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Stable Subclones of the Chondrogenic Murine Cell Line MC615 Mimic Distinct Stages of Chondrocyte Differentiation

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ABSTRACT

Fourteen stable subclones derived from the murine chondrogenic cell line MC615 were established and characterised regarding their differentiation stages and responsivity to BMP2. Based on their gene expression profiles which revealed remarkable variances in Col2a1 and Col10a1 expression, subclones could be grouped into at least three distinct categories. Three representative subclones (4C3, 4C6 and 4H4) were further characterised with respect to gene expression pattern and differentiation capacity. These subclones resembled (i) weakly differentiated chondrogenic precursors, strongly responding to BMP2 stimulation (4C3), (ii) collagen II expressing chondrocytes which could be induced to undergo maturation (4C6) and (iii) mature chondrocytes expressing Col10a1 and other markers of hypertrophy (4H4). Interestingly, BMP2 administration caused Smad protein phosphorylation and stimulated Col10a1 expression in all clones, but induced Col2a1 expression only in precursor-like cells. Most remarkably, these clones maintained a stable gene expression profile at least until the 30th passage of subconfluent culture, but revealed reproducible changes in gene expression and differentiation pattern in long term high density cultures. Thus, the newly established MC615 subclones may serve as a potent new tool for investigations on the regulation of chondrocyte differentiation and function. J. Cell. Biochem. 108: 589–599, 2009.

KEY WORDS: CHONDROCYTE; BMP2; COLLAGEN; HYPERTROPHY

During formation of the skeleton in vertebrate development, chondrocytes undergo a distinct series of differentiation steps, beginning with the differentiation of mesenchymal precursor cells to chondrocytes and ending with maturation towards hypertrophic chondrocytes. The first step, early chondrogenesis, is characterised by condensation of mesenchymal progenitor cells and their overt differentiation into chondrocytes [reviewed in Hall and Miyake, 2000]. During further development chondrocytes of the cartilage anlagen in long bones, ribs and vertebrae further differentiate into prehypertrophic and hypertrophic chondrocytes, preparing the cartilage anlage for replacement by bone in a complex process summarised as endochondral ossification [Poole, 1991]. Chondrocyte maturation towards terminally differentiated hypertrophic is also associated with a decrease in cell proliferation [reviewed in Beier et al., 1999]. Terminally differentiated hypertrophic chondrocytes either undergo apoptosis or survive temporarily as bone-forming, post-hypertrophic chondrocytes in the cartilaginous core of newly formed endochondral bone trabecules [Kirsch and von der Mark, 1991; Roach and Erenpreisa, 1996; Ducy and Karsenty, 1998; Delise and Tuan, 2002 for review].

Each step of chondrocyte differentiation during chondrogenesis and endochondral ossification is tightly regulated in a coordinate manner by a multitude of growth factors and hormones, which include factors of the TGFβ/BMP family, FGF, and Wnt factors,

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hedgehog factors in a regulatory feed back loop with PTHrP, IGF-1 and others [for review see Ballock and O'Keefe, 2003; Provot and Schipani, 2005; Goldring et al., 2006]. The same factors are also responsible for development of articular cartilage in the postnatal joint and control of the concerted balance of anabolic and catabolic events [Poole et al., 2001]. In particular, factors of the BMP family are key regulators of various stages on chondrogenic differentiation and cartilage development [Yoon and Lyons, 2004]. BMP-2, -4 and-6 have been shown to induce ectopic cartilage and endochondral bone formation, and to stimulate chondrogenic differentiation of mesenchymal precursor cells in vitro [Wozney et al., 1988; Luyten et al., 1994; Majumdar et al., 2001]. BMP activation of chondrogenic cells involves both BMPR1a and BMPR1b, which induce expression of Sox9, l-Sox5 and Sox6, the major transcription factors regulating cartilage-specific matrix genes including Col2a1, Col11a1, aggrecan and others [de Crombrugghe et al., 2001; Lefebvre and Smits, 2005; Yoon et al., 2005]. Also chondrocyte maturation to hypertrophic cells, including expression of Ihh, type X collagen and alkaline phosphatase, is stimulated by BMP factors, in particular by BMP-6 [Terkeltaub et al., 1998; Ito et al., 1999; Grimsrud et al., 2001].

Although the general hallmarks of cartilage development and homeostasis have been unravelled, the molecular mechanisms regulating the various steps of chondrocyte differentiation and expression and synthesis of cartilage specific matrix components are not yet fully understood. Major obstacles in analysing such mechanisms are problems in the isolation of homogeneous chondrocyte populations at distinct differentiation stages, particular from small animals, and the well-known instability of the chondrocytic phenotype of primary cartilage cells grown in vitro [Holtzer et al., 1960]. This has hampered not only in vitro studies on cartilage development using chondrocytes in culture, but also the analysis of molecular aspects of degenerative cartilage diseases. In the past, several chondrogenic cell lines have been introduced and successfully used to investigate aspects of chondrocyte responses to growth factors, signalling pathways, and transcriptional mechanisms (e.g. T/C28a4, MCT, rat chondrosarcoma, HCS-2/8, MC615 and ATDC5) [Kimura et al., 1979; Takigawa et al., 1989; Atsumi et al., 1990; Mallein-Gerin and Olsen, 1993; Lefebvre et al., 1995; Loeser et al., 2000]. Although most cell lines also exhibit phenotypic changes including altered gene expression, morphology, and matrix synthesis, valuable information on molecular mechanisms of chondrocyte regulation have been extracted using these chondrogenic lines. Cell lines like ATDC5 and MC615 have the potential to differentiate and mature in vitro under certain conditions [Atsumi et al., 1990; Mallein-Gerin and Olsen, 1993; Shukunami et al., 1996]. Moreover, as in primary chondrocytes and mesenchymal progenitor cells, gene expression and phenotype of the MC615 cell is sensitive to BMP2 [Valcourt et al., 1999, 2002; Grimsrud et al., 2001; Majumdar et al., 2001].

In recent studies we observed that MC615 cells grown in monolayer have the tendency to develop heterogeneity in morphology and molecular response to BMP2 after repeated passaging. In order to generate stable cell lines, we subcloned MC615 cells and obtained different stable subclones which exhibited distinct phenotypic features and gene expression profiles. Here we present the characterisation of three of these subclones (4C3, 4C6 and 4H4), each expressing a distinct profile of cartilage–related genes encoding extracellular matrix proteins, growth factors and transcription factors, with a distinct maturation potential. In many aspects these three subclones represent chondrocytes at different stages of their differentiation, ranging from chondrogenic precursor cells to proliferating chondrocytes and to prehypertrophic-like chondrocytes.

