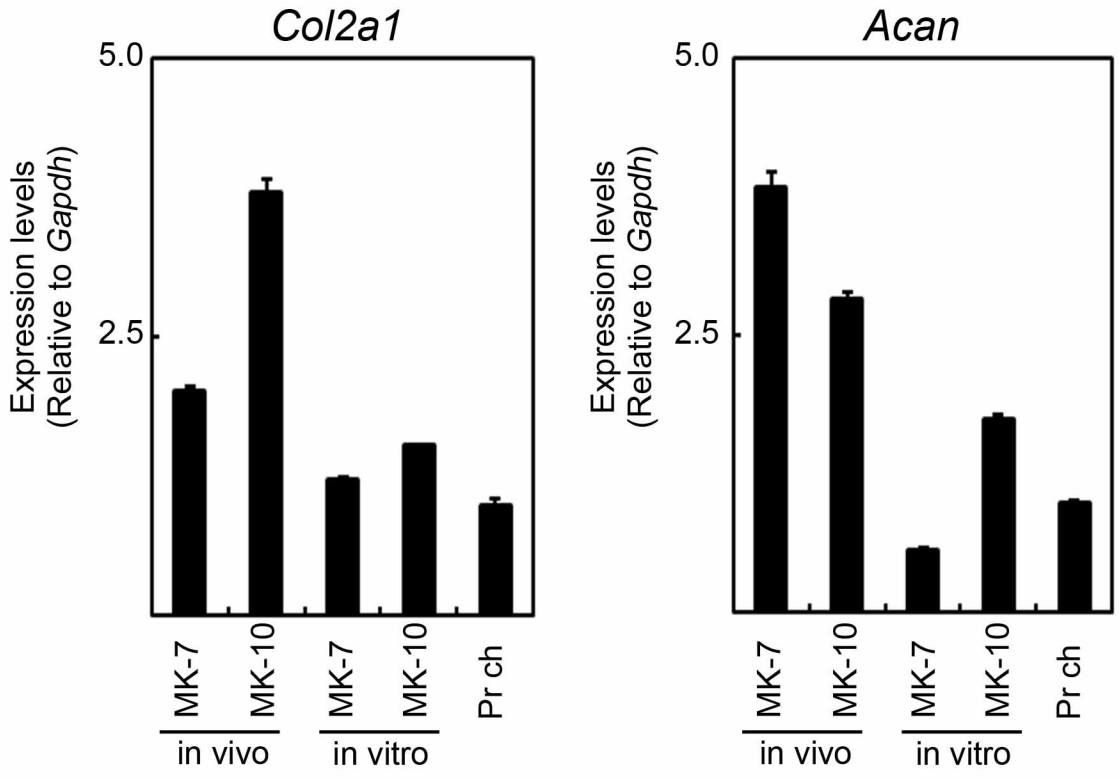




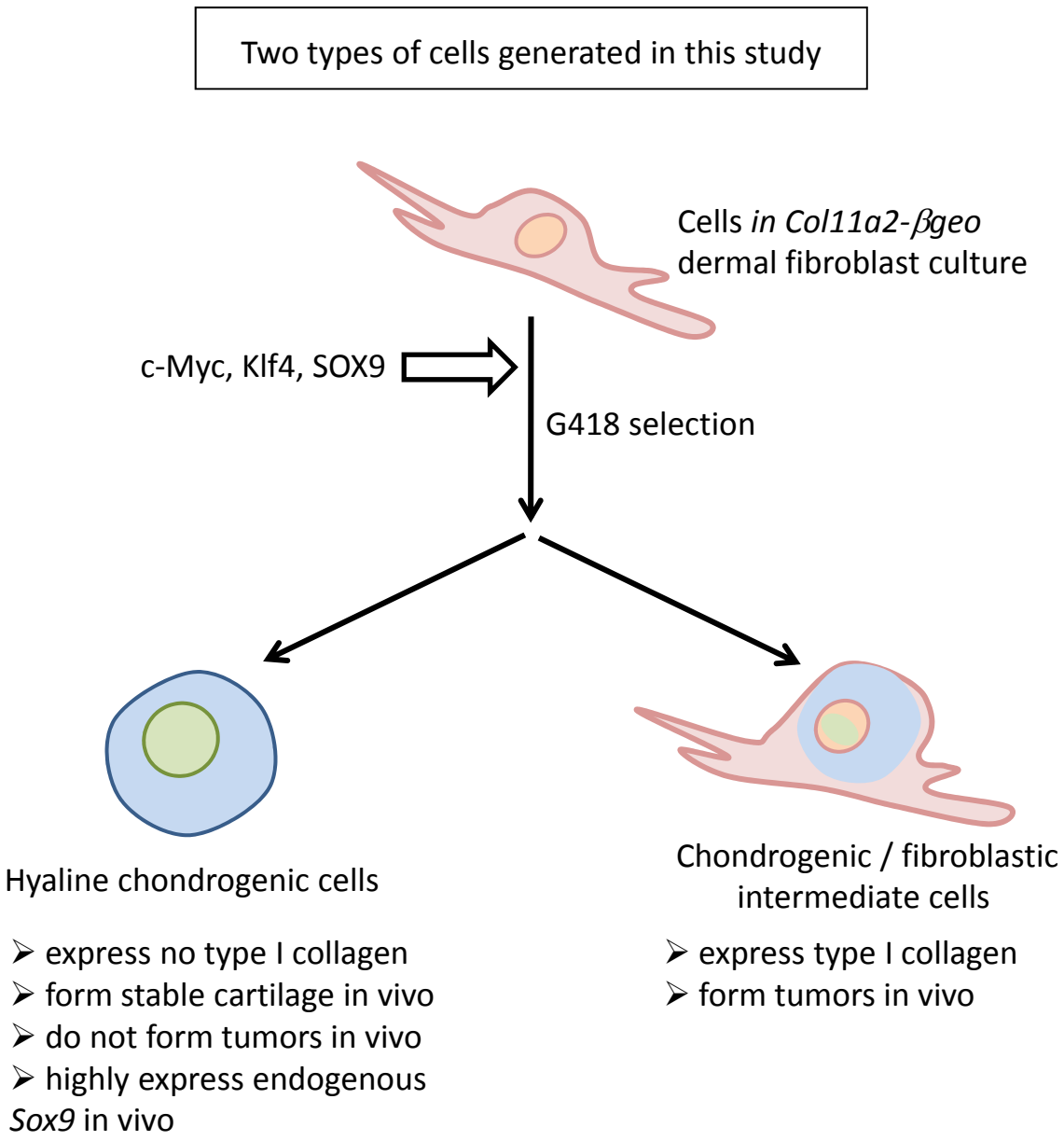
Supplemental Figure 4 Cartilaginous tissue formation in nude mice
Histology of injected sites at 4 weeks after subcutaneous injection of MK-1, -3, -7, -10 and -15 cells. Semiserial sections were stained with toluidine blue and immunostained with anti-GFP antibodies. Magnifications of boxed regions in top panels are shown in bottom panels. GFP-positive cells appeared to represent implanted cells. Bars: (top panels) 200 μm; (bottom panels) 100 μm. Each top panel of histology form MK-7 cell injection (left top) is assembled from two images.



Supplemental Figure 5 Characterization of cartilaginous tissue generated by MK-5 cell injection into nude mice.
(A) Induced chondrogenic cells produce hyaline cartilage in vivo as indicated by absence of type I collagen. Histological sections were stained with Safranin O and immunostained with anti-type I collagen antibodies (green). *Left*, as for control, humeral primordial hyaline cartilage at 14.5 dpc expresses no type I collagen and is distinct from other surrounding connective tissues such as muscle, ligament, adipose tissue, tendon, vesseles, and dermis which strongly express type I collagen. *Right*, the cartilage generated 4 weeks after injection of MK-5 cells into nude mice expressed no type I collagen. The surrounding host subcutaneous tissues strongly expressed type I collagen. Bars: 500 μm. Each right panel is assembled from 2 images.
(B) *Left panels*, histological sections from nude mice that were sacrificed at 1 (left panels), 3 (middle panels), and 4 (right panel) weeks after subcutaneous injection of MK-5 cells. Semiserial sections were stained with safranin O (top row) and immunostained with anti-BrdU antibodies (bottom row). Arrowhead in the right bottom panel indicates the single BrdU-positive cell in this field. Bar: 100 μm. *Right graph*, the average numbers of BrdU-positive cells per total cells. For controls, the primordial cartilage of the proximal humerus of 14.5 dpc mouse embryos and articular cartilage of proximal tibia of 3-week-old mice were used. Error bars indicate means ± SD (n = 3).