MATERIALS AND METHODS

CELL CULTURES

Subcloning: For subcloning the MC615 cell line was used. This cell line is derived from limb chondrocytes of 14-day-old mouse embryos which have been immortalised by infection with a recombinant retrovirus transducing the large T (tumour) antigen of simian virus 40 (SV40) [Mallein-Gerin and Olsen, 1993].

MC615 cells were kept in confluent culture in DMEM/F12 1:1 medium with 10% FCS and 0.5 U/ml Penicillin/Streptomycin. Cartilage-like nodules were picked, dissociated, and individual subclones were obtained by limiting dilution. Clones were propagated in DMEM/F12 1:1 medium with 10% FCS and passaged before reaching confluence.

Prolonged Culture of Subclones: For long term experiments cells between passage 20–30 were seeded at 2.0×10^4 per cm^2 and grown for the indicated time periods in medium with 10% FCS in the absence or presence of 10 mM β-glycerophosphate and 50 μg/ml ascorbate or with ITS (40 μg/ml insulin, 20 μg/ml holotransferrin, 27 ng/ml sodium selenite) as indicated, without further passaging. Medium was changed every 2 or 3 days. Cells were harvested 2–3 days after the last medium change.

BMP2 Stimulation of Subclones: For BMP2 stimulation 2.0×10^4 cells/cm^2 were seeded in medium containing 10% FCS. The next day cells were washed with PBS and incubated with medium containing 1% FCS and the indicated amounts of recombinant BMP2 and Twisted Gastrulation (TSG). For RNA extraction cells were lysed 48 h after addition of BMP2.

DETECTION OF Smad PROTEIN PHOSPHORYLATION BY WESTERN BLOTTING

Cells were incubated with BMP2 and TSG for 1 h and were and lysed in Laemmli buffer. Sample preparation, SDS–PAGE, Western blotting and immunodetection were performed as described before using rabbit anti-phospho-Smad (1, 5, 8) antibody (Cell Signaling Technology, Inc., Danvers, MA) [Laemmli, 1970; Schmidl et al., 2006].

PROLIFERATION ASSAY

Cellular growth was measured using a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The protocol used during this work was adapted from Mosmann [1983]. At day zero, 625 cells per well were seeded in triplicates in 96-well format. At each time point 20 μl MTT solution (5 mg/ml MTT in PBS) was added to 100 μl medium per well. After incubation for 2 h at 37°C, cells were lysed in 100 μl DMSO per well and formazan-specific absorption at 550 nm was measured as means of viable cell counts. Reference absorbance was determined at 670 nm.

RT-PCR AND REAL-TIME RT-PCR

Total RNA was isolated from cells using the RNeasy-Kit (Qiagen) including a DNase treatment to remove any contaminating genomic DNA. Reverse transcription (RT) was performed with 1 μg total RNA by using Superscript II RNase-H-reverse Transcriptase (Invitrogen).

Real-time RT-PCR was performed by the SYBR-Green PCR assay as described before [Schmidl et al., 2006].

RT-PCR was carried out with the primers listed below:

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Cells were fixed with ice cold methanol at −20°C for 10 min. After washing with PBS and blocking in 5% BSA for 30-min, type II collagen and type X collagen were detected with undiluted supernatant of hybridomas CIIE8 and X53, respectively [Holmdahl et al., 1986; Girkontaite et al., 1996], using the M.O.M. Kit (Vector Laboratories) and Texas Red Avidin according to manufacturers instructions.

Gene	5′primer	3′primer	Temp. (°C)	Size (bp)	Figure 3 cycle	Figure 5 cycle
Actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT	58	138	18	nd
Aggrecan	GAAGAGCCTCGAATCACCTG	ATCCTGGGCACATTATGGAA	58	133	28	27
BMP2	GTTTGGCCTGAAGCAGAGAC	GTCGAAGCTCTCCCACTGAC	58	441	nd	32
BMP4	GTAACCGAATGCTGATGGT	GGCAGTTCTTATTCTTCTTCC	58	914	nd	24
BMP6	AGCTTGCAAGAAGCATGAGC	CTCGGGATTCATAAGGTGGA	58	186	nd	36
BMP7	ATTGCACCTGAAGGCTATGC	AGAGGACAGAGATGGCGTTG	58	179	30	30
BMPRIa	GGAAATGGCTCGTCGTTGTA	ATTATGGGCCCAACATTCTG	58	210	nd	24
BMPRIb	GTGCCCAGTGACCCTTCTTA	TCATAAGCTTCCCCATCTGC	58	122	nd	32
BMPRII	AGGCCCAATTCTCTGGATCT	CACTGCCATTGTTGTTGACC	58	209	nd	24
Bsp	CGTGCCACTCACTCGAGCCAGGACT	GGGGAGGGGGCTTCACTGATGGTAGTAATAAT	61	1043	nd	30
Col1a1	CCCCACCCCAGCCGCAAAGAGT	CAGGGGGACCAGGAGGACCAGGAAGT	61	456	nd	22
Col2a1	AGAACAGCATCGCCTACCTG	CTTGCCCCACTTACCAGTGT	58	161	27	18
Col9a1	GTGGTCCTCCAGGAAGAGGT	TGCTCACAGAACCCAGGAG	58	182	nd	27
Col10a1	CATAAAGGGCCCACTTGCTA	CAGGAATGCCTTGTTCTCCT	58	98	30	30
Col11a1	GCTTTGTATGATGGCTGTGC	ATCCAAGAAAGCAAGCTGGA	58	167	nd	24
Crtl1	TCAGGAACTACGGGTTTTGG	AAGCTTCCAGGCAGCAAATA	58	192	28	24
Cyclophilin	CCACCGTGTTCTTCGACAT	CAGTGCTCAGAGCTCGAAAG	58	114	nd	22
Decorin	TGAGCTTCAACAGCATCACC	AAGTCATTTTGCCCAACTGC	58	182	nd	27
FGFR3	GGATTTAGACCGCATCCTCA	GGGTGAACACCGAGTCATCT	58	135	nd	32
Gapdh	ATCACTGCCACCCAGAAGAC [Valcourt et al., 1999]	ATGAGGTCCACCACCCTGTT [Valcourt et al., 1999]	58	443	nd	21
IGF2	TCCGAGAGGGACGTGTCTAC	CGTTTGGCCTCTCTGAACTC	58	195	nd	32
Matrilin I	CAACCAGATTGGCAAGAAGC	CCAGGCCACCAGAGTTCTTA	58	190	nd	27
Mmp13	AAAGATTATCCCCGCCTCAT	TGGGCCCATTGAAAAAGTAG	58	104	nd	27
Osteocalcin	CAAGTCCCACACAGCAGCTT [Desbois et al., 1994]	AAAGCCGAGCTGCCAGAGTT [Desbois et al., 1994]	58	370	nd	28
Osteopontin	TTGCAGGACTAACTACGACCAT	GTATTCCTGCTTAACCCTCACTA	56	985	nd	27
Phex	GTGCTCACAGTCCTCCACAA	GAAGGCAGAAACCAGCACTC	60	152	nd	35
PTHR	CCGGATCGCACCCAGCCTGGCGCTCCTTCT	ATGCCAGCAGTCCAGCCCCTT GATGCCCAGTCACA	61	374	nd	30
Runx2	ATACCCCCTCGCTCTCTGTT	AGGTTGGAGGCACACATAGG	58	94	nd	24
Smad1	TACTGGCGCAGTCTGTGAAC	GGGGTGCTGGTAACATCCT	58	128	nd	28
L-Sox5	GACGGCAAGAAACTGCGTAT	GATGGGGATCTGTGCTTGTT	58	106	nd	32
Sox6	GGATTGGGGAGTACAAGCAA	CACCTGTTCCTGTGGTGATG	58	106	nd	32
Sox9	AGGAAGCTGGCAGACCAGTA	TGTAATCGGGGTGGTCTTTC	58	158	nd	28

NORTHERN BLOTTING AND HYBRIDISATION

Northern blot analyses were carried out as described before [Stock et al., 2003]. Briefly, total RNA was resolved on a 1% formaldehyde-agarose gel and transferred onto a Roti®-Nylon membrane (Carl Roth GmbH, Karlsruhe, Germany) using 10× SSC. Blots were hybridised with ^{32}P-dCTP-radiolabelled cDNA probes in Church buffer (500 mM phosphate buffer pH 7.2, 7% SDS, 1 mM EDTA, 100 μg/ml Salmon sperm DNA) over night at 65°C, and after washing exposed to phosphoimager plates. cDNA probes for detection of aggrecan and fibromodulin transcripts encompassing nucleotides 1318–1653 of the murine aggrecan mRNA sequence (Genbank: NM_007424) and nucleotides 669–926 of the murine fibromodulin mRNA sequence (Genbank: X94998), respectively, were generated by RT-PCR.

Probes recognising Gapdh, galectin-3 and Col10a1 and Col2a1 transcripts were prepared as previously described [Stock et al., 2003; Schmidl et al., 2006; Surmann-Schmitt et al., 2008]. Note that the Gapdh hybridisation in the 4C6 panel of Figure 5A has already previously been shown in Surmann-Schmitt et al. [2008].

ALCIAN BLUE AND FAST RED STAINING

Cells were fixed with ice cold methanol at −20°C for 10 min.

Alcian Blue: After washing with PBS, cells were stained with 1% Alcian Blue in 3% acetic acid for 1 h at room temperature. Afterwards cells were washed with 70% ethanol.

Fast Red: After washing with TBS, alkaline phosphatase activity was detected with Fast Red/Naphthol tablets without levamisole (Sigma catalogue numbers F5146 and N8518) according to the manufacturer's instructions.

RESULTS

COLLAGEN EXPRESSION PATTERNS AS MARKERS OF CHONDROCYTE DIFFERENTIATION DIFFER BETWEEN MC615 SUBCLONES

To obtain homogenous cell populations with differentiated chondrocytic phenotypes, MC615 cells were kept in confluent culture for 2 weeks; developing cartilage nodules were picked, dissociated, and single cell clones were isolated by a limiting dilution series. Fourteen subclones were propagated. In subcon-

TABLE I. Expression Patterns of Cartilage Markers Differ Between Subclones of MC615 Cells

Clone	*Col2a1* [%cyclo.]	*Col10a1* [%cyclo.]
5F12	2.82	0.0008
5F8	0.84	0.0016
4C3	6.93	0.0063
4E3	0.68	0.0118
4D5	20.31	0.0145
5A2	46.65	0.0793
4C9	11.27	0.0880
4F6	27.74	0.1700
4B5	246.23	0.1760
4D7	84.09	0.2022
4C6	110.96	0.2167
4G11	21.76	0.2489
4C1	20.31	0.3644
4H4	81.22	3.8473

Relative expression levels of Col2a1 and Col10a1 of subconfluent cultures in medium containing 1% FCS were determined by real-time RT-PCR and are denoted as percentage of cyclophilin A (cyclo.) expression for standardisation. RNA was extracted 48 h after plating.

fluent cultures (48 h after seeding) Col2a1 and Col10a1 expression were analysed by real-time RT-PCR as marker genes for proliferating and hypertrophic chondrocytes, respectively, revealing major differences between the clones (Table I). Most strikingly, clone 4H4 showed dramatically higher levels of Col10a1 expression compared to the other clones, and clones 4B5 and 4C6 expressed high levels of Col2a1, whereas other clones, for example 4C3, 4E3 and 5F8, exhibited only marginal collagen expression levels.

MC615 SUBCLONES DIFFER IN MORPHOLOGY AND PROLIFERATION

In monolayer culture the MC615 subclones differed in morphology (Fig. 1) and growth behaviour (Fig. 2). Cells corresponding to clones with low basal collagen expression, for example 4C3 and 5F8, displayed a more fibroblast-like appearance in subconfluent cultures and did not substantially change cell shape after reaching confluence in medium containing 10% FCS. In contrast, clones expressing high amounts of Col2a1, for example 4C6, 4B5 as well as 4H4 exhibiting the highest amount of Col10a1, appeared polygonal in subconfluent cultures and rounded up during confluent culturing to a cobble stone-like pattern, characteristic for primary chondrocytes. Clone 5A2 with medium Col2a1 and Col10a1 expression showed initially a stellate fibroblastic cell shape but rounded up to some extent in confluent cultures. In other clones there was, however, no strict correlation between collagen gene expression and cell morphology.

Cell growth of the subclones 4C3, 4C6 and 4H4 was analysed by measuring cell numbers using a colorimetric assay. Figure 2 shows that 4C3 cells grew as fast as the parent cell line MC615, whereas the rate of cell division of 4C6 cells was lower; 4H4 displayed the lowest growth rate. This is in accordance with the more advanced stage of maturation of 4C6 and 4H4 cells indicated by their relatively high expression levels of types II and X collagens.