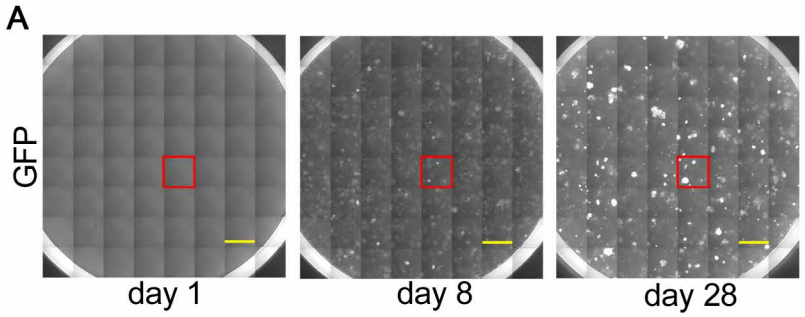


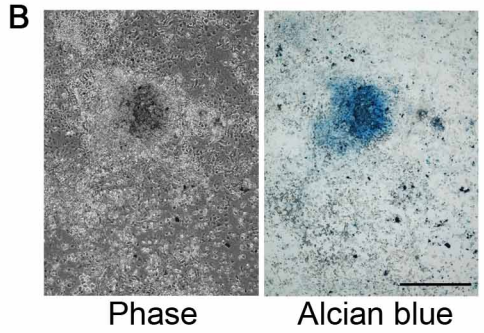
Supplemental Figure 6 Chondrocyte-marker gene expression was increased after implantation compared with that in monolayer culture.
RNA samples were extracted from cartilage tissue generated in nude mice by subcutaneous injection of MK-7 (16 weeks after injection) and MK-10 (8 weeks after injection) and from monolayer culture of MK-7 and MK-10 cells. *Col2a1* and *Acan* mRNA levels were determined with real-time RT-PCR analysis. Individual RNA expression levels were normalized to respective *Gapdh* expression levels. in vivo, RNA samples from cartilage tissue generated in nude mice by subcutaneous injection; in vitro, RNA samples from monolayer culture; Pr Ch, primary chondrocytes. Error bars indicate means ± SD (n = 3).

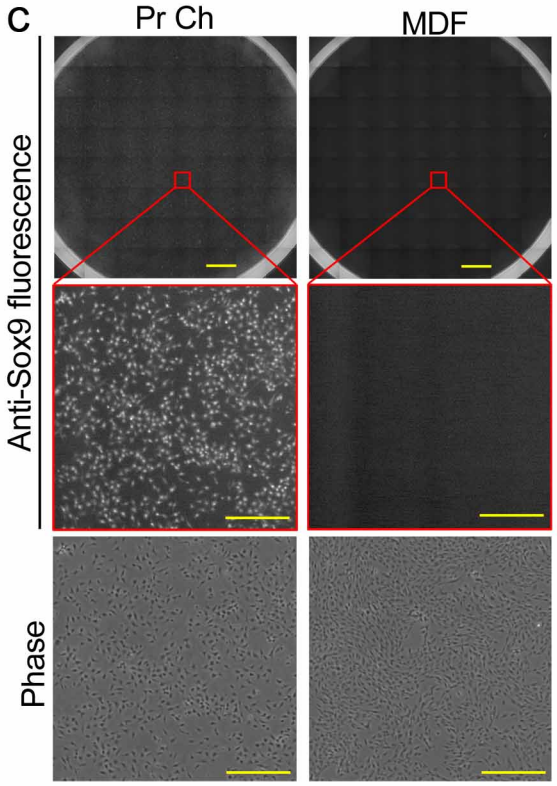


Supplemental Figure 7 Schematic representation of induced cells generated in this study