4C3, 4C6 AND 4H4 MAINTAIN A STABLE PHENOTYPE DURING 30 PASSAGES IN SUBCONFLUENT CULTURES

In order to assess their phenotypic stability, MC615 subclones 4C3, 4C6 and 4H4 were cultured for 30 passages, and the expression

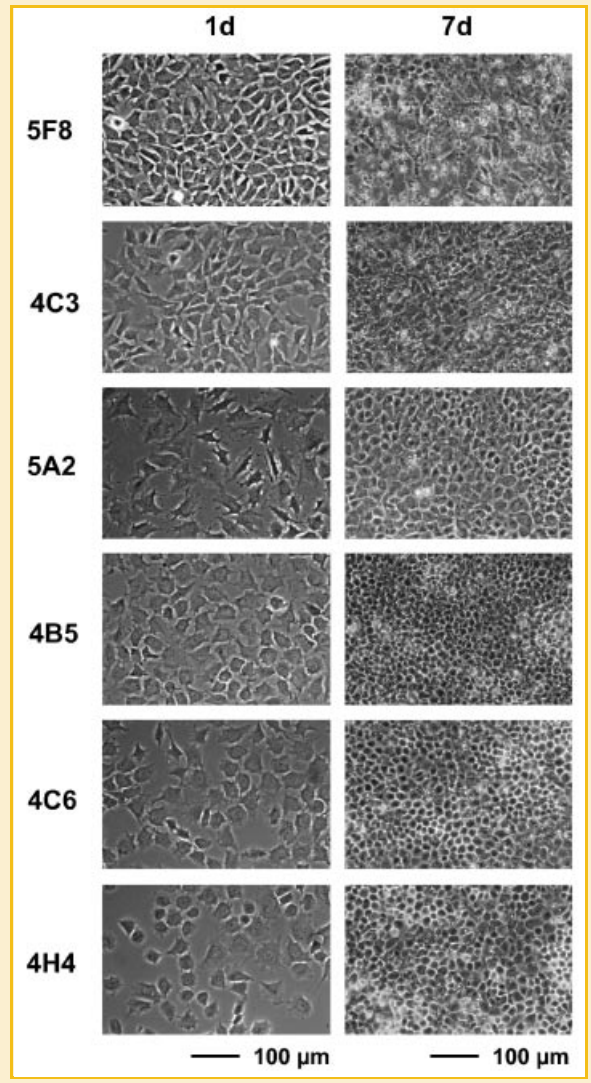

Fig. 1. MC615 subclones differ in cell morphology. Differences in cell morphology of subconfluent (1 day) and confluent (7 days) cultures grown under 10% FCS were visualised by phase contrast.

levels of genes typically involved in chondrocyte differentiation were analysed by RT-PCR after passage 20 and 30 (Fig. 3). The expression patterns for Col10a1, Col2a1, cartilage link protein and aggrecan of each subclone did not change between passage 20 and 30, indicating phenotypic stability of the isolated clones over many passages when kept in subconfluent cultures. In addition, the relative levels of gene expression of these cartilage markers observed in the three clones were generally consistent with the expression profiles observed at the first passage (see Table I). Cartilage link protein (CRTL1) mRNA was restricted to 4C6 and 4H4 cells, similarly as Col2a1, while aggrecan was expressed at similar levels in all clones including 4C3. Interestingly, only clone 4C3 expressed BMP7 (see also Fig. 5).

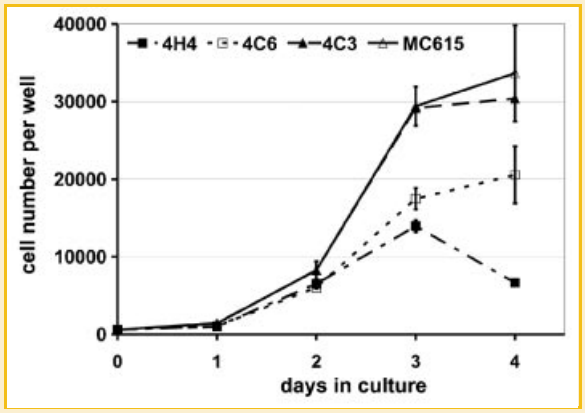

Fig. 2. MC615 subclones differ in their growth behaviour. Cell number and viability of clone 4C3, 4C6 and 4H4 were assayed by MTT and compared with the parent cell line.

MC615 SUBCLONES 4C6 AND 4H4 BUT NOT 4C3 DEPOSIT DISTINCT CARTILAGINOUS EXTRACELLULAR MATRICES IN CONFLUENT CULTURES WITHOUT FURTHER STIMULATION

The polygonal, cobble-stone like cell shape of 4C6 and 4H4 cells, their expression of cartilage specific matrix genes and the formation of nodular condensations during prolonged confluent cultures pointed to a cartilaginous differentiation and maturation of these cells. Therefore the deposition of extracellular matrix by the three subclones was analysed with regard to collagen type II, collagen type X, proteoglycans and alkaline phosphatase content during a period of up to 4 weeks without passaging (Fig. 4). Immunofluorescence analysis revealed extracellular deposition of collagen type II in confluent cultures of 4C6 and 4H4 cells, with a maximum after 3 and 4 weeks, respectively (Fig. 4A). Likewise Alcian Blue staining for proteoglycans was positive in these clones, with the first positive nodules detectable approximately after 1 week of culturing (Fig. 4C). While the deposition of type X collagen marking hypertrophic cells was only observed in clone 4H4 after 4 weeks of confluent cultivation (Fig. 4B), alkaline phosphatase, another marker characteristic of chondrocytic maturation was present in both 4H4 and 4C6 prolonged cultures (Fig. 4D). In contrast, in 4C3 confluent cultures only few Alcian Blue positive nodules developed after 4 weeks and the cultures remained negative for alkaline phosphatase activity and extracellular collagen deposition.

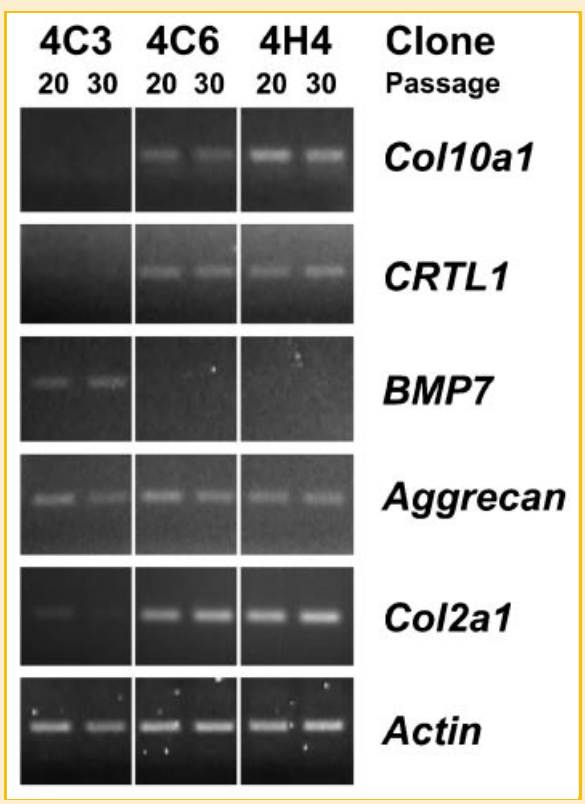

Fig. 3. 4C3, 4C6 and 4H4 maintain a stable phenotype over 30 passages. Cells were kept in subconfluent cultures for 30 passages, each passage for 2–4 days. After 20 and 30 passages total RNA was isolated and analysed by RT-PCR.