Transduction of c-Myc, Klf4, and SOX9 into dermal fibroblast culture produced hyaline chondrogenic cells that do not express type I collagen and chondrogenic/fibroblastic intermediate cells that do express type I collagen. Induced cell lines such as MK-7, MK-10 and MK-5 subclones, which formed homogenous cartilage in vivo and did not produce tumor for a prolonged period of time, might be hyaline chondrogenic cells. Type I collagen-expressing cells that composed tumors in vivo might be chondrogenic/fibroblastic intermediate cells.

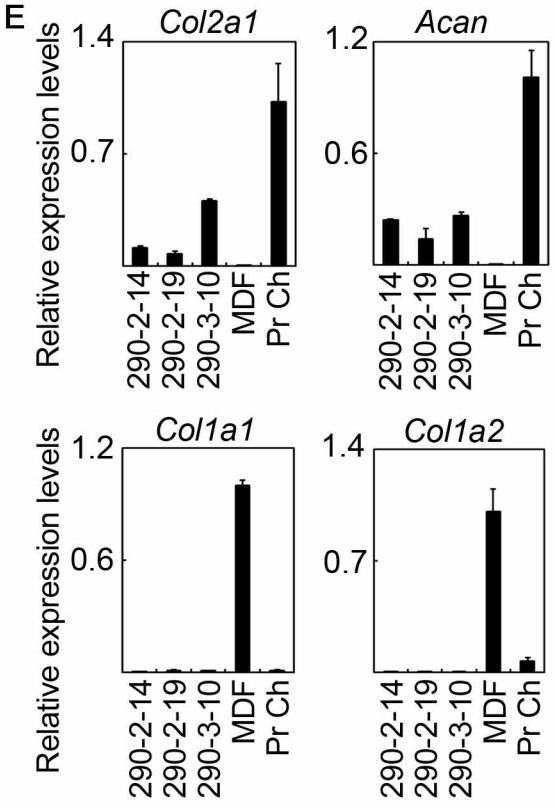






D Frequencies of cells showing immunoreactivities against anti-Sox9 antibodies

	total cell number	positive cell number	%
Pr Ch dish #1	77910	67126	86.2
Pr Ch dish #2	71003	59205	83.4
Pr Ch total	148913	126331	84.8
MDF dish #1	175345	3	0.0017
MDF dish #2	167800	4	0.0024
MDF total	343145	7	0.0020



Supplemental Figure 8 Induction of *Col11a2-Egfp-Ires-Puro*–positive cells in MDF culture by transduction of c-Myc, Klf4 and SOX9 and immunofluorescence staining of MDFs with anti-Sox9 antibodies.

(A)After an overnight incubation in the retrovirus-containing medium, *Col11a2-Egfp-Ires-Puro* transgenic MDFs were replated into 6-well plates (day 1) and subjected to timelapse GFP observation by Biostation CT (Nikon). Each whole well was scanned with 8 × 8 images, and tiling images were reconstituted. Tiling GFP images of whole dishes on day 1, 8, and 28 are shown. Magnifications of boxed regions in panels correspond to the lower panels in Figure 8C, respectively. Bars: 4 mm.

(B)Alcian blue staining of MDF culture 29 days after transduction of c-Myc, Klf4 and SOX9. Bar: 500 μm.

(C)Immunofluorescence staining of MDFs and primary chondrocytes in 6-well plates with anti-Sox9 antibodies. Each whole well was scanned with 8 × 8 images, and tiling images were reconstituted (top panels). Magnifications of the boxed region in the top panels are shown in the middle panels. Top and middle panels are fluorescent images (Alexa Fluor). Bottom panels are phase images corresponding to the middle panels, respectively. Bars: 4 mm in the top row and 0.5 mm in the middle and bottom rows.

(D)Frequencies of cells showing immumoreactivities against anti-Sox9 antibodies. Cell numbers were counted using images collected in (C) with CL-Quant software (Nikon). Two wells of a 6-well plate were analyzed for primary chondrocytes and MDFs respectively. Total cell numbers represent numbers of cells recognized in phase images. Positive cell numbers represent numbers of cells showing immunofluorescence with anti-Sox9 antibodies.

(E) Real-time RT-PCR analysis of chondrocyte- and firbroblast-marker gene expression in induced cells with dox-inducible lentiviral vectors, MDFs, and primary chondrocytes (Pr Ch). Induced cells were maintained in DMED + 10% FBS in the presence of 1 μg/ml dox. Error bars indicate means ± SD (n = 3).

Supplemental Tables

Supplemental Table 1. Results of subcutaneous injection of MK cell lines or subclones into nude mice

Sacrifice after injection (weeks)	Cell lines or subclones	Number of injected sites	Number of sites with tissue[1]	Number of sites with tumor[2]	Cartilage homogeneity (%)[3]
4	MK-1	2	2	0	10
4	MK-3	4	4	0	10
4	MK-4	4	0	3	5
4	MK-5	4	4	0	100
4	MK-7	4	3	0	100
4	MK-10	4	3	0	70
4	MK-15	4	3	0	0
8	MK-5	3	0	3	5
8	MK-7	3	3	0	100
8	MK-10	3	3	0	100
12	MK-10	6	5	0	100
16	MK-7	3	3	0	100
8	MK-5-2	4	4	0	80
8	MK-5-3	4	4	0	80
8	MK-5-4	4	4	0	80
12	MK-5-2	3	3	0	80
12	MK-5-3	3	3	0	80
12	MK-5-4	3	2	0	80
4	290-2-14	8	0	0	-
4	290-2-19	8	0	0	-

[1]Number of sites where tissue were histologically recognized

[2]Number of sites where tumors were macroscopically recognized

[3]Ratio of cartilaginous area / (cartilaginous + noncartilaginous area) in the histological sections

Supplemental Table 2. Sequences of primers for factors

Primer	Sequence
Primers specific for retroviral transgene transcripts	
Klf4 Tg RT S	GACCACCTTGCCTTACACA
Klf4 Tg RT AS	CCCTTTTTCTGGAGACTAAAT
c-Myc Tg RT S	TCGCTACCATTACCAGTTG
c-Myc Tg RT AS	CCCTTTTTCTGGAGACTAAAT
Oct3/4 Tg RT S	TCCCATGCATTCAAACTG
Oct3/4 Tg RT AS	CCCCTGTTGTGCTTTTAATC
Sox2 Tg RT S	CCATTAACGGCACACTGC
Sox2 Tg RT AS	CCTTACGCGAAATACGGG
SOX9 RT S	CTGGGAACAACCCGTCTACA
SOX9 RT AS	CACCAGACCAACTGGTAATG
Primers specific for dox-inducible lentiviral transgene transcripts	
hcMYC LeTg S	CGACGAGAACAGTTGAAACAC
hcMYC LeTg AS	AGAGGGTTAGGGATAGGCTTA
hKLF4 LeTg S	CCCGTTCCAGTGCCAAAA
hKLF4 LeTg AS	CCACTGTGCTGGATATCAACC
hSOX9 LeTg S	ACCACCAGAACTCCAGCTC
hSOX9 LeTg AS	CACTGTGCTGGATATCAGACC
Primers specific for endogenous transcripts	
cMyc endo S	TGACCTAACTCGAGGAGGAGCTGGAATC
cMyc endo AS	AAGTTTGAGGCAGTTAAAATTATGGCTGAAGC
Klf4 endo S	GGCGAGAAACCTTACCACTGT
Klf4 endo AS	TACTGAACTCTCTCTCCTGGCA
Sox9 endo S	AGCTCACCAGACCCTGAGAA
Sox9 endo AS	TCCCAGCAATCGTTACCTTC
Primers common for both endogenous and retroviral transgenic transcripts	
cMyc total S	CAGAGGAGGAACGAGCTGAAGCGC
cMyc total AS	TTATGCACCAGAGTTTCGAAGCTGTTCG
Klf4 total S	GTGCCCCGACTAACCGTTG
Klf4 total AS	GTCGTTGAACTCCTCGGTCT