DISTINCT DIFFERENTIATION BEHAVIOUR OF 4C3, 4C6 AND 4H4 CELLS

To characterise the differentiation and maturation behaviour of the 4C3, 4C6 and 4H4 subclones, cells were grown for 4 weeks in confluent cultures without passaging, and expression levels of 36 genes involved in chondrocyte differentiation were analysed every week by Northern blotting (Fig. 5A) and RT-PCR (Fig. 5B). Genes in the RT-PCR panel are subdivided into group I consisting of genes coding for cartilage matrix proteins and related transcription factors; group II includes genes associated with hypertrophic maturation and osteoblastic differentiation, and group III covers genes of the BMP and other signalling pathways.

Clone 4C3 showed only a slight induction of Col2a1 and other cartilage matrix genes (Col11a1, aggrecan, decorin, Crtl1), indicating only partial chondrogenic differentiation, which is in accordance with the weak increase in Alcian Blue staining (Fig. 4C). Genes of group II were expressed at low levels and did not change with time in clone 4C3. In line with an early differentiation status of these cells, clone 4C3 also displayed the highest expression of BMP7 and IGF2, both being highly expressed in chondrocyte precursor cells [Shinar et al., 1993; Lyons et al., 1995].

In contrast, in clone 4C6 expression of most cartilage matrix genes (group I) was high after 1 week of confluent culture and declined after 3 weeks (exception: decorin); expression of Col10a1, Bsp, Ocn, Mmp13, Phex and PTHR (group II) increased with time, pointing to hypertrophic maturation of these cells. Remarkably, expression of BMP2 paralleled this process leading to high expression levels of BMP2 in prolonged cultures of 4C6 (Fig. 5B).

Clone 4H4 displayed continuously high levels of group I cartilage matrix genes (exception: Col9a1) and BMP6, while group II genes increased with time (Fig. 5B). In Northern blot analysis, this clone exhibited already in the first week highest expression of marker genes for hypertrophic chondrocytes like Col10a1, galectin-3 (Gal3) and fibromodulin (Fmod), which were augmented with time in culture. Similarly, RT-PCR analysis revealed that expression levels

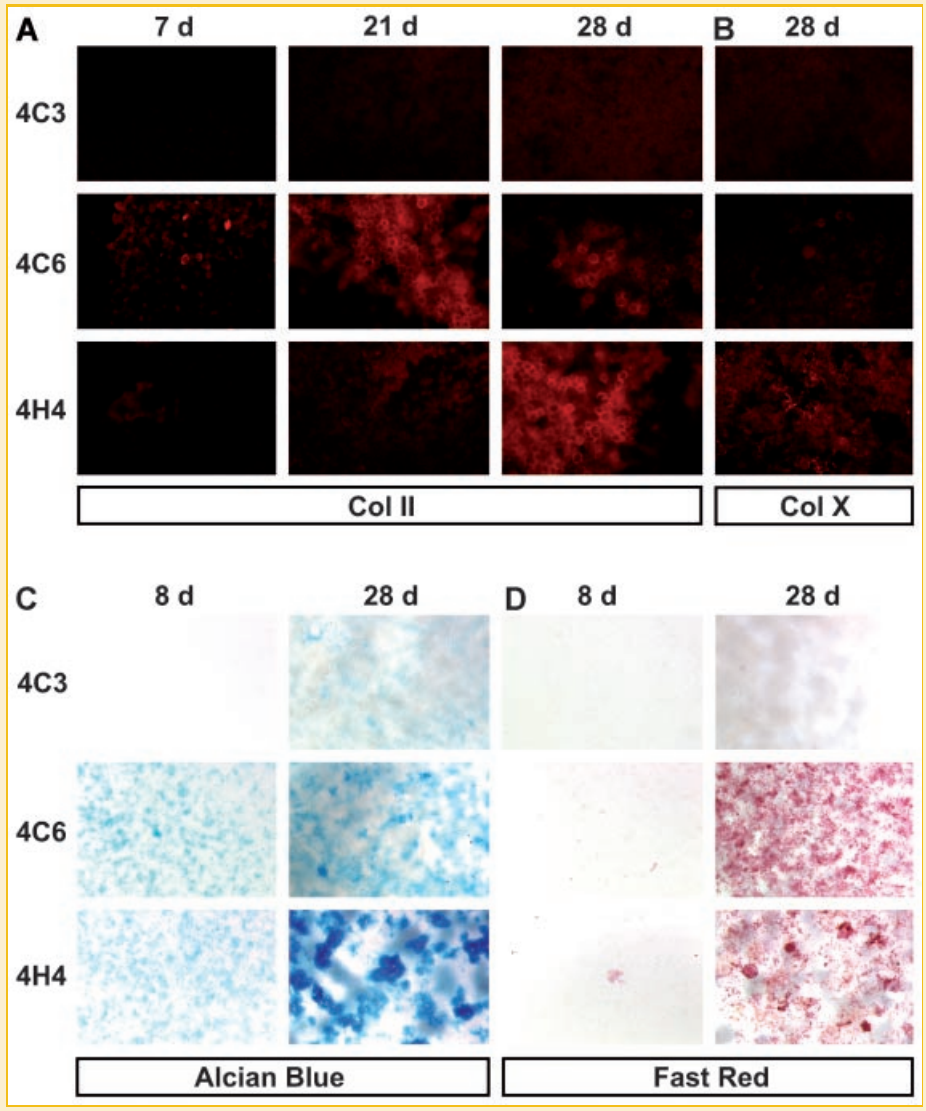

Fig. 4. MC615 subclones 4C3, 4C6 and 4H4 develop distinct extracellular matrices upon prolonged confluent culture. Extracellular deposition of collagen type II (A) and collagen type X (B) was analysed by immunofluorescence at the indicated time points. Alcian Blue staining (C) indicates proteoglycan rich cartilage-like matrices produced by clone 4C6 and 4H4. Hypertrophic maturation of 4C6 and 4H4 was further assayed by Fast Red/Naphthol staining for Alkaline Phosphatase activity.

of BSP (bone sialo protein), Ocn (osteocalcin), and PTHR (PTHrP receptor 1) in 4H4 cells increased with time in culture, indicating hypertrophic differentiation (Fig. 5B). Interestingly, despite high levels of Col10a1, only minute amounts of MMP13 were expressed in clone 4H4, even less than in 4C6, indicating that clone 4H4 chondrocytes were not terminally differentiated hypertrophic chondrocytes. Together with the immunofluorescence data these findings support the concept of an early hypertrophic character of clone 4H4.

The expression levels of some marker genes including Sox6, Sox5, Col1a1, osteopontin, BMP4, BMPRIa, BMPRII and Smad1, 5, 8 as well as ALK2 appeared to be in the same range in the three sublines and remained constant during the culture period (Fig. 5B and data not shown), but altogether the expression profiles suggest distinct differentiation capabilities of each clone.

CHONDROGENESIS OF 4C3 AND MATURATION OF 4C6 AND 4H4 IS ENHANCED BY β-GLYCEROPHOSPHATE/ASCORBATE BUT NOT BY INSULIN

Since insulin is a known inducer of chondrogenesis and hypertrophic maturation in chondrocytes, we analysed the effect of insulin on differentiation of MC615 subclones 4C3, 4C6 and 4H4. Cells treated with ITS (insulin, transferrin, and sodium selenite) or only with TS (transferrin and sodium selenite) in medium containing 10% FCS for 32 days differentiated in a similar manner as described above; no difference was detected in cartilage matrix gene expression and Alcian Blue staining between insulin treated and control cells (Supplementary Fig. 1 and data not shown).

The combination of ascorbate (AA) and β-glycerophosphate (β-GP) has been shown to maintain the chondrocyte phenotype and to support deposition and mineralisation of extracellular matrix in

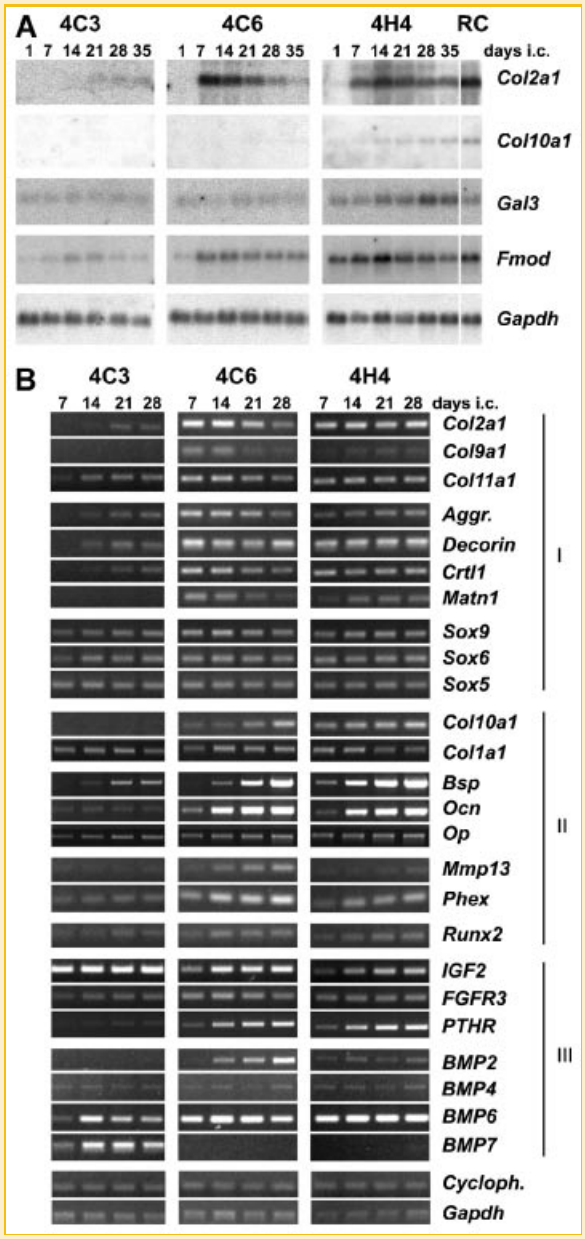

Fig. 5. 4C3, 4C6 and 4H4 cells exhibit distinct patterns of gene expression and chondrogenic differentiation during prolonged culture. Gene expression profiles of clones 4C3, 4C6 and 4H4 during prolonged confluent culture in the presence of 10% FCS were determined by Northern blotting (A) and RT-PCR (B) (the same RNA samples, extracted after 1, 7, 14, 21, 28 and 35 days in culture were used for A and B). For better overview genes in the RT-PCR panel were grouped as follows: (I) genes of cartilage matrix components and their transcription factors, (II) genes associated with hypertrophic maturation or osteogenesis and (III) genes of signalling pathways involved in cartilage differentiation.

chondrocyte cultures [Daniel et al., 1984; Reiter et al., 2002]. When treated with this combination, cultures of clone 4C3 showed a dramatic increase in Alcian Blue positive cells after 1 week (Fig. 6A), correlating with the induction of aggrecan and Col2a1 expression as determined by Northern blotting (Fig. 6B). In clones 4C6 and 4H4 the intensity of Alcian Blue staining was also enhanced by β-GP/AA. Interestingly, expression of Col10a1 was upregulated, whereas aggrecan and Col2a1 expression declined in these two clones after β-GP/AA administration, pointing to an accelerated maturation. Thus, β-GP/AA promoted chondrogenesis and maturation in the MC615 subclone system.

MC615 SUBCLONES RESPOND TO BMP2 IN A DIFFERENTIATION STAGE-DEPENDENT MANNER

Chondrocyte differentiation as well as collagen expression levels are highly influenced by BMP signals [Valcourt et al., 1999, 2002]. Since BMP2 expression was induced in the maturation process of 4C6 and BMP2 responsivity was reported for the parent cell line, we tested the response of different subclones to BMP2 stimulation. Cells were cultured with medium containing 1%FCS with or without recombinant BMP2 and the BMP antagonist twisted gastrulation (TSG) [Schmidl et al., 2006]. In all clones analysed, phosphorylation of Smad1, 5, 8 was detected by Western blotting after BMP2 administration. This effect was blocked by addition of TSG (Fig. 7C). Likewise, activation of BMP signalling led to an induction of Col10a1 expression, which was attenuated by TSG. Interestingly, however, only clones with low basal collagen expression, for example 5F8, 4E3 and 4C3, responded to BMP2 with an upregulation of Col2a1 mRNA expression that was impaired by TSG. In contrast Col2a1 expression of clones with higher basal collagen expression, for example 4B5, 4C6 and 4H4, remained largely unaffected (Fig. 7A,B). The slight decrease of Col2a1 mRNA levels observed in clone 4B5 upon BMP2 administration may reflect a dose-dependent inhibition of Col2a1 by BMP2 as reported before [Valcourt et al., 1999].