Supplemental Table 3. Sequences of primers for maker genes

Primer	Sequence
Marker gene expression analysis	
Gapdh RT S	GAGATGATGACCCTTTTGGCT
Gapdh RT AS	TCAAGGCCGAGAATGGGAAG
Sox5 RT S	TTTTCCCAACAAGCCTCACTC
Sox5 RT AS	TTGCCATCGACTTCCATTGTG
Sox6 RT S	TCATCCCGGCCTAAGACA
Sox6 RT AS	ACAGGGCAGGAGAGTTGAG
Col2a1 RT S	TTGAGACAGCACGACGTGGAG
Col2a1 RT AS	AGCCAGGTTGCCATCGCCATA
Col11a1 RT S	ATGAGTATGCACCTGAGGAT
Col11a1 RT AS	GGAGTCTCAGTCTGGTAAGGTT
Col11a2 RT S	GACTGTAAGAAGCGAGTTACC
Col11a2 RT AS	GCCTTCAAAGACTTCATCG
Col10a1 RT S	TTCTGCTGCTAATGTTCTTGACC
Col10a1 RT AS	GGGATGAAGTATTGTGTCTTGGG
Mmp13 RT S	CTTCTTCTTGTTGAGCTGGACTC
Mmp13 RT AS	CTGTGGAGGTCACTGTAGACT
Col1a1 RT S	GCAACAGTCGCTTCACCTAC
Col1a1 RT AS	GTGGGAGGGAACCAGATTG
Col1a2 RT S	TCGGGCCTGCTGGTGTTCGTG
Col1a2 RT AS	TGGGCGCGGCTGTATGAGTTCTTC
Acan RT S	CCCTCGGGCAGAAGAAAGAT
Acan RT AS	CGCTTCTGTAGCCTGTGCTTG
Bisulfite genomic sequencing	
Col1a1-DM-S	TTGGTTTATGTAGATTTGGGG
Col1a1-DM-AS	TCCACAAAACTAAACATATCTAAACC
Col1a2-Me-S2	GGATTGGATAGTTTTTGTTTTT
Col1a2-Me-AS2	AAAACCCAAACCTACCTTATTT

Supplemental Table 4. Antibodies

Antibody	Cat. No.	Dilution
Anti-type I collagen (rabbit polyclonal)	Abcam (ab34710)	1/1000
Anti-type II collagen (anti-mouse monoclonal)	Thermo (#MS-235-P0)	1/200
Anti-type II collagen (goat polyclonal)	Santa Cruz (sc-7764)	1/100
Anti-aggrecan (rabbit polyclonal)	Santa Cruz (sc-25674)	1/200
Anti-Sox9 (rabbit polyclonal)	Santa Cruz (sc-20095)	1/200
Anti-type X collagen (anti-mouse monoclonal)	Quartett (2031501001)	1/200
Anti-Klf4 (rabbit polyclonal)	Santa Cruz (sc-20691)	1/200
Anti-cMyc (rabbit polyclonal)	Santa Cruz (sc-764)	1/200
Anti-Oct3/4 (mouse monoclonal)	Santa Cruz (sc-5279)	1/200
Anti-Sox2 (goat polyclonal)	Santa Cruz (sc-17320)	1/200
Anti-GFP (rabbit polyclonal)	Cell signaling (#2555)	1/800
anti-β-actin (rabbit polyclonal)	Cell Signaling (#4967)	1/1000
Alexa Fluor 546 goat anti-rabbit	Invitrogen (A11010)	1/2000
Alexa Fluor 546 donkey anti-goat	Invitrogen (A11057)	1/2000
Alexa Fluor 568 rabbit anti-mouse	Invitrogen (A11061)	1/2000