DISCUSSION

The analysis of several subclones of the chondrogenic cell line MC615 demonstrated a number of features that were immanent to all subclones analysed. Most notably, all clones analysed remained phenotypically stable for at least 30 passages. Furthermore, the maturation behaviour of the subclones followed similar rules. Thus, chondrogenic differentiation and maturation of clones analysed was promoted by β-glycerophosphate (β-GP) and ascorbate (AA) but not by insulin. Moreover, BMP2 stimulation resulted in phosphorylation of Smad proteins and in increased Col10a1 expression.

However, the generated MC615 subclones differed in their expression levels of cartilage collagens and other chondrocyte marker genes, and thus could be classified into three groups: (1) Chondrogenic precursor-like cells (e.g. 5F8, 4C3) characterised by low Col2a1 and Col10a1 expression levels, (2) Differentiated, proliferating chondrocytes (e.g. 4B5, 4C6) with high Col2a1 and low Col10a1 levels and (3) Hypertrophic-like chondrocytes (4H4) with high Col2a1 and high Col10a1 expression levels [DeLise et al., 2000].

Three clones representing these categories were chosen for extensive analysis regarding differentiation status, gene expression profile, their capacity to assemble extracellular cartilage matrix, and their response to BMP2.

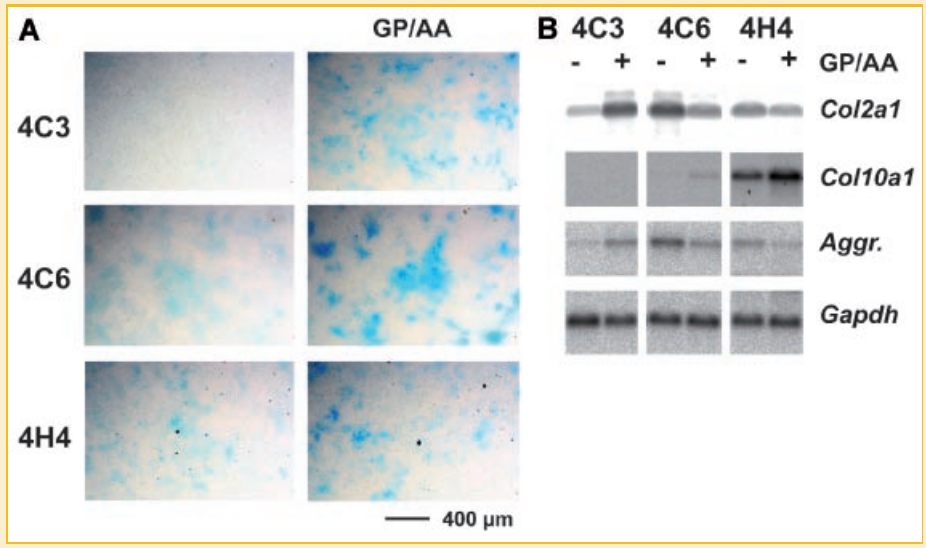

Fig. 6. Chondrogenesis and maturation of MC615 subclones are accelerated by β-glycerophosphate/ascorbate. A: Alcian Blue staining after 7 days of culture in the presence of 10% FCS pointing to enhanced chondrogenesis in clone 4C3 upon β-glycerophosphate/ascorbate administration. B: Northern blotting of 8-day cultures displayed upregulation of Col2a1 and aggrecan in 4C3, whereas these chondrocytic markers were downregulated in 4C6 and 4H4. Col10a1 was strongly upregulated in clone 4H4, and weakly induced in 4C6. GP/AA: β-glycerophosphate/ascorbate.

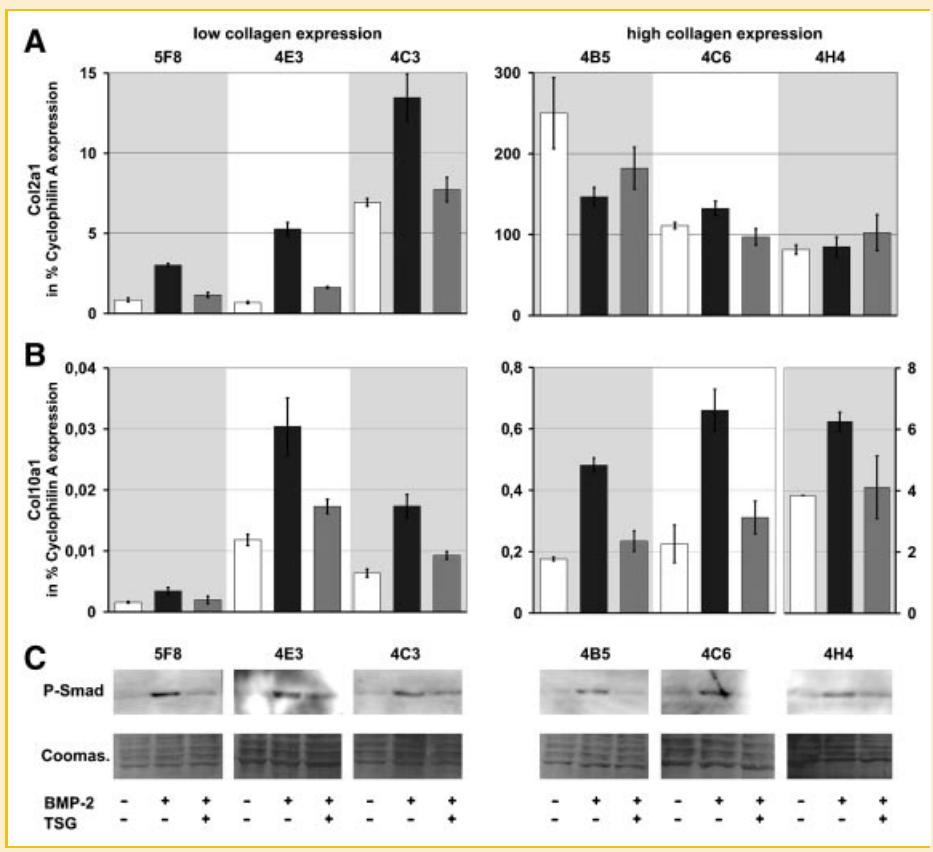

Fig. 7. MC615 subclones are BMP2 responsive in a differentiation stage-dependent manner. Col2a1 (A) and Col10a1 (B) expression was analysed after 48 h of incubation in medium containing 1% FCS in the absence (white bars) or presence of 100 ng/ml BMP-2 alone (dark bars) or combined with 2µg/ml TSG (grey bars) by real-time RT-PCR. Phosphorylation of Smad1, 5 and 8 (P-Smad) was determined after 1 h incubation by western blotting (C) in the same clones as above. Coomassie staining (Coomas.) of the PVDF membrane served as loading control. BMP2 induced Smad phosphorylation and Col10a1 expression in all clones but Col2a1 was only upregulated in clones with low basal collagen expression (e.g. 5F8, 4E3, 4C3). The BMP modulator TSG (twisted gastrulation) largely neutralised the BMP activity in all cell lines.

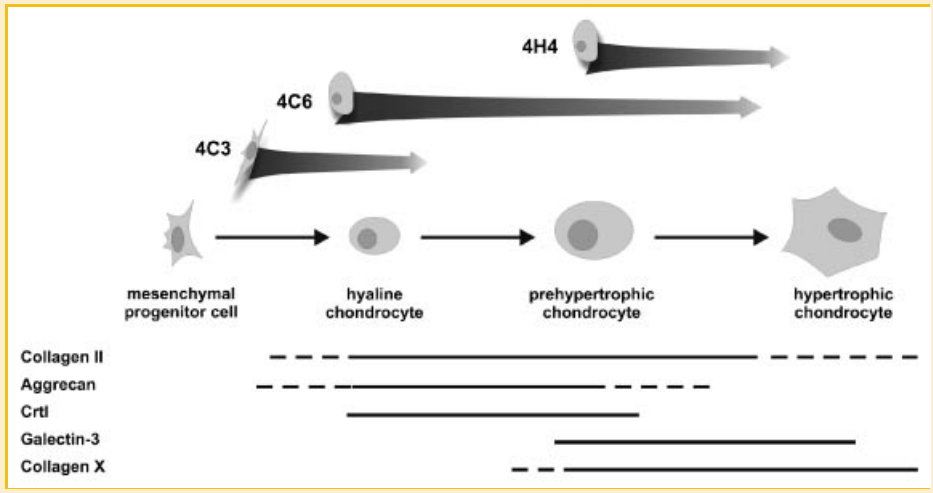

Fig. 8. Subclones of the chondrogenic cell line MC615 exhibit distinct differentiation stages.

The expression profile of 4C3 cells, representing group one subclones, with low levels of Col2a1, Col10a1 and Col11a1 and other cartilage genes such as aggrecan or matrilin 1 was similar to primary chondroprogenitor cells such as embryonic limb bud mesenchyme cells [Goldring et al., 2006]. Furthermore 4C3 cells expressed BMP7 (osteogenin) in contrast to 4C6 and 4H4 cells. BMP7 is beside BMP2 a strong inducer of osteogenic and chondrogenic differentiation and is also expressed in the limb bud mesenchyme as well as in the perichondrium [Ripamonti and Reddi, 1992; Helder et al., 1995; Luo et al., 1995; Lyons et al., 1995; Shintani and Hunziker, 2007]. Moreover, the fibroblast-like morphology of 4C3 cells appears to be more similar to that of undifferentiated (prechondrogenic) mesenchymal limb bud cells than that of differentiated rounded chondrocytes. The higher proliferation rate in comparison to 4C6 and 4H4 cells also points to a rather undifferentiated phenotype, since differentiation/maturation stage and proliferation rate of chondrogenic cells correlates reciprocally [reviewed in Beier et al., 1999]. Interestingly, only minor spontaneous chondrogenic differentiation was observed in confluent cultures of 4C3 cells, but it was strongly induced by BMP2 administration. Chondrogenesis and cartilage matrix synthesis were also enhanced by β-GP/AA, which promote collagen synthesis and stabilize the extracellular matrix, thereby providing an environment favouring matrix mineralisation [Daniel et al., 1984; Zimmermann et al., 1992].

As a typical representative of group two, subclone 4C6 was chosen. 4C6 cells exhibit typical features of a chondrocyte such as cobble stone-like cell shape in monolayer culture, low proliferation rate and a high expression level of Col2a1 and other cartilage-related matrix genes [Goldring et al., 2006]. The intermediate growth rate of 4C6 cells in comparison to 4C3 and 4H4 cells also indicates that these cells are in a differentiation stage in between the stages of those subclones. Spontaneous differentiation and maturation was detectable in confluent cultures with extracellular deposition of a cartilage-like matrix. Strong upregulation of Col10a1, MMP13, Ocn and Bsp mRNA levels, genes associated with maturation of chondrocytes further indicates the ability of 4C6 cells to mature in confluent cultures [de Crombrugghe et al., 2001; Wagner and Karsenty, 2001; Goldring et al., 2006]. This maturation process could also be accelerated by βGP/AA treatment.

Clone 4H4, which is the only member of group three, resembles a mature, hypertrophic chondrocyte with high Col10a1 levels, a rounded morphology and the lowest growth rate, indicating the most advanced maturation stage of the clones analysed. During prolonged confluent culture expression of most cartilage genes remained unchanged in this clone. In contrast genes related with hypertrophy and mineralisation, such as PTHRI, galectin-3, Col10a1, Ocn, Bsp and Phex were strongly upregulated pointing to further maturation of 4H4 cells [Vortkamp et al., 1996; Colnot et al., 1999; Wagner and Karsenty, 2001; de Crombrugghe et al., 2001; Stock et al., 2003; Miao et al., 2004; Goldring et al., 2006]. Interestingly, MMP13, a marker of late hypertrophic chondrocytes, was only weakly expressed after 4 weeks in culture, indicating that the expression of genes considered characteristic of late hypertrophic chondrocytes such as Col10a1, osteocalcin and MMP13 were uncoupled in differentiated 4H4 cells.

These results indicate that the three subclones of MC615 mark three distinct differentiation/maturation stages of chondrogenic cells, with 4C3 displaying the most undifferentiated and 4H4 exhibiting the most mature phenotype, as summarised in Figure 8.

Another interesting difference between these three clones was observed in their response to BMP2. Phosphorylation of BMP-related Smad1, 5 and 8 proteins was observed in all three clones after BMP2 stimulation, as it has been previously demonstrated for the MC615 cell line [Schmidl et al., 2006]. However, the resulting regulation of cartilage-associated collagen genes differed between the subclones. Thus, in the precursor-like clone 4C3 both Col2a1 and Col10a1 were induced upon BMP2 stimulation, marking a chondrogenic differentiation similar to stimulation of chondrogenesis with βGP/AA. In contrast, clone 4C6—the model for a proliferating chondrocyte—exhibited only a moderate induction of

Col2a1 by BMP2 but a pronounced stimulation of Col10a1 expression, indicating maturation. Clone 4H4, the most mature of the three clones, did not change Col2a1 expression upon BMP2 administration, but still activated Col10a1 expression. The distinct response of the three clones to differentiation stimuli was also reflected in the βGP/AA experiment where only 4C3 showed induction of early chondrocyte markers like Col2a1 and aggrecan, whereas in 4C6 and 4H4 these markers decreased and only the maturation marker Col10a1 was induced.

Taken together these three cell lines represent potentially useful new cell culture systems to study various aspects of chondrocyte differentiation and function.

